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Ph.D. in Sustainable agricultural and forestry systems and food security

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Adaptive physiological responses of tomato plants to combined abiotic stress and biostimulant application.

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Abstract

Tomato is one of the most cultivated crops in the world. Due to the antioxidant and anti-cancer properties of lycopene and other compounds, tomato consumption and production is still on the rise. However, its productivity is greatly compromised by a wide range of abiotic stresses and, therefore, the production of stress-tolerant tomato lines and the identification of novel strategies to increase stress tolerance are key challenges for modern agriculture. The presence of adverse environmental factors such as extreme temperatures, salinity or drought causes morphological, physiological and biochemical changes in tomato plants. The biotechnological and agronomical methods used to increase tomato tolerance to various abiotic stresses include the selection of tolerant genotypes and the use of management practices, such as the application of biostimulants. An in-depth study of the physiological responses of tomato plants to abiotic stress and to biostimulant application was performed. The first aim of this research was to investigate the mechanisms that control plant physiological responses to high temperature stress, drought and combined stresses in different tomato genotypes in order to select those tolerant to abiotic stress. A second aim was the identification of strategies to increase tomato growth and final yield under stress. To this aim, we focused on the protein hydrolysate-based biostimulant and investigated its ability to induce better performances in plants under heat, drought and combined stresses in different environmental conditions. As for the first aim, the first part of this research focused on the eco-physiological screening of several tomato genotypes under elevated temperatures (**Chapter 2**) that allowed the selection of two genotypes potentially tolerant to heat stress (LA3120, E42). The response of the selected genotypes was further tested in a growth chamber to better investigate their responses to combined stresses, such as high temperatures and water shortage (50% of water requirements) (**Chapter 3**). As for the second aim of this thesis, the response of different genotypes grown in open field under elevated temperatures after application of a protein hydrolysate-based biostimulant was analysed. This additional analysis allowed to demonstrate that the use of the biostimulant by fertigation led to better plant performances under elevated temperatures (**Chapter 4**). The adaptive physiological response to single and combined stresses and biostimulant treatment was also investigated under controlled conditions in the selected genotypes E42 and LA3120 (**Chapter 5**). Considering that plants grown in open field are subjected to a higher number of different variables compared with controlled environments, in the final part of this work, the performances of the genotype E42 exposed to water deficit and treated with the novel protein hydrolysate biostimulant were evaluated under open field conditions. This final experiment allowed to demonstrate the positive effect of the biostimulant on final yields under water deficit and in different field trails (**Chapter 6**). Our findings contributed to a better understanding of

the morphological and physiological effects of combined abiotic stresses on tomato crop. Additionally, the results obtained in this thesis further demonstrate the effects of protein hydrolysate-based biostimulants on improving plant performances under abiotic stresses. Altogether, results obtained in this thesis provide novel solutions to increase final yields in plants facing the future climate changes.

Chapter 1. General introduction

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Tomato (*Solanum lycopersicum* L.) is one of the most important and widespread crops in the world. It originated in western South America, and domestication is thought to have occurred in Central America (Kimura & Sinha 2008). Given its importance as a food product, tomato has been inserted in different breeding programs to improve yield, fruit quality and resistance to biotic and abiotic stresses. Moreover, the tomato has been broadly used as a research material, presenting interesting features that other model plants, such as rice and Arabidopsis, do not have. For example, tomato plants produce fleshy fruits, botanically called berries, important for the human diet, and it is the only model plant with sympodial shoots and compound leaves. Almost all of these attributes are agronomically important. Currently, 13 wild tomato species are known (Peralta et al. 2005; Spooner et al. 2005). Most of them can be crossed with the cultivated tomato being diploid (2n = 24) and provide a source of variation for desirable traits such as increased productivity, fruit quality and resistance to pathogens and abiotic stresses. For all these reasons, tomato is an ideal model plant species for plant research for all fleshy fruit plants. In this thesis tomato has been used as a model plant to investigate the adaptive physiological responses of plants to combined abiotic stresses and biostimulant application. Considering that tomato is a Solanaceous species and that is similar to other important horticultural plants including potato, eggplant, peppers and tobacco, the know-how obtained from this thesis can be easily applied to other vegetable crops.

1.1 – Effect of abiotic stresses on tomato plants growth and development

1.1.1 Heat stress

One of the main abiotic stress plants face is heat stress. Heat stress causes a series of biochemical, morphological, physiological, and molecular changes that adversely affect plant development (Fig. 1). The consequences of heat stress can range from increased leaf transpiration under moderate stress up to cause cell damages and plant death under thermal stress (Wahid et al. 2007). The susceptibility of plants to elevated temperatures is considered to be linked to phenological aspects and the extent of damage to the crop is related to the time at which plants are exposed to heat stress and to the length reduction of the actual growing season (Cleland et al. 2007). Given the current predictions on the global average temperature rise, certainly plant's phenology will be affected (Piao et al. 2019). An accelerated growth rate will lead to a decrease of the duration of the plant’s cycle, time reduced to assimilate resources and consequently a reduction in yield. At the same time, the increase in short episodes of high temperature (heat waves) is expected to affect yield, regardless of any substantial variation in mean air temperature (Wheeler et al. 2000). It is known that heat stress inhibits seed germination (Toh et al. 2008), shortens the length of the vegetative cycle (Haque et al. 2014), affects

the number and fertility of flowers, also causing the sterility of pollen (Prasad et al. 2014). Indeed, the reproductive phase of plants is known to be particularly sensitive to abiotic stresses (Farooq et al. 2009). In particular, it has been demonstrated that, in tomatoes, when temperatures exceed 35 °C, all the reproductive stages, from pollen formation and viability to fruit set, are adversely affected (Sato et al. 2004), causing yield reductions. From a physiological point of view, photosynthesis in the higher plants is certainly the most sensitive process to heat since, generally, the optimal temperature range for photosynthesis is considered to be between 25 °C and 30 °C (Khavari & Ra 1980). The major problems of heat stress are at the level of the photochemical reactions in the thylakoid and of carbon metabolism in the chloroplast stroma (Wise et al. 2004) and include the reduction of the chlorophyll content (Farooq et al. 2011) and the alterations in the content and activity of antioxidant molecules (Venkatesh & Park 2014). Also in tomato, the physiological responses to heat stress at different developmental stage of the plant include a decrease in chlorophyll content, net photosynthetic rate (P_N) and Fv/Fm values (Zhou et al. 2017). The decrease of photosynthesis under heat stress is also attributed to increases in the rate of photorespiration (Walker et al.2016). Thermal stress leads to less CO_2 solubility than O_2 and an increase in the maximum rate of oxygenation of the Rubisco enzyme. Under these conditions, the enzyme Rubisco binds to O_2 instead of CO_2, leading to the production of a toxic compound, which must then be recycled by photorespiration (Betti et al. 2016). Any effect on photosynthesis leads to a reduction in crop growth and productivity. Hence, one of the aims of this thesis was to carefully investigate the effects of heat stress on both the photosynthetic performances and growth parameters with a particular attention of the effect on final yield and yield components in tomato.

A further important consequence of high temperature is the alteration of the oxidative metabolism, which causes membrane instability. In particular, the accumulation of reactive oxygen species (ROS), such as H_2O_2, in both chloroplasts and mitochondria, can have negative impacts, such as severe DNA damage, the autocatalytic peroxidation of lipids and membrane pigments, the loss of semi-permeability membranes and the breakdown of photosynthetic pigments and decreased enzyme activity (Wang et al. 2014). The imbalance between the production of ROS and antioxidant defense of the plant is believed to lead to oxidative stress. Finally, heat stress increases the leaf senescence in plants, causing a reduction in the number of photosynthetic pigments. The progressive loss of green (i.e. chlorophyll) in a leaf leads to an overall reduction of the photosynthetic leaf area.

Climate change is exacerbating and/or increasing the frequency of high temperature shocks in the Mediterranean basin, where tomato is mostly cultivated, pointing to the need for developing novel tomato varieties with enhanced tolerance to this stress (Zhou et al., 2020a, Zhou et al., 2020b). Therefore, one of the goals of this thesis was the identification of tomato genotypes that are tolerant

to heat stress and the analysis of the different strategies activated in different genotypes in response to this stress.

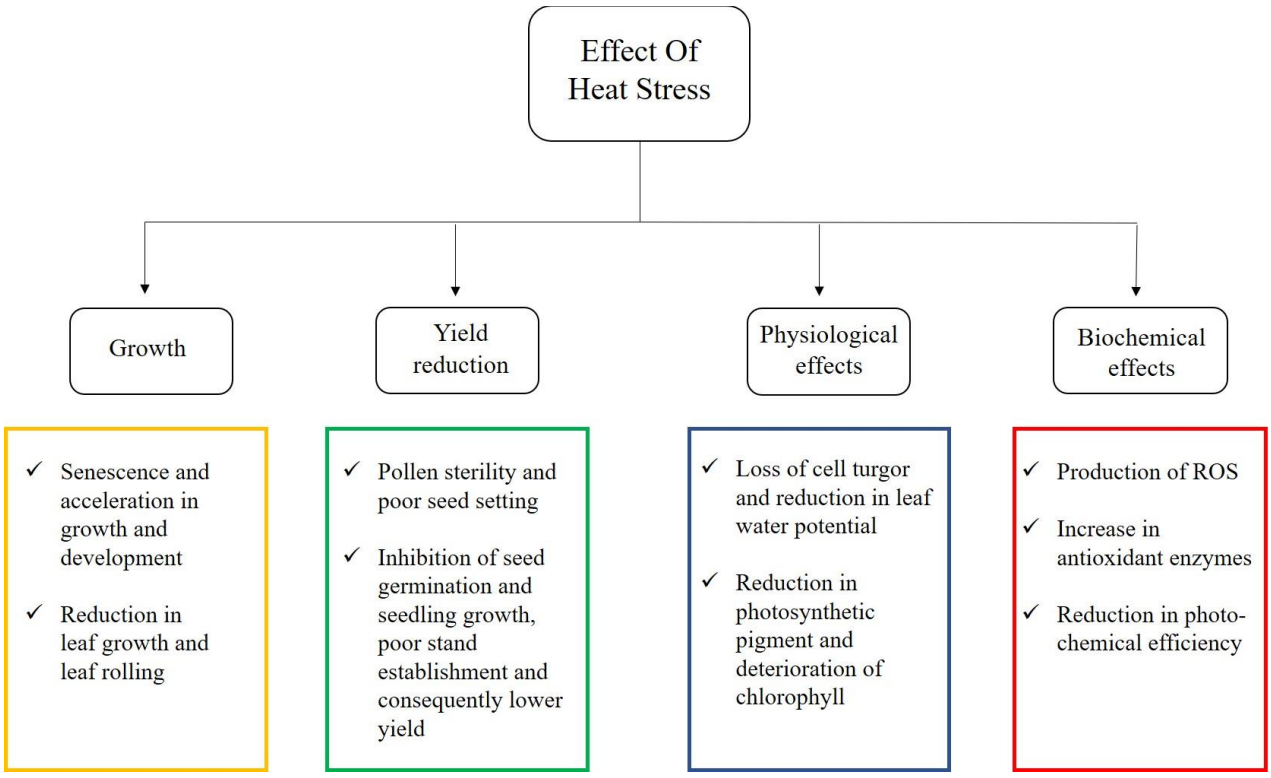

Figure 1. Main effects of heat stress on plant growth, physiological and biochemical processes, and yield related factors.

1.1.2 Drought stress

In any agronomic year, short periods of transitional drought and long periods of occasional drought are frequent. Drought stress involves many aspects of the plant and generally involves changes at a morphological, ecological, biochemical, physiological and molecular level (Bhargava et al. 2013; Farooq M. et al. 2009, Shao et al. 2008, Fig.2). Moreover, it also has negative effects on the quality and productivity of crops (Nezhadahmadi et al. 2013). The response to drought stress in crops may range from partial stomatal closure under moderate stress to drying and death of the plant at the wilting point (Parkash & Singh 2020). Usually, drought stress in plants, from agronomic viewpoints, occurs when transpiration from the leaf surface is higher than the water absorbed by the roots (Salehilisar et al. 2012). The signs of drought in crop vary depending on the plant species, developmental stage and growth conditions. Broadly, drought symptoms involve loss of leaf turgor, drooping, wilting, etiolation, yellowing, premature leaf senescence and at last, under extreme conditions, death of plant (Arbona et al. 2013, Jaleel et al. 2009). The growth and development of

plants depend on cell division and differentiation, and water stress causes a loss of turgidity, a disorder at the level of enzyme activity and a consequent reduction of energy to be used during photosynthesis (Ding et al. 2013). In addition, the root-to-shoot ratio of plants considerably increase under dehydration conditions, and the total biomass of the plants is reduced considerably (Franco 2011, Zare M. 2011). This reduction of the size of the plant causes a low photosynthesis rate in higher plants subjected to drought. The reduction of the photosynthetic process is also attributed to the reduction of the diffusion of CO_2 through stomata (Chaves et al. 2009), to the increase of oxidative stress, to the decrease of photosynthesic foliar area and to premature foliar senescence, decreasing chlorophylls content and the synthesis of photosynthetic enzymes (Lawson & Blatt 2014). Regulation of water loss through stomata is an early response of the plant to drought, as they regulate gas exchange in plants and CO_2 absorption and water loss pass through stomatal pores. A decrease in photosynthesis can also be linked to non-stomatal factors, including a constrain in the carbon metabolism. The reduction of intracellular CO_2 concentration (Ci) is associated to a decrease in Rubisco activity, due to the restriction of CO_2 availability for carboxylation (Sharkey 1990) or to the reduction in Ribulose-1,5-bisphosphate (RuBP) regeneration as a result of a reduction in ATP synthase activity (Tezara et al.1999). It is known that plants under stress, initially, respond with a decline of one or several physiological functions (alarm phase). This is followed by the activation of stress coping mechanisms which can lead to a restitution of the previous physiological functions (resistance process) (Lichtenthaler and Rinderle 1988). However, according to the authors, when subjected to a long-term or high intensity stress, the adaptation capacity of the plant is surpassed, causing irreversible damage and finally cell death (exhaustion phase). Still, when the stress factors are removed before the exhaustion phase becomes permanent, the plants can regenerate and move to a new physiological stage (regeneration phase). Different physiological responses occur in tomato plants according to the duration and intensity of the applied water stress, such as molecular mechanisms governing the timing of stomata closure, modulation of photosynthetic performances, accumulation of osmolytes and growth retardation, for this reason in this thesis experiments were conducted both under prolonged water stress and/or drought.

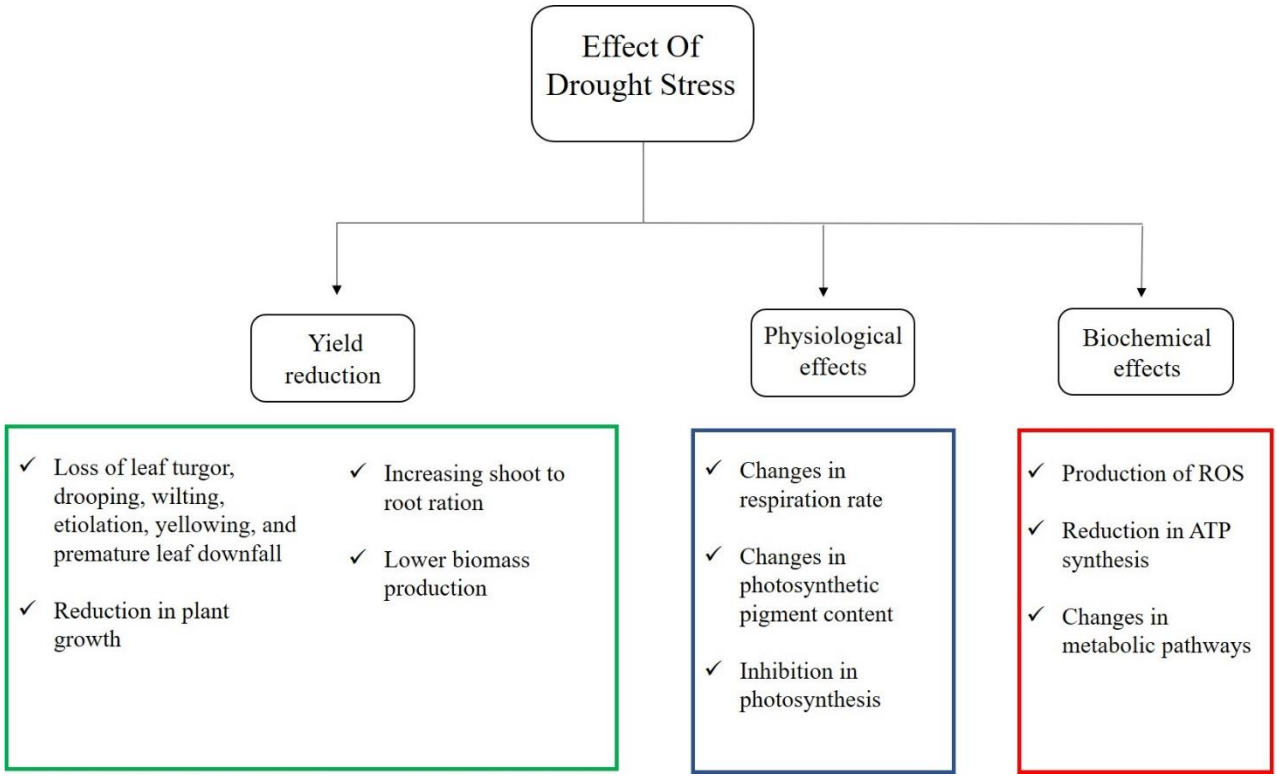

Figure 2. Main effects of drought stress on physiological and biochemical processes, and yield related factors.

1.1.3 Combined stresses

Abiotic stresses have been largely investigated during the past decades, by subjecting plants to individual and independent stress imposition. Yet, under realistic field conditions, abiotic stresses generally appear recurrently in combination during the growing seasons. Several works have concluded that each combination of two or more stresses imposes a specific set of responses on the plant (Rizhsky et al. 2004, Pandey et al. 2015, Prasch & Sonnewald 2013). There are different opinions reported in the literature and several studies report that the interactions between stresses have higher negative effects on crop productivity than single stress applied individually (Mittler & Blumwald 2010, Suzuki et al. 2014). Examples of these negative effects have been reported for drought combined with cold stress (Sales et al. 2013), heat stress with high CO_2 (Wang et al.2016) and waterlogging combined with salinity (Alhdad et al.2013). On the other hand, other papers have reported that some stress combinations can have a favorable effect on plant (reviewed in Suzuki et al.2014), and examples include combination of ozone with drought (Iyer et al. 2013) or salinity with heat stress in tomato (Rivero et al. 2013). Given the ongoing climate change, it is expected that the incidence of drought combined with heat waves will be the most imminent stress combination to be addressed, stressing the importance of further studying this interaction. High temperatures can increase evaporation of water from soil and increase water loss from plants and consequently enhance

plant exposure to water stress and consequent crop yield reductions (Shao et al., 2008, Trenberth et al., 2014). More severe depletion of photosynthetic rates, enhanced production of ROS, reduction of the PSII photochemical efficiency and decrease of Rubisco activity are among the physiological responses reported when plants are subjected to drought and heat stress combinations (Zandalinas et al. 2018, Mittler 2006). However, the effect of physiological responses to combined stress depends on plant species and genotype. Recently, Zhou et al. (2019) observed that when tomatoes were subjected to combined stress, the leaf water loss and the dry matter accumulation significantly decreased, on the other hand, combined stresses reinforced the negative impact of individual stresses. Understanding the response to combined abiotic stress, a quite common condition in agricultural systems, will be important for the selection and breeding of tolerant tomato genotypes and to maintain crop production in the most critical production areas.

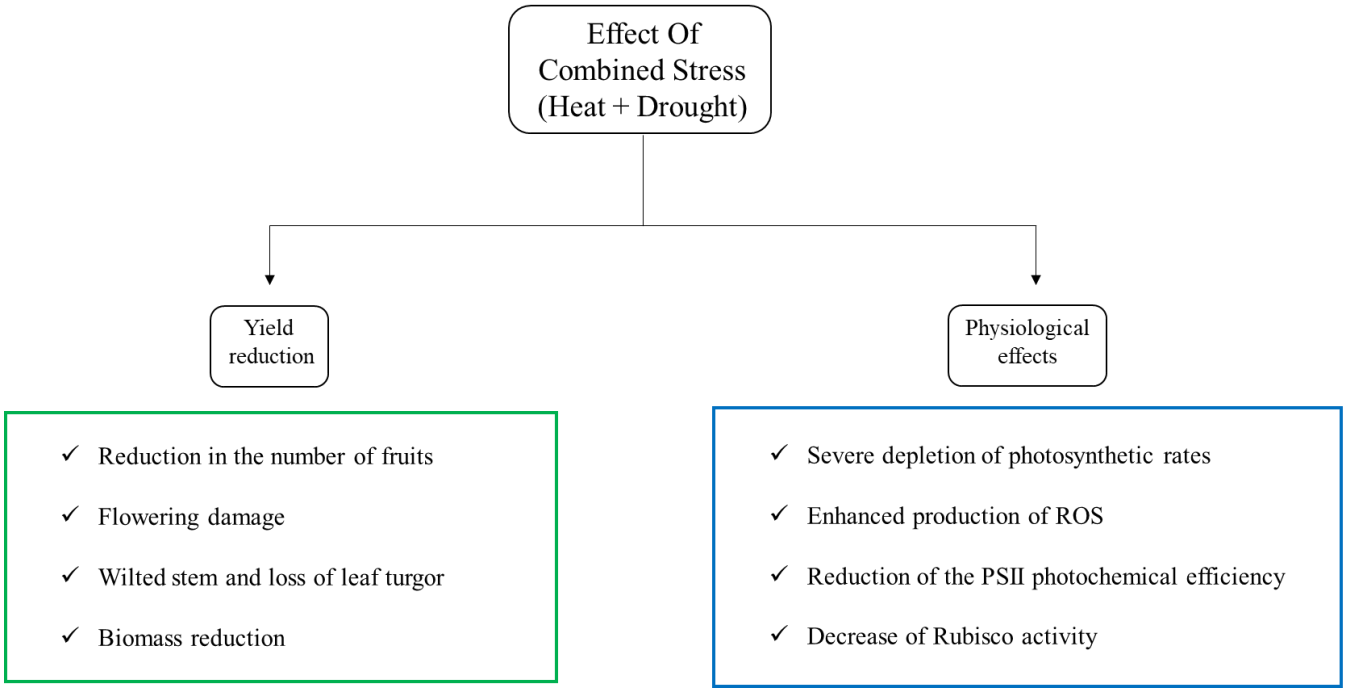

Figure 3. Main effects of drought stress on physiological and biochemical processes and yield related factors.

1.2 Plant biostimulant

Considering the importance of tomato crop, the development of new management practices to enhance tolerance to abiotic stresses, including heat and drought stresses, could contribute to global food production. The use of biostimulants is proposed as an innovative solution to address the novel challenge to improve the sustainability of agricultural systems and reduce the use of chemical fertilizers. Already in 1933, the concept of biostimulant began to be used in plant research, where the term "biogenic stimulants" were indicated as those derivatives of biological organisms, including

plants, which could have beneficial effects on plant metabolism and also on metabolic and energetic processes of animals and also of humans. Subsequently, these substances were examined only for plants, and several studies tried to identify "organic acids that had stimulating effects on enzyme activity" (Yakhin et al. 2017). Over the years there have been different definitions of biostimulants: allelopatic preparations, plant strengtheners, metabolic enhancers, plant growth promoters, metabolic antiperspirants are only some of the definitions used. Biostimulants are defined according to the European Biostimulant Industry Consortium (EBIC) as "substances or organisms, other than fertilisers and pesticides, whose function, when applied to plants, rhizosphere, seeds or growing media, is to modify physiological processes in order to improve/increase nutrient uptake, the efficiency of the nutrients themselves, tolerance to abiotic stress and the quality of crops" (Goñi et al. 2018; Biostimulants.eu/). Biostimulants are therefore a set of compounds or organisms that cannot be traced back to fertilizers or pesticides, whether biological or not. Instead, they have the function of activating or favoring resources already present in the plant-soil system. An initial definition that highlights the differences between biostimulant and fertilizer is given by du Jardin (2015): "materials that, in minimal quantities, promote the growth of plants". The term plant conditioner is also used as a synonym to clarify how it increases the efficiency of the absorption of nutrients from the soil and tolerance to stress (Du Jardin 2012). The use of biostimulants can increase yields, increase the concentration of foliar pigments, secondary metabolites and vitamins, increase the resistance to numerous biotic and abiotic stress of cultivated plants, reduce fertilizer delivery and encourage agriculture close to organic farming, thereby reducing environmental contamination. Additional benefits include increased germination rate, improved root depth, and reduced post-transplant stress (Fig.3). For example, it has been demonstrated that an enzymatically hydrolyzed animal protein-based biostimulant may exert a positive effect on tomato plants under water stress (Casadesús et al. 2019), or foliar application of seaweed sap enhances yield and quality in tomato (Zodape et al. 2011). In another work, perennial ryegrass (*Lolium perenne* L.) treated with hydrolyzed amino acids improved photosynthetic efficiency compared to non-treated plants at high temperatures (Botta 2012). With the increase in research and products, any type of substance or organism which is beneficial for plants is considered as a biostimulant, but without being a fertilizer, a soil improver or a pesticide. This includes growth promoting bacteria (Pgprs) and fungi already used in biological control, but which also have biostimulant properties (for example those belonging to the genus Trichoderma spp.) (López-Bucio et al. 2015). Fungi and beneficial bacteria fall under the term according to a lot of dedicated literature, even if they have existed for a long time in the market as biofertilizers or Ppps (Plant Protection Products) (du Jardin 2015). Among different biostimulant categories, protein hydrolysates seem to be promising, since they contain high amounts of molecules

such as amino acids, small peptides and osmoactive compounds which are beneficial for plant growth and development under abiotic stress condition (Van Oosten et al. 2017). For example, the application of an extract from moringa (Moringa oleifera Lam.) increased yield and growth in tomato, basil, cabbage and pepper, as well as the quality of tomato, lettuce, radish, spinach, rocket and pepper (Zulfiqar et al. 2020). To improve the tolerance to abiotic stress the use of protein hydrolysates and plant-based biostimulants has been widely investigated, even if it is still unclear the functional cause-effect relationship and to what extent these compounds are able to improve the physiological performances of tomato plants under combined stresses. On that account, in this work we chose to use a novel protein hydrolysate-based biostimulant to improve tomato plant performance under heat, drought and combined stresses.

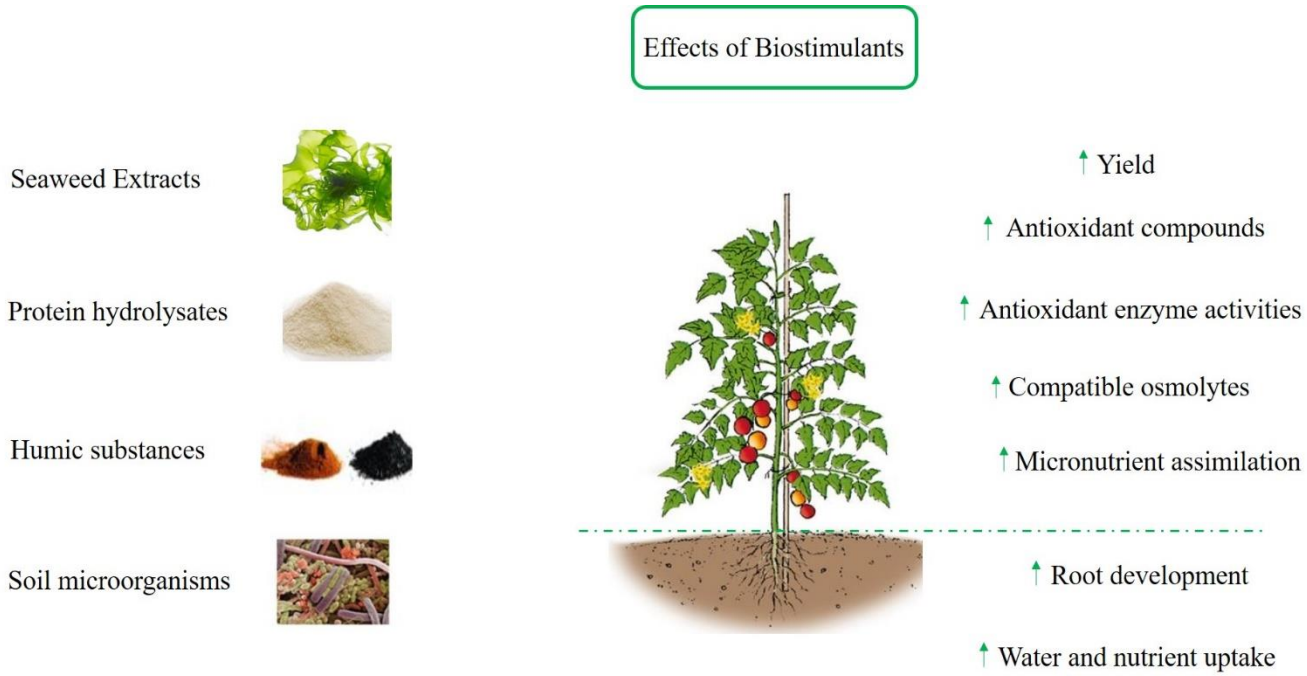

Figure 3. Overview of the effects of biostimulant application on plants.

1.3 Aim of the research

This PhD project aims to advance the understanding of plant responses to individual and combined abiotic stresses in tomato plants. Therefore, the main aims of this research are:

1:to verify the effects of heat and drought stress on different tomato genotypes

2: to identify the physiological and molecular mechanisms activated under combined (heat + drought) abiotic stresses

3: to link physiological responses and agronomic performances of tomato plants treated with a plant-derived protein hydrolysate

1.4 References

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Chapter 2. Eco-Physiological Screening of Different Tomato Genotypes in Response to High Temperatures: A Combined Field-to-Laboratory Approach

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Abstract: High temperatures represent a limitation for growth and development of many crop species. Several studies have demonstrated that the yield reduction of tomato under high temperatures and drought is mainly due to a photosynthetic decline. In this paper, a set of 15 tomato genotypes were screened for tolerance to elevated temperatures by cultivating plants under plastic walk-in tunnels. To assess the potential tolerance of tomato genotypes to high temperatures, measurements of chlorophyll fluorescence, pigments content and leaf functional traits have been carried out together with the evaluation of the final yields. Based on the greenhouse trials, a group of eight putative heat-sensitive and heat-tolerant tomato genotypes was selected for laboratory experiments aimed at investigating the effects of short-term high temperatures treatments in controlled conditions. The chlorophyll fluorescence induction kinetics were recorded on detached leaves treated for 60 min at 35 °C or at 45 °C. The last treatment significantly affected the photosystem II (PSII) photochemical efficiency (namely maximum PSII quantum efficiency, Fv/Fm, and quantum yield of PSII electron transport, ΦPSII) and the non-photochemical quenching (NPQ) in the majority of genotypes. The short-term heat shock treatments also led to significant differences in the shape of the slow Kautsky kinetics and its significant time points (chlorophyll fluorescence levels minimum O, peak P, semi-steady state S, maximum M, terminal steady state T) compared to the control, demonstrating heat shock-induced changes in PSII functionality. Genotypes potentially tolerant to high temperatures have been identified. Our findings support the idea that chlorophyll fluorescence parameters (i.e., ΦPSII or NPQ) and some leaf functional traits may be used as a tool to detect high temperatures-tolerant tomato cultivars.

2.1 Introduction

Increasing atmospheric temperatures, which are expected to rise by 2–4.8 °C in the next few decades, can compromise crop productivity in numerous regions worldwide (Stocker et al. 2013, Vitale et al. 2011, Sehgal et al. 2018, Zhou et al.2018). Indeed, elevated temperatures can induce a series of physiological responses with consequent decreases of crops yields and quality (Rigano et al. 2016, Zhou et al. 2016). Tomato (*Solanum lycopersicum*), being an excellent source of health-promoting compounds, is one of the most important crops cultivated worldwide and its heat sensitivity varies among different genotypes (Zhou et al. 2018, Francesca et al. 2020, Zhou et al. 2017). Generally, the optimal temperature range for photosynthesis is considered to be between 25 °C and 30 °C (Zhou et al. 2016). The rising of average temperatures due to the ongoing climate change will cause extensive

productivity losses in Mediterranean areas, where tomato is traditionally cultivated [Mathur et al. 2014, Carvalho et al 2016, Giorio et al. 2018). In this framework, it becomes important to perform studies that are able to identify the most promising genotypes able to face heat stress. The relationship between gas exchange and crop yield has been largely studied in tomato, suggesting leaf transpiration as the most reliable indicator for yield prediction under drought (Patanè et al. 2016)]. However, beside gas exchange, other photosynthesis related parameters (Brestic et al.2016) should be taken into account to build a "eco-physiological identity card" for different genotypes. Chlorophyll fluorescence represents a good tool to detect plant health status, the occurrence of damage rapidly and accurately within photosystem II (PSII), and to study heat tolerance *in vivo* [Zhou et al. 2016, Brestic et al.2016, Poudyal et al. 2019). The decline of maximum quantum efficiency of PSII (Fv/Fm), as well as the increase of non- photochemical quenching (NPQ), are two heat-affected fluorescence parameters (Zhou et al.2015) related to photoinhibition and photoprotection mechanisms in response to high temperatures [Rigano et al. 2016, Carvalho et al. 2016, Arena et al. 2008). Also, transient changes in fluorescence intensity (Kautsky phenomenon) have been demonstrated to be particularly suitable for the screening of physiological parameters in plants. The shape of fluorescence curves changes significantly when plants switch from a healthy status to stress, giving precious information on plant capability to overcome the stress (Tyystjarvi et al. 1999). Indeed, physiological screening techniques may complement phenotypic measurements and, therefore, increase the efficiency of the selection of tolerant genotypes (Sharma et al.2014). Currently, the majority of the experiments on tomato responses to heat stress have been carried out in controlled chambers and only few studies have been performed in the field (Giorio et al. 2018, Poudyal et al. 2019, Prasanth et al.2017). On one hand, with the field approach, it is possible to screen a high number of different genotypes. On the other hand, it is not easy to separate the effects of heat stress from other environmental variables, such as light or water depletion, in inducing the plant specific responses being examined (Sharma et al. 2014). For these reasons, field experiments should be complemented by laboratory studies in which the use of novel screening methods, such as chlorophyll fluorescence imaging, may provide further information for the characterization of tomato genotypes best suited to different environmental conditions. This study aimed to evaluate the responses to elevated temperatures in tomato genotypes in a combined field-to-laboratory approach. First, we investigated the photosynthetic efficiency by fluorescence emission measurements and leaf structural traits of different tomato varieties grown in Mediterranean agro-ecosystems during summer in the field. These trials allowed us to identify functional parameters correlating with final crop yields in different genotypes. After, a group of heat-tolerant and heat-sensitive tomato genotypes were selected based on crop yield and photosynthesis-related parameters. These selected genotypes were further characterized in the laboratory, analyzing

the Kautsky fluorescence induction curve after short-term heat treatments to obtain "a signature of photosynthesis". More specifically, the shapes of the curves changed when plants were subjected to stress. The presence and the timing of the appearance of specific fluorescence transients (for definitions, see (Stirbet et al. 2014) were calculated in order to assess the heat stress-induced changes in PSII functionality among cultivars. These laboratory analyses allowed us to validate this easy and very quick protocol as an alternative method for the selection of potentially heat-tolerant tomato genotypes. The combined field-to-laboratory approach provides additional information which could help plant biologists and breeders with characterizing responses to high temperatures in *Solanum lycopersicum*

2.2 Results

2.2.1 Greenhouse Trials: Correlations Between Physiological Parameters and Yields

Fifteen tomato genotypes, differing in geographical origin, were grown at supra-optimal temperatures in order to identify the high and low performers in field environmental conditions. The genotypes were selected on the basis of crop yield and eco-physiological indices. During anthesis, maximum air temperatures inside the greenhouse were in the range of 24–43 °C. Significant differences between each genotype *vs.* the control genotype JAG8810 were recorded for leaf functional traits, chlorophylls and carotenoids contents and final yields, as reported in Table 1. The hybrid JAG8810 had a good yield under high temperatures (from Monsanto, unpublished results). Therefore, this line may be considered as a positive control which is able to resist extreme high temperatures.

Table 1. Leaf traits (DW = dry weight of individual leaves, LA = leaf area, SLA = specific leaf area), total carotenoids, chlorophylls (Chl a and Chl b) and crop yield *per* plant (YP) evaluated on tomato genotypes grown under a plastic walk-in tunnel. Values are means ± standard deviation. ANOVA with Least Significant Difference (LSD) post-hoc test was used to compare each genotype *vs*. the control genotype JAG8810. The asterisks indicate statistically significant differences at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Genotypes.	DW (g)	LA (cm2)	SLA (cm2 g−1)	Carotenoids (mg 100 g−1)	Chl a (mg 100 g−1)	Chl b (mg 100 g−1)	YP (kg pt−1)
E7	0.04 ± 0.10	11.41 ± 1.71	278.17 ± 63.62 *	28.76 ± 0.07 ***	104.82 ± 0.13 ***	36.53 ± 0.29 ***	0.98 ± 0.00 **
E8	0.10 ± 0.04	14.37 ± 2.86	155.73 ± 36.29	41.30 ± 0.25 ***	169.30 ± 0.27 ***	76.05 ± 0.46 ***	0.77 ± 0.17 ***
E17	0.08 ± 0.02	17.76 ± 2.37	231.60 ± 45.53	44.67 ± 0.23 ***	177.92 ± 0.29 ***	75.18 ± 0.77 ***	1.26 ± 0.17 **
E36	0.14 ± 0.03 ***	20.09 ± 3.53 **	146.83 ± 15.50	39.94 ± 0.10 ***	159.32 ± 0.37 ***	67.97 ± 0.17 ***	0.94 ± 0.22 **
E37	0.15 ± 0.04 ***	13.12 ± 2.52	92.48 ± 17.82**	34.40 ± 0.03 ***	133.61 ± 0.64 ***	54.61 ± 0.64 ***	0.88 ± 0.17 ***
E42	0.10 ± 0.03	18.02 ± 3.11	188.57 ± 37.70	43.02 ± 0.54 ***	174.35 ± 1.62 ***	76.43 ± 0.83 ***	2.25 ± 1.04
E45	0.16 ± 0.02 ***	27.58 ± 4.78 ***	171.16 ± 26.94	36.61 ± 0.04 *	142.81 ± 0.36 ***	59.02 ± 0.35 **	2.24 ± 1.04
E53	0.07 ± 0.02	12.50 ± 2.19	210.79 ± 62.56	34.37 ± 0.06 ***	132.38 ± 0.10 ***	53.01 ± 0.26 ***	1.32 ± 0.28 **
E76	0.11 ± 0.01**	21.17 ± 2.25 **	210.79 ± 62.56	44.63 ± 0.12 ***	171.90 ± 0.06 ***	68.26 ± 0.37 ***	0.64 ± 0.01 ***

E107	0.07 ± 0.01	13.87 ± 2.95	194.36 ± 30.79	36.43 ± 0.04 **	134.20 ± 0.28 ***	48.49 ± 0.44 ***	0.42 ± 0.12 ***
IL12-4-SL	0.10 ± 0.02	21.00 ± 1.83 **	217.16 ± 48.16	39.71 ± 0.07 ***	149.58 ± 0.11 ***	56.68 ± 0.20 ***	2.77 ± 0.24 *
JAG8810	0.07 ± 0.01	13.46 ± 1.86	203.15 ± 55.39	42.96 ± 0.29	161.63 ± 1.51	60.49 ± 1.25	2.99 ± 0.5
LA2662	0.12 ± 0.01 **	21.59 ± 2.07 **	190.13 ± 24.57	36.98 ± 0.03 ***	143.90 ± 0.42 ***	58.78 ± 0.47 **	1.97 ± 0.66
LA3120	0.13 ± 0.03 *	20.57 ± 1.14 ***	166.53 ± 36.45	39.30 ± 0.13 ***	155.43 ± 0.23 ***	65.78 ± 0.60 ***	1.91 ± 0.98
M82	0.15 ± 0.03 ***	24.37 ± 4.93 ***	163.26 ± 12.14	32.87 ± 0.14 ***	121.82 ± 0.23 ***	44.88 ± 0.20 ***	3.25 ± 0.6 *

Leaf dry weight (DW) and leaf area (LA) of genotypes E36, E45, E76, LA2662, LA3120 and M82 were significantly higher compared to JAG8810. The genotype E7 was the only one with SLA values higher than JAG8810. The pigments content (chlorophyll a and b and carotenoids) of all genotypes was significantly lower than JAG8810, whereas only the M82 genotype showed a crop yield higher than JAG8810, considered as the control. Chlorophyll fluorescence parameters (Fv/Fm, ΦPSII and NPQ) are shown in Figure 1.

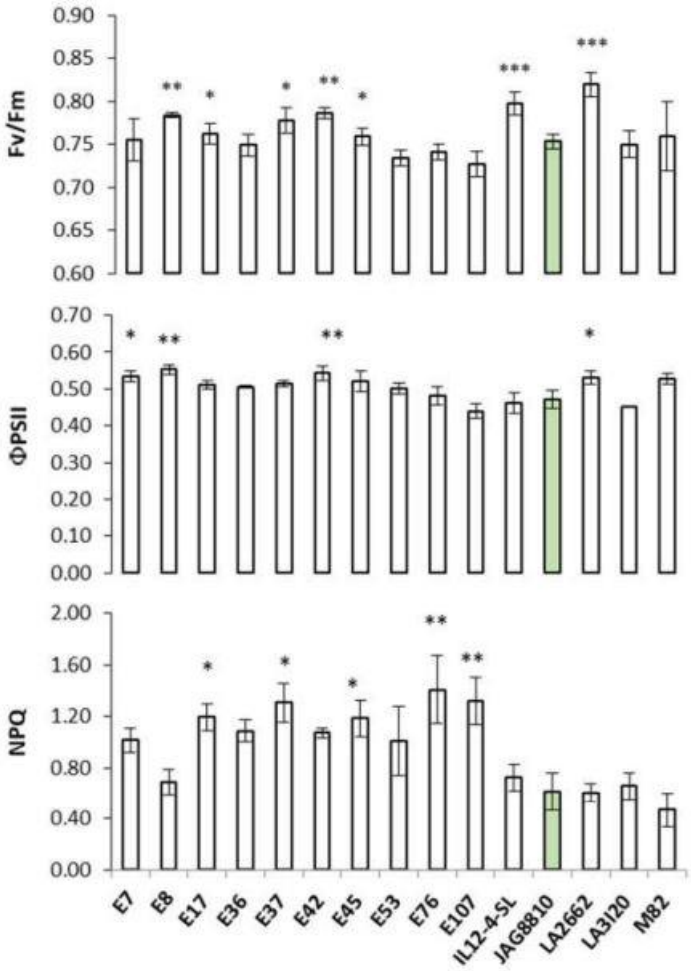

Figure 1. Maximum quantum yield of photosystem II (PSII) (Fv/Fm), effective quantum yield of PSII (ΦPSII), and non-photochemical quenching (NPQ) measured on different tomato genotypes grown under a plastic walk-in tunnel. Bars are means ± standard error (n = 5). ANOVA with LSD post-hoc test was used to compare each genotype *vs.* the control

genotype JAG8810 (green column). The asterisks indicate statistically significant differences at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Based on field screening, the photochemical parameters differed among genotypes; more specifically, seven genotypes, namely E8, E17, E32, E37, E42, IL12-4-SL and LA2662, showed higher Fv/Fm values compared to JAG8810. The genotypes E8, E42 and LA2662 also exhibited higher ΦPSII compared to the control genotype. Conversely, E76 and E107 genotypes showed the highest NPQ values compared to the control and the other genotypes. The correlations among the analyzed physiological and structural parameters are reported in Table 2.

Table 2. Pearson's correlations between physiological parameters, pigment content and crop yield production (* $p < 0.05$; ** $p < 0.01$). YP = yield *per* plant; Fv/Fm = maximum quantum yield of PSII; ΦPSII= effective quantum yield of PSII; NPQ = non-photochemical quenching; DW = leaf dry weight; SLA= specific leaf area; LA = leaf area; Chl a = chlorophyll a; Chl b = chlorophyll b; Car = carotenoids.

	YP	Fv/Fm	ΦPSII	NPQ	DW	SLA	LA	Chl a	Chl b	Car
YP	1	0.254	−0.010	−0.647 **	0.192	0.026	0.376	−0.017	0.036	−0.048
Fv/Fm		1	0.506	−0.266	0.304	−0.405	0.256	0.219	0.281	0.167
ΦPSII			1	−0.115	0.161	−0.124	0.002	−0.022	0.139	−0.147
NPQ				1	−0.068	−0.049	−0.183	0.106	0.035	0.154
DW					1	−0.650 **	0.744 **	0.155	0.221	0.11
SLA						1	−0.149	−0.226	−0.307	−0.167
LA							1	0.251	0.212	0.281
Chl a								1	0.970 **	0.983 **
Chl b									1	0.909 **
Car										1

Crop yield was negatively correlated with NPQ. Leaf area was positively correlated with leaf DW. The maximum quantum efficiency of PSII (Fv/Fm) differed among genotypes, although no significant correlation was found between crop yield and Fv/Fm. No correlations were found between crop yield and chlorophylls or carotenoids content. Based on crop yield (YP) and NPQ (higher YP and lower NPQ, Table 1 and Figure 1), the top five performers (genotypes IL12-4-SL, JAG8810, LA3120, LA2662 and M82) along with the low performer (genotype E107), were chosen for further analyses.

2.2.2 Chlorophyll a Fluorescence Measurements on Detached Leaves: Heat Shock Treatment at 35 °C and 45 °C

Chlorophyll fluorescence transients and derived parameters were measured as described in the Materials and Methods section on detached leaves from the selected genotypes and from an additional two tomato varieties, BG1620 and E41, which are supposedly heat-sensitive from previous studies carried out in our laboratory.No significant differences in the chlorophyll fluorescence parameters

after a 60-min heat shock at 35 °C were found between control and treated leaves (data not shown). Conversely, heat shock at 45 °C for 60 min resulted in significant damage to PSII compared to control (Figure 2).

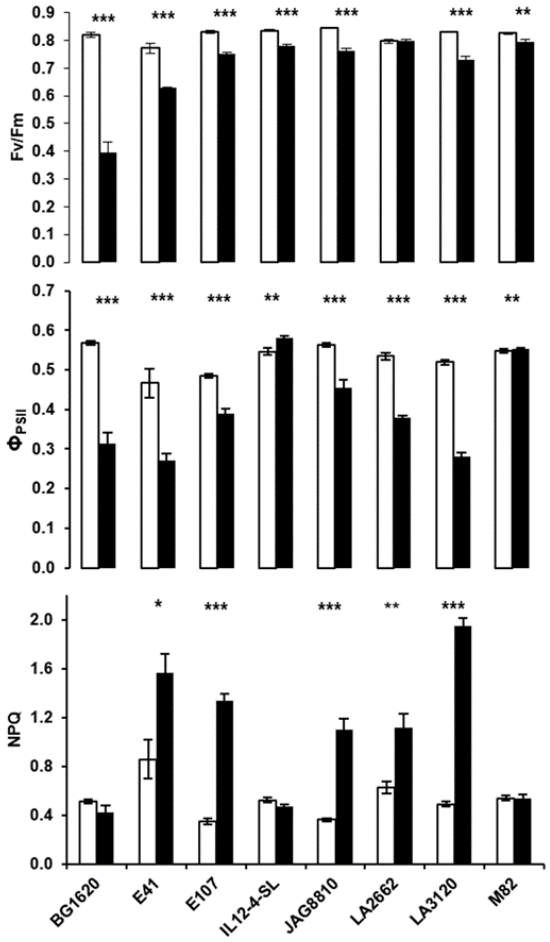

Figure 2. Maximum quantum yield of PSII (Fv/Fm), effective quantum yield of PSII (ΦPSII) and non- photochemical quenching (NPQ) in detached tomato leaves of different tomato genotypes following short term heat treatment for 60 min at 45 °C (solid bars), compared with the respective non-treated control (open bars). Bars are means ± standard error (n = 5). The asterisks indicate statistically significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) according to Student's t-test.

The short-term heat shock treatment led to a reduction in maximum PSII quantum efficiency (Fv/Fm) in almost all genotypes. Interestingly, the highest reductions in Fv/Fm were recorded in the two supposedly heat-sensitive genotypes, BG1620 (−52%) and E41 (−19%), that were more affected by heat stress compared to the genotype LA3120 (−12% Fv/Fm reduction). Contrastingly, the Fv/Fm ratio was little or not affected by the heat shock treatments in M82 (−4%) and LA2662 (0%) genotypes, which were among the top performers in the field trial. The quantum efficiency of PSII electron transport (ΦPSII) was also affected by the heat shock treatment. For most genotypes, a significant reduction in the ΦPSII was recorded and LA3120, BG1620, and E41 were found to be the most sensitive genotypes with a decrease of −46%, −45% and −42% compared to the control,

respectively. The M82 and IL12-4-SL genotypes were little or not affected by the heat shock treatment. As a consequence of the heat shock treatment, the NPQ values increased significantly in most genotypes compared to their respective controls, but not in BG1620, IL12-4-SL and M82.

2.2.3 Heat-Induced Changes in Shape of Kautsky Kinetics

The shape of slow Kautsky kinetics and the derived parameters clearly showed that the effects of heat treatment vary with genotype. As an example, in Figure 3, the slow Kautsky kinetics of the least heat sensitive (IL12-4-SL) and the most heat sensitive (E107) genotype are reported. Heat treatment leads to a reduction of the P peak in both genotypes compared to non-stressed controls. This decline appears more pronounced for the heat sensitive genotype E107. The comparison among genotypes showed that the M and S chlorophyll fluorescence signals were missing in heat-treated samples and resulted in the absence of P/S, P/M, and S/M ratios compared to respective controls (Table 3*)*.

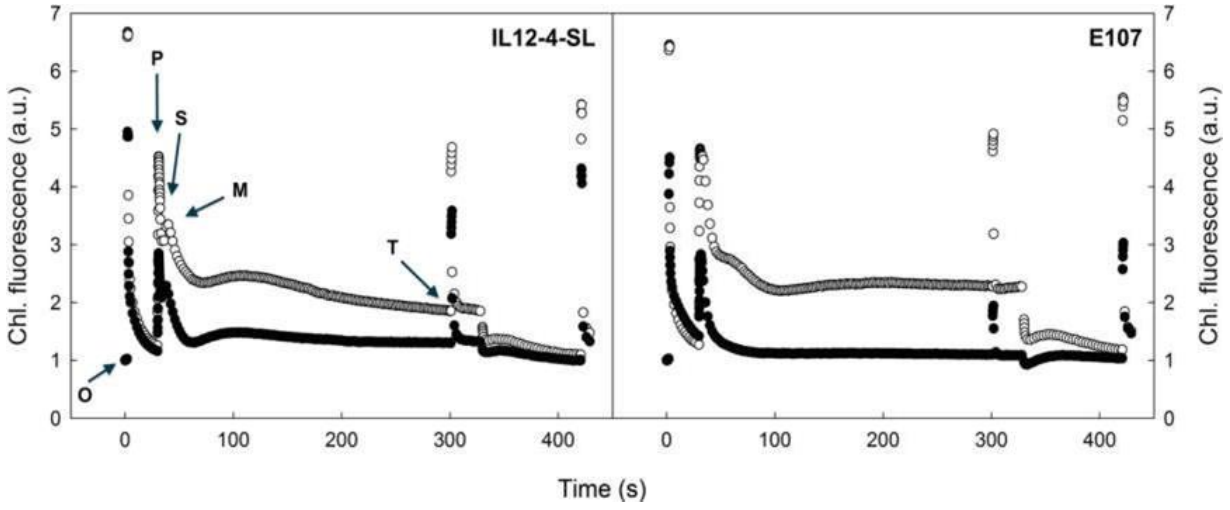

Figure 3. The effect of heat shock (60 min at 45 °C) on the shape of slow Kautsky kinetics in tomato plants. Non-treated controls are indicated by open symbols (□), heat shock-treated leaves by solid symbols (□). Less sensitive (IL12-4-SL) and substantially sensitive genotypes (E107) are presented. The curves are means of at least 4 replicates (leaves). Data are normalized to background chlorophyll fluorescence (F0). The chlorophyll fluorescence levels: O, fluorescence minimum; P, fluorescence peak; S, semi-steady state; M, fluorescence maximum; T, terminal steady state are indicated

Table 3. Heat-induced changes in chlorophyll fluorescence parameters characterizing the shape of slow Kautsky kinetics recorded for 8 tomato genotypes. Values are means of 5 replicates. Standard deviation (not shown here) was under 4% of means. Asterisks indicate the statistical significance of the difference in heat treated leaves compared to their respective non-treated control (* 0 to 50%; ** 50 to 100%; *** over 100%). O (origin) = minimum fluorescence level (also termed F0); P = peak fluorescence level reached after 1–2 s of actinic light exposure; S = semi-steady state of fluorescence emission; M = maximum of fluorescence; T = terminal steady state chlorophyll fluorescence of the slow Kautsky kinetics; Fp = fluorescence peak; Fs= fluorescence steady state; Rfd = relative fluorescence decline (vitality index); *tP* = time at which P fluorescence peak is reached; *tS* = time at which S fluorescence level is reached; *tM* = time at which M fluorescence level is reached; *tT* = time at which T fluorescence level is reached, Dip = decrease

Control								
	BG1620	E41	E107	IL12-4-SL	JAG8810	LA2662	LA3120	M82
O/P	0.229	0.242	0.212	0.22	0.201	0.239	0.194	0.207
P/S	1.81	1.167	1.065	1.517	1.405	1.242	1.193	1.673
P/M	1.669	-	1.059	1.389	1.318	-	1.163	1.486
S/M	0.922	-	0.994	0.916	0.938	-	0.975	0.888
P/T (steady state)	2.665	3.101	2.101	2.482	2.428	2.887	2.694	2.801
M/T (steady state)	1.597	-	1.985	1.787	1.842	-	2.316	1.886
Rfd = (Fp−Fs) /Fs	1.665	2.101	1.101	1.482	1.428	1.887	1.694	1.801
tP	1.12	1.12	0.96	1.2	1.04	1.04	1.28	1.04
tS	6.08	3.36	4.08	4.08	4.08	4.08	−29.940	6.08
tM	10.08	-	2.72	10.08	8.08	-	6.08	12.08
tT	270.06	270.06	270.06	270.06	270.06	270.06	270.06	270.06
Dip at	26.08	-	52.08	42.08	-	48.08	86.08	38.08
Heat Treated (60 min at 45 °C)								
	BG1620	E41	E107	IL12-4-SL	JAG8810	LA2662	LA3120	M82
O/P	0.713 ***	0.483 **	0.341 **	0.344 **	0.366 **	0.252 *	0.379 **	0.316 **
P/S	-	-	-	1.280 *	0.936 *	-	-	0.855 *
P/M	-	-	-	1.166 *	0.929 *	-	-	0.853 *
S/M	-	-	-	0.911 *	0.993 *	-	-	0.997 *
P/T (steady state)	1.658 *	2.729 *	2.680 *	2.250 *	2.407 *	2.779 *	2.881 *	2.302 *
M/T (steady state)	-	-	-	1.929 *	2.591 *	-	-	2.700 *
Rfd = (Fp-Fs)/Fs	0.658 **	1.729 *	1.680 **	1.250 *	1.407 *	1.779 *	1.881 *	1.302 *
tP	1.280 *	1.280 *	1.760 **	1.120 *	1.280 *	1.04	1.28	1.04
tS	-	-	-	4.08	2.240 ***	-	-	3.36
tM	-	-	-	8.08	2.400 ***	-	-	4.08
tT	255.060 *	270.060 *	245.060 *	260.060 *	245.060 *	260.060 *	270.060 *	250.06 *
Dip at	-	-	-	34.080 *	40.08	-	-	40.08 *

It may be generalized that for all tomato genotypes the shape of the slow Kautsky kinetics was affected mainly during the early phase (i.e., within the first 60 sec after the actinic light was switched on). The most pronounced change was found in the O/P ratio in BG1620 which showed a relative change of about 200%, while other genotypes showed a much lower variation (LA2662: 5.4%). Apart from the shape of the slow Kautsky kinetics and ratio values, the time at which the P, S, and M points were reached differed within treatments. Heat treatment led to an increase in tP in four cultivars. However, tP showed no change in three genotypes, and a decrease in the IL12-4-SL genotype.

2.3 Discussion

To date, there is a lack of knowledge about how differently sensitive/tolerant tomato genotypes would respond to heat events. In heat-sensitive tomato genotypes, high temperatures are responsible for the decrease in photosynthesis and overall crop yield (Zhou et al. 2016). In this paper, a set of eco-physiological parameters have been proposed to screen the most promising tomato genotypes to be cultivated under elevated temperatures. A combined field/laboratory experimental approach was performed. Firstly, a field trial was carried out under a plastic walk-in tunnel to assess the field

performances of tomato genotypes cultivated at high temperatures (up to 43 °C) in terms of crop yield and physiological traits. In the second part of this study, the heat-resistant and heat-sensitive genotypes were tested in the laboratory to analyze their responses to short-term heat shock and to investigate the photochemical behavior related to their different field performance. We suppose that at the temperatures considered in this study, the photosynthetic apparatus was not damaged but rather regulated in the different genotypes, contributing to the degree of their sensitivity or resistance to heat. Our field studies indicated that tomato genotypes with higher yields also had lower NPQ values. Non-photochemical quenching is considered a key mechanism in photoprotection against light and temperature stress in higher plants [Arena et al.2008, Vitale et al. 2008). High NPQ is often associated with conformational changes within PSII, which transiently depresses CO_2 fixation (Kromdijk et al. 2016). In our trials, the top performers in terms of yield (genotypes IL12-4-SL, M82, LA2662, LA3120 and JAG8810) also showed the lowest NPQ values. Our data suggest that under experimental field conditions, these tomato genotypes are more efficient in transferring the light excitation energy to CO_2 fixation, thus producing a higher photosynthetic carbon gain. Contrastingly, the highest NPQ values measured in the low performing genotypes (E37, E76 and E107) correspond to more intense thermal energy dissipation, leading to lower CO_2 assimilation and crop yield. In heat-tolerant tomato genotypes, the NPQ protection is likely activated less promptly than in the heat-sensitive genotypes. Conversely to NPQ, the Fv/Fm ratio and ΦPSII did not show any correlation with crop productivity, suggesting that the higher Fv/Fm values in some genotypes may indicate a better photosynthetic performance that is not always related to a higher crop yield. These data are in contrast with findings of many authors, who demonstrated that the Fv/Fm ratio is one of the fluorescence parameters most affected by high temperatures and used it as an index for screening tomato genotypes under heat stress (Poudyal et al. 2019, Zhou et al. 2015, Sharma et al. 2015). Indeed, our data indicated that the high temperatures experienced by plants in the field did not compromise the quantum yield of PSII in any genotype. Based on the results of Hückstädt et al, it may be hypothesized that the detrimental effect of the high diurnal temperatures on photosystems has been compensated for by the optimal temperatures (Zhou et al. 2016) reached in the greenhouse during the night (see Figure 4). Elevated temperatures could also determine a loss in the amount of photosynthetic pigments. Therefore, the capacity of some genotypes to maintain higher pigments content under heat stress, as well as to adjust some leaf functional traits (i.e., SLA, LA, RWC), are considered key heat tolerance- linked traits (Zhou et al. 2016, Nankishore et al. 2016, Wang et al. 2016, Ahammed et al. 2018). For example, a higher SLA can be essential to obtain a higher potential evaporative demand and a more extensive foliar display to capture more light (Wang et al. 2016). However, in this work, no significant correlation was found between final crop yield and pigments content or final crop yield and SLA,

indicating that these traits are not automatically linked to crop productivity. Therefore, these traits were not considered for the selection of the heat-tolerant and heat-sensitive genotypes in this study. On the basis of field trials, the tomato genotypes IL12-4-SL, JAG 8810, LA3120, LA2662 and M82 were selected as the top performers in terms of yield and low NPQ values, whereas the genotype E107 was selected as a low performer considering the low photochemical efficiency and crop yield. The heat sensitive BG1620 and E41 genotypes were added to the list of genotypes to be further studied in the laboratory. For these tomato genotypes, the chlorophyll fluorescence transient analysis (slow Kautsky kinetics) was performed in response to heat treatments to separate the effects of the high temperature from other environmental constraints in the field. In a previous study on tomato, plants exposed to 42 °C for 24 h showed a decline of net photosynthesis, maximum PSII photochemical efficiency and electron transport rate (Pan et al. 2018). Consistent with these findings, our results further demonstrated that a short-term (60 min) heat shock at 45 °C was sufficient to cause significant effects on the photochemistry in detached tomato leaves. Interestingly, the Fv/Fm ratio was found to be most affected in the heat-sensitive genotype BG1620 and in the low performer E107 genotype. An Fv/Fm reduction of only 8% was registered in the genotype LA2662, which was selected as heat-tolerant in the field experiment. These data are also in agreement with Sharma et al. who measured a reduction of Fv/Fm in detached wheat leaves at 45°C and Camejo et al. who reported an Fv/Fm decrease in a heat-susceptible tomato cultivar and no changes in heat-tolerant cultivar. We supposed that the Fv/Fm and PSII decreases in the sensitive (BG1620 and E41) genotypes could be due to heat-induced structural modifications in PSII, particularly D1 protein oxidative degradation. It has been demonstrated that heat stress may cause cleavage of the reaction center- binding protein D1 and induce dissociation of a manganese-stabilizing 33 kDa protein from the PSII reaction center complex (Yamane et al. 1998). Such oxidative damages have a strong positive relationship with the accumulated levels of reactive oxygen species (ROS) and lipid peroxidation under heat stress (Yamashita et al.2008). However, it cannot be excluded that the significant reduction of Fv/Fm observed in some genotypes may also be associated with photoprotection mechanisms, as indicated by NPQ values that peaked in correspondence to the reduction in Fv/Fm. Simultaneously, protective mechanisms are activated to protect the D1 protein, such as the expression of heat shock proteins (HSPs) like HSP21 that directly binds D1 to shield it against damage (Wang et al.2018). However, this does not seem to be the case for the genotype BG1620, which was the most affected by the severe decline of photochemical and non- photochemical processes. The decrease in Fv/Fm values in detached tomato leaves that were heat-treated at 40 °C was also reported by Willits and Peet (Willits et al. 2001). In our work, a heat shock treatment at 35 °C on detached leaves was similarly tested with no significant effects on the photochemical efficiency of PSII (data not shown), supporting the idea

that such responses depend on the severity of the heat stress applied (Sharma et al.2014). The analysis of the shape of the slow Kautsky kinetics and the parameters calculated from its significant time points (chlorophyll fluorescence levels O, P, S, M, T) revealed that heat shock treatment led to significant differences compared with the control. Polyphasic changes in chlorophyll fluorescence signal in the PSMT part of the Kautsky kinetics represent the combined effect of photochemical and non-photochemical processes taking place in the chloroplast (Riznichenko et al. 1996). As the main changes happened during the first 60 s of actinic light exposure, they might be attributed to the interperiod of balancing the rate of primary photochemical processes in PSII to the rate of CO_2 assimilation. Since the S and M chlorophyll fluorescence levels were generally missing in heat-treated tomato leaves, the processes responsible for S and M interstates (i.e., the processes regulating the Calvin– Benson cycle of CO_2 fixation, such as limitations in NADP+, phosphate pool equilibration, and transmembrane ΔpH formation (Stirbet et al. 2014)) were overwhelmed by a strong non-photochemical quenching that was activated by the heat shock treatment. This may be a consequence of the heat-induced thermal dissipation of absorbed light energy, such as state 1 to state 2 transitions causing preferential excitation of PSI and structural changes in thylakoid membranes, as reported by Marutani et al. The activation of these protective mechanisms may, however, lead to PSI damage in high temperature treated plants due to over-reduction of the acceptor side of PSI (Brestic et al. 2016). The changes observed during the transition from P to S phase of the Kautsky kinetics indicate the actual proportion between the mechanisms involved in photochemical and non-photochemical quenching (Papageorgiou et al. 2014). Since the parameters derived from the slow Kautsky kinetics responded to heat treatment, it might be concluded that they have a high potential in the evaluation of heat effects on the chloroplast function of tomato, as shown for light stress and leaf age effects by Nesterenko et al. Many studies support the idea that a sustained increase in leaf photosynthesis can also lead to an increase in total biomass production (Brestic et al. 2018). Overall, in this work, several useful photosynthetic parameters were identified, which could be essential to detect and describe high temperature-tolerant tomato cultivars. These parameters could be used as an effective tool for the prompt identification of tomato genotypes tolerant to high temperatures.

2.4 Materials and methods

2.4.1 Plant Material and Growth Conditions

Fifteen genotypes have been tested in the field. Of these genotypes, eleven genotypes (marked as E## in Table 4) were selected based on their different productivity (demonstrated in a previous experiment conducted in the Campania region in the year 2016 (Ruggieri et al. 2019)). The genotypes JAG8810 (Monsanto F1 hybrid), LA2662 (Saladette) and LA3120 (Malintka) were reported to have high fruit

productivity under high temperatures (JAG8810, from Monsanto, unpublished results; LA2662 and LA3120, Tomato Genetic Resources Center). The JAG8810 hybrid may be considered as a positive control which is able to resist extreme high temperatures. The M82 and the IL12-4-SL genotypes, previously selected and characterized in a recent paper (Rigano et al. 2018), were added to the list of genotypes to be tested because their physiological response to elevated temperatures was unknown. One additional tomato variety, BG1620 (kindly provided by Prof. G. Pevicharova, MVRCI Bulgary), was also added to the set of genotypes to be analyzed. The genotypes BG1620 and E41 are supposedly heat-sensitive based on previous analyses carried out in our laboratories (unpublished data).

Table 4. Tomato genotypes analyzed in this study.

No.	Genotype	Origin	Common Name
1	E7	Italy	Corbarino PC04
2	E8	Italy	Corbarino PC05
3	E17	Italy	Pantano Romanesco
4	E36	Italy	Riccia San Vito
5	E37	Italy	Siccagno
6	E41	Italy	Parmitanella
7	E42	Italy	PI15250
8	E45	Italy	SM246
9	E53	South America	Latin American cultivar (Honduras)
10	E76	URSS	Black Plum
11	E107	Europe	E-L-19, Spain
12	JAG8810	-	Monsanto F1 hybrid
13	M82	California	M82
14	IL12-4-SL	Italy	IL12-4-SL
15	LA2662	-	Saladette
16	LA3120	-	Malintka
17	BG1620	Bulgary	-

Tomato plants were grown in the year 2017 in Battipaglia (Salerno, Italy) (40°23′03 N, 17°21′17 E, 72 m a.s.l.) in a Mediterranean or Csa climate according to the Köppen classification scheme (Peel et al. 2007), under walk-in thermal polyethylene tunnels. During the whole cultural cycle, climatic data were recorded using the weather station VantagePro2 from Davis Instrument Corp. The maximum and minimum temperatures during anthesis are reported in Figure 4. Spatial variation in temperature within the walk-in tunnels was found to be minimal.

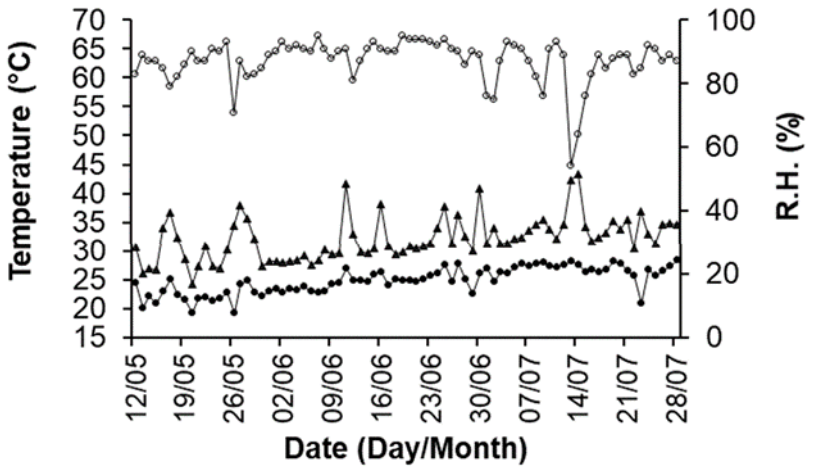

Figure 4. Relative humidity (R.H.; open symbols, right axis) and maximum and minimum air temperatures (solid symbols, left axis) during May–July 2017 inside a greenhouse in the experimental field at Battipaglia (Salerno, Campania region, Italy).

The seeds of all genotypes were first rinsed and soaked in distilled water and then kept for 4 days in 8.5 cm diameter Petri dishes over 3 layers of filter paper saturated with distilled water. After germination, the seeds were sown in seed trays kept in the greenhouse. Seedlings were transplanted in April under plastic walk-in tunnels. Plants were grown following the standard cultural practices of the area. Insecticides and fungicides were applied to the plants according to general local practices and recommendations. Urea phosphate fertilizer (40 kg ha^{-1}) was applied to the soil before transplanting. Tillage treatments included plowing that was followed by one/two milling. Weeding and ridging were also carried out. Through fertirrigation, recommended levels of N (190 kg ha^{-1}) and K (20 kg ha^{-1}) were applied. During cultivation, plants were irrigated as required. All genotypes were grown according to a completely randomized experimental design with three replicates and 10 plants *per* replicate. Total fruit number and fresh weight were measured at the end of growth season to evaluate the crop yield *per* plant (YP). The crop yield was measured at the red fruit ripe stage.

2.4.2 Functional Leaf Trait Analysis

The measurements of leaf area (LA), specific leaf area (SLA) and leaf dry weight (DW) were performed on the fourth leaf from the apex in each plant. Five leaves for each genotype were sampled from 5 different plants. LA was measured using ImageJ 1.45 software for image analysis (Schneider et al. 2012). The leaves were then dried at 70 °C and their DW was measured after 48 h. SLA was calculated as the ratio of leaf area to leaf dry weight and expressed as cm2 g^{-1} DW according to Cornelissen et al.

2.4.3 Chlorophyll Fluorescence Emission Measurements in the Field

Chlorophyll fluorescence parameters were measured on fully expanded leaves (the fourth leaf from the apex) using a portable FluorPen FP100 Max fluorometer, equipped with a Photosynthetically Active Radiation (PAR) sensor (Photon Systems Instruments, Drásov, Czech Republic) following the procedure reported by Sorrentino et al. (Sorrentino et al. 2018). Five replicate measurements for each genotype were taken as follows. The ground state fluorescence (F0) was induced by an internal Light Emitting Diode (LED) blue (1–2 μmol photons m^{-2} s^{-1}) on 30-min dark-adapted leaves of plants moved into a dark room. The maximum fluorescence level in the dark-adapted state (Fm), was triggered by a 1 s saturating light pulse of 3000 μmol photons m^{-2} s^{-1}, and the maximum quantum efficiency of PSII (Fv/Fm) was calculated according to Equation (1).The fluorescence readings in the light were taken using an open leaf-clip, allowing for measurements of the steady state fluorescence level (Fs) at an ambient light Photosynthetic Photon Flux Density (PPFD) of 150–200 μmol m^{-2} s^{-1} in the PAR spectrum and of the maximum fluorescence level in light-adapted leaves (F'm) measured after a saturating light pulse. The quantum yield of PSII electron transport (ΦPSII) was calculated according to Equation (2) (Genty et al. 1989). Non-photochemical quenching (NPQ) was calculated using Equation (3) (Bilger et al. 1990).

$$Fv/Fm = (Fm - F0)/Fm \quad (1)$$

$$\Phi PSII = (F'm - Fs)/F'm \quad (2)$$

$$NPQ = (Fm - F'm)/F'm \quad (3)$$

2.4.4 Determination of Total Chlorophylls and Carotenoids Content

Following the chlorophyll fluorescence field measurements, the same leaves were excised, stored in a cool box and transferred to the laboratory for the determination of photosynthetic pigments content (chlorophyll a, chlorophyll b and carotenoids) according to the method described by Rigano et al. 2016. One gram of leaf sample was extracted with 16 mL of acetone/hexane (40/60, *v*/*v*) with a T-25 Ultra-Turrax Homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany). The homogenate was then centrifuged at 5000 rpm for 5 min at 4 °C and supernatants were collected and stored at −20 °C prior to spectrophotometric analysis. Pigment contents were calculated in mg on 100 g of leaf fresh weight. Three separate biological replicates for each sample and three technical assays for each biological repetition were measured.

2.4.5 Chlorophyll a Fluorescence—Heat Treatments and Laboratory Measurements

Chlorophyll fluorescence transients and numeric parameters were measured with a FluorCam (Photon Systems Instruments, Brno, Czech Republic) on detached leaves from genotypes selected from the

in-field analyses. Two additional tomato varieties, BG1620 and E41 (supposedly heat- sensitive based on previous analyses carried out in our laboratories (unpublished data), were also added to the set of genotypes to be studied in this research. Whole compound leaves from field grown tomato plants were sampled in the morning, between 2 and 3 h after dawn (8:00 to 9:00 a.m.). Fully expanded leaves (the fourth leaf from the apex) were excised at the base of the petiole from each plant using a sharp blade and the cut base was immediately immersed in distilled water in a 50 mL test tube in order to prevent dehydration. The sampled leaves were then temporarily stored in a dark cool box and transferred to the laboratory for the short-term high temperature treatments and chlorophyll fluorescence experiments. In the laboratory, single leaflets were excised from the tomato compound leaves and placed in 9.0 cm diameter Petri dishes over water saturated filter paper. Two groups of leaf samples were selected for each genotype: one group was kept at the laboratory room temperature (25–26°C) in the dark and assumed as control treatment, while another group was placed in a thermostatic cabinet set, either at 35°C or at 45°C, for 1 h in the dark and was considered the heat treatment. At the end of the heat treatment, leaf samples were adapted at room temperature for 20 min in the dark prior to performing the fluorescence measurements. The same procedure was replicated on different leaves and repeated for each tomato genotype. The whole experiment was carried out subsequently on leaf samples treated either at 35 °C (lower heat stress level) or at 45 °C (higher heat stress level), plus the respective controls. The temperature conditions in this experiment were chosen to represent the optimal temperature range for tomato (within 25 – 30 °C (Zhou et al. 2016)) and the maximum air temperature of 33–43 °C encountered by tomato genotypes in the walk-in tunnels during the experimental period. Chlorophyll fluorescence transients (slow Kautsky kinetics supplemented with quenching analysis) were measured with a Handy Fluor Cam FC-1000H imaging fluorimeter (Photon Systems Instruments, Drásov, Czech Republic) controlled by the FluorCam7 software (Photon Systems Instruments, Drásov, Czech Republic).The experimental protocol started with the measurement of the ground state (minimum) fluorescence level (F0, O) when the samples were exposed to low intensity measuring light flashes, followed by a saturating pulse of light (960 ms, 2400 μmol m−2 s−1) to induce maximum chlorophyll fluorescence (Fm, P). After 27 s of dark adaptation, the samples were exposed to actinic light (200 μmol m−2 s−1) for 5 min until steady state chlorophyll fluorescence (Fs) was reached. At this point, another saturating pulse of light induced the maximum chlorophyll fluorescence of the light-adapted sample (F’m). The maximum quantum efficiency of PSII (Fv/Fm), the quantum efficiency of PSII electron transport (ΦPSII) and non-photochemical quenching (NPQ) were calculated using the FluorCam7 software, according to Equations (1) – (3) reported above, respectively.

2.4.6 Analysis of Kautsky Kinetics Shape in Response to Heat Treatment

Slow Kautsky kinetics is routinely used to evaluate the sensitivity of plants to a wide variety of stressors (Baker et al. 2004, Rohacek et al. 2008). In this study, the analysis of kinetic fluorescence shape, i.e., the presence and the time of appearance of specific fluorescent transients (O, P, S, M and T), was utilized to assess heat stress-induced changes in PSII functionality in tomato. For individual Kautsky kinetics recorded after a heat treatment (see above), chlorophyll fluorescence levels O, P, S, M and T were identified, as well as the times at which they were reached. Effects of experimental temperature on the O-, P-, S-, M-, and T-derived parameters were then evaluated for the individual genotypes and the genotype- dependent responses of the parameters were characterized.

2.4.7 Statistical Analysis

Statistical analysis was performed on all measured traits using SPSS 23 Software (IBM SPSS Statistics, USA). Analysis of variance (ANOVA) was used to check for significant differences between each genotype vs. the control genotype (JAG8810) and where significant differences were found, the least significant difference (LSD) at the 0.05, 0.01 or 0.001 level of probability was calculated and used to compare the mean values. Student's t-test was performed to check for differences between control and heat-treated samples in the case of detached leaf experiments. Pearson's correlation coefficient was used to test associations between tomato yield and other variables.

2.5 Conclusions

Due to ongoing climate change, the screening and identification of the most promising tomato cultivars able to maintain elevated productivity under heat stress becomes a priority for farmers and producers to avoid significant losses of crop yield. In our experiments, heat tolerant and heat sensitive tomato cultivars were identified and characterized using a correlative approach combining different field and laboratory methods based on functional leaf traits, crop yield and photochemical indexes. The three main outcomes emerging from this work include the confirmation that some parameters linked to chlorophyll fluorescence emission can be used to phenotype heat tolerance in tomato, both in the field and in the laboratory. Secondly, we demonstrated that the detached leaf method can be used as an easy, quick and valid alternative for the selection and characterization of potentially heat-tolerant tomato genotypes. The advantage of a laboratory approach that implements field measurements is that it is possible to separate the effects caused by heat treatments from the other related environmental factors such as high light, low relative humidity and limited water supply. Finally, we identified five tomato genotypes (JAG8810, LA3120, LA2662, IL12-4-SL and M82) as

promising genotypes that are potentially tolerant to elevated temperatures. These genotypes also represent a valuable resource to be used in future works aiming to assess the underlying physiological mechanisms for variability in photosynthetic responses among different cultivars.

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Eco-Physiological Screening of Different Tomato Genotypes in Response to High Temperatures: A Combined Field-to-Laboratory Approach

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Abstract: High temperatures represent a limitation for growth and development of many crop species. Several studies have demonstrated that the yield reduction of tomato under high temperatures and drought is mainly due to a photosynthetic decline. In this paper, a set of 15 tomato genotypes were screened for tolerance to elevated temperatures by cultivating plants under plastic walk-in tunnels. To assess the potential tolerance of tomato genotypes to high temperatures, measurements of chlorophyll fluorescence, pigments content and leaf functional traits have been carried out together with the evaluation of the final yields. Based on the greenhouse trials, a group of eight putative heat-sensitive and heat-tolerant tomato genotypes was selected for laboratory experiments aimed at investigating the effects of short-term high temperatures treatments in controlled conditions. The chlorophyll fluorescence induction kinetics were recorded on detached leaves treated for 60 min at 35 °C or at 45 °C. The last treatment significantly affected the photosystem II (PSII) photochemical efficiency (namely maximum PSII quantum efficiency, F_v/F_m, and quantum yield of PSII electron transport, Φ_{PSII}) and the non-photochemical quenching (NPQ) in the majority of genotypes. The short-term heat shock treatments also led to significant differences in the shape of the slow Kautsky kinetics and its significant time points (chlorophyll fluorescence levels minimum O, peak P, semi-steady state S, maximum M, terminal steady state T) compared to the control, demonstrating heat shock-induced changes in PSII functionality. Genotypes potentially tolerant to high temperatures have been identified. Our findings support the idea that chlorophyll fluorescence parameters (i.e., Φ_{PSII} or NPQ) and some leaf functional traits may be used as a tool to detect high temperatures-tolerant tomato cultivars.

Keywords: heat stress; tomato genotypes; photosynthesis; crop yield; chlorophyll a fluorescence; *Solanum lycopersicum*

1. Introduction

Increasing atmospheric temperatures, which are expected to rise by 2–4.8 °C in the next few decades, can compromise crop productivity in numerous regions worldwide [1–4]. Indeed, elevated temperatures can induce a series of physiological responses with consequent decreases of crops

Chapter 3. Physiological responses to combined water and heat stress that facilitate adaptation in tomato genotypes.

Manuscript in preparation

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ABSTRACT

Climate change is increasing the frequency of high temperature shocks and water shortage, pointing to the need for developing novel varieties with enhanced tolerance to co-occurring stresses. Understanding the mechanisms engaged by crops to withstand combined abiotic stresses is therefore pivotal. Two tomato genotypes, a heat-tolerant accession of *S. lycopersicum* (LA3120) and a novel genotype (E42), selected within a collection of landraces from the Campania region (Italy) as a stable yielding genotype under high temperatures, were exposed to drought (heat and water shortage). Combined abiotic stresses had a severe impact on plant growth parameters and on the reproductive phase of development, with a predominant role played by heat stress compared to water deficit. Activation of antioxidant defence mechanisms seemed to be critical for both genotypes to counteract combined limited water availability and heat stress. Growth parameters and leaf gas exchange measurements revealed that the two genotypes used different physiological strategies to overcome individual and combined stresses, with E42 having a more efficient capability to utilise the limiting water resources. A Genotyping-by-Sequencing approach allowed us to explore the genetic variability of the tested genotypes in order to identify the candidate genes regulating abiotic stress tolerance in the selected genotypes. Altogether, results obtained in this paper further demonstrate how new tomato genetic resources can be a valuable source of traits for adaptation to water stress and high temperatures and should be used in future breeding programs to improve the tolerance to abiotic stress in commercial varieties.

3.1 Introduction

Plants are continuously subjected to many abiotic and biotic stresses, from seed germination through to the whole life cycle (Deryng et al., 2014), which are now intensified by climate change. In particular, water and heat stress are two of the most critical abiotic stresses limiting crop growth and productivity worldwide; especially in arid or semi-arid areas (Vitale et al., 2009, Deryng et al., 2014, Hussain et al., 2019; Arena et al., 2020; Madhava et al., 2006). In the past few year drought, *i.e.* the combination of high temperature and water scarcity, caused global losses in crop production for ~ $ 30 billion (Gupta et al., 2020). Improving crop production under water-limitation (Farooq et al., 2009) and elevated temperatures (Wahid et al., 2007) is therefore a primary goal in agriculture (Rigano et al., 2016). There are many other factors, including high temperature, high intensity of light, and dry wind, which can increase evaporation of water from soil and lead to drought. These factors can also

increase water loss from plants and consequently enhance plant exposure to water stress (Shao et al., 2008, Trenberth et al., 2014). High temperatures accelerate rapid water loss from plant and soil surface, which can cause water stress and consequent crop yield reductions (Nankishore and Farrel, 2016; Hussain et al., 2019). Furthermore, it has been shown that with temperatures above 35 °C both the formation and viability of pollen are highly compromised, causing an additional reduction in final yield (Olivieri et al., 2020). The simultaneous occurrence of high temperature and soil water depletion may result in a range of morphological, anatomical, physiological and biochemical adjustments in plants in order to counteract these constraints (Chaves et al., 2009). Plant responses to these abiotic stresses and the extent of damages vary depending on species, growth stage and the severity of the stress applied (Fahad et al., 2017).

One of the physiological processes most sensitive to water and heat stress in plants is photosynthesis. Indeed, under prolonged drought, stomatal conductance limits CO_2 uptake and heat stress might affect biochemical reactions of the photosynthetic machinery (Zhou et al., 2017) limiting the plant carbon gain (Hussain et al., 2019). It is demonstrated that drought causes photoinhibition of photosystem II (PSII) (Arena et al., 2008a; Vitale et al., 2008) and lead to a reduction in both photosynthetic electron chain functionality and Rubisco activity (Zhou et al., 2018a), but also may trigger mechanisms of damage repair (Murata et al., 2007; Arena et al., 2008b). A further important consequence of high temperature and water deprivation is the alteration of the oxidative metabolism, which causes membrane instability. In particular, the accumulation of reactive oxygen species (ROS), such as H_2O_2, may induce lipid peroxidation of cellular membranes, breakdown of photosynthetic pigments and decreased enzyme activity (Zhou et al., 2019; Demirel et al., 2020). In order to counteract the production of ROS, antioxidant defence mechanisms are normally activated which comprise the action of enzymatic antioxidants including ascorbate peroxidase (APX), peroxidase (POD) and catalase (CAT) and non-enzymatic antioxidants such as ascorbic acid (AsA) and glutathione (GSH) (Zhou et al., 2020a).

Tomato (*Solanum lycopersicum* L.) is one of the most cultivated vegetable crops worldwide. It has an optimal growth diurnal temperature range of 25-30 °C and is known to be sensitive to both water shortage and heat, although its sensitivity varies among genotypes (Arena et al., 2020; Francesca et al., 2020; Rigano et al., 2016; Zhou et al., 2016; Zhou et al., 2020a; Zhou et al., 2018b; Zhou et al., 2018c). Climate change is exacerbating and/or increasing the frequency of high temperature shocks and water shortage in the Mediterranean basin, pointing to the need for developing varieties with enhanced tolerance to naturally co-occurring stresses (Zhou et al., 2020a, Zhou et al., 2020b). Indeed, despite a comprehensive literature on plant response to single stresses, the response to multiple stresses is rarely addressed (Zhou et al., 2020b). In this regard, novel tolerant genotypes should be

identified to improve the traditional varieties and also to investigate physiological mechanisms controlling tolerance to combined abiotic stresses. In order to characterize these genotypes, rigorous phenotyping under controlled conditions combined with whole-genome genotyping must be carried out. Currently, genotyping can be realized using the Next Generation Sequencing technologies. These technologies, including Genotyping-by-sequencing (GBS), are powerful tools that offer a solution for identifying DNA polymorphisms greatly reducing the sequencing costs with results that can be easily applied in plant breeding. If the whole genome sequence of reference organisms is already available, an approach like RAD-seq (Restriction Site-Associated DNA sequencing) is highly cost-effective and can sample about 200,000 SNPs (Single Nucleotide Polymorphisms) with the same coverage depth and at nearly 35-fold lower costs compared to the Whole Genome Sequencing of the same number of individuals (Scheben et al.,2017).

The aims of this study were to phenotype two tomato genotypes subjected to individual and combined heat stress and limited water availability and analyse the different strategies activated in different genotypes in response to stress. These landraces were also genotyped in order to exploit their genetic variability. Herein, the mutations (SNPs and InDels) identified were annotated and a prediction of their effect on protein functions and structures was carried out. The phenotypic and genotypic data recorded in the present work were used for identifying candidate genes associated with thermotolerance. Results here reported can lead to further understand the response to combined abiotic stress, a quite common condition in agricultural systems and could be used for the selection and breeding of tolerant tomato genotypes able to maintain stable crop production in the most critical production areas.

3.2 Material and Methods

3.2.1. Plant material and experimental design

One tomato genotype selected at the University of Naples, Department of Agricultural Sciences, named E42, and one heat-tolerant tomato accession, named LA3120 (Tomato Genetics Resource Centre, TGRC, University of California, CA, USA) were used in this work. Seeds were sown in seed tray and, after 20 days, the seedlings were transferred to plastic pots (21 cm diameter) with commercial substrate in two controlled growth chambers located at the Department of Agricultural Science, University of Naples (Italy). The climate settings of the chambers were 29/24 °C day/night in the control chamber, while in the other chamber (hot chamber) the temperatures were 35/30° C day/night. Plants were grown in a completely randomized block with three replicates per genotype and 5 plants per treatment in each replicate. The experiment included 4 treatments:

1. Control: 29/24 °C, 100% irrigation

2. Water deficit: 29/24 °C, 50% irrigation
3. Heat stress: 35/30° C, 100% irrigation
4. Combined stress: 35/30° C, 50% irrigation

The climate control was supported by the Arduino Mega 2560 system. The temperature of the two rooms was measured by the DHT11 sensor every 5 minutes and the data were saved on SD (Secure Digital) slot. When the temperature exceeded 30 °C in the Control chamber, the refrigeration system was activated until reaching a temperature of 25 °C. In the Hot chamber, the heating was activated when the temperature was below 30 °C until reaching the temperature of 32 °C. The automatic irrigation system was based on nine soil moisture sensors for each room. Micro-flow irrigation was applied using self-compensating 4l/hour drippers. The percentage of humidity (% v/v) of the substrate in the 100% and 50% irrigation treatments was calculated by averaging the content on five pots. The irrigation intervention was carried out when the average percentage content of water contained in the substrate fell below 20%. In each irrigation 34 mL and 17 mL of water per pot were delivered in the 100% and 50% treatment, respectively. After a pause time of 30 minutes, the Arduino read the average value of the soil moisture sensors for each chamber and, if the average value of the readings for each chamber was less than the set point, it activated the irrigation pump again for 15 minutes. The system was set up to provide a maximum of three irrigations per day per single room. The software was written in Arduino IDE (native environment for Arduino programming).

3.2.2. Plant biomass and leaf functional traits determination

Plants were harvested three weeks after stress treatments and were separated into shoot and root by cutting at the cotyledonary node. Shoot fresh weight was determined and all leaves from each plant were counted for leaf number. Root fresh weight was determined, and the roots were cleaned from the ground and weighed. The root/shoot ratio was calculated using the following formula: fresh weight of root/fresh weight of shoot. Fruits fresh weight and fruit number were also determined.

The leaf functional traits, namely leaf area (LA) and specific leaf area (SLA) were determined on five well-exposed and fully expanded leaves per genotype per treatment, following the methods reported in Cornelissen et al. (2003). For the measurements, the fourth leaf from the apex in each plant was chosen. The leaf area (LA) was measured by the program Image J 1.45 (Image Analysis Software) and expressed in cm^2. The specific leaf area (SLA) was calculated as the ratio between leaf area and leaf dry mass ($cm^2\ g^{-1}$). The leaf dry mass (DW) was obtained drying the leaves in the stove at 70 °C for 48 h.

3.2.3. Pollen viability

Pollen viability was analyzed using three flowers per plant sampled from three different plants per replicate. In the laboratory, pollen grains were spread on microscope slides. One droplet of DAB solution (SIGMA) was added on each pollen sample; slides were gently warmed with a gas lighter and mounted with a cover slip (Dafni, 1992). Scoring was made using an LEITZ Laborlux12 microscope.

3.2.4. Leaf gas exchanges and chlorophyll a fluorescence emission measurements

Leaf gas exchange and chlorophyll a fluorescence emission measurement were simultaneously performed by using the Li6400 portable photosynthesis system (Licor, Lincoln, Nebraska, USA) integrated with Li6400-40 Leaf Chamber Fluorometer, which acts as both a leaf cuvette and light source/pulse-amplitude modulated (PAM) fluorometer. Measurements were carried out in the morning (09:00-11:30 a.m.) on fully expanded mature leaves at the following environmental parameters: Photosynthetic Photon Flux Densities (PPFD) of 1000 μmol photons m^{-2} s^{-1}, 360 μmol CO_2 mol^{-1}, RH 50-55%, and two regimens of fixed temperatures at 25 °C (considered as ambient control) and at 35 °C (considered as heat treatment). Net photosynthesis (A_N), stomatal conductance (g_s) and transpiration (E) were calculated according to von Caemmerer and Farquhar (1981) by the software operating in Li6400. The steady-state fluorescence (F_s) and the maximal fluorescence (F'_m) upon illumination were measured by applying a 0.8 s-saturating flash of 7,000 μmol photons m^{-2} s^{-1} and the quantum yield of PSII electron transport (Φ_{PSII}) was calculated as reported in Maxwell and Johnson (2000). The instantaneous water use efficiency (WUE_i) was calculated as A_N/E ratio.

3.2.5. Photosynthetic pigment content analyses

The evaluation of total carotenoids and chlorophylls was carried out according to the method reported by Wellburn (1994) and by Zouari et al. (2014), as modified by Rigano et al. (2016). To obtain the lipophilic extract, 0.30 grams of sample were extracted with 24 mL of acetone/hexane (40/60, v/v). The mixture was centrifuged at 15,000 rpm for 5 min at 4°C. Supernatants were collected and stored at -20°C until analyses. For carotenoid and chlorophylls a and b determination, the absorbance of lipophilic extracts was read at 470, 663, and 645 nm, respectively. Results were converted into mg/100 g FW. Three separated biological replicates for each sample and three technical assays for each biological repetition were measured.

3.2.6. Hydrogen peroxide, malondialdehyde, ascorbic acid and glutathione determination

Quantification of H_2O_2 content was carried out by using a colorimetric method (Sergiev et al.,1997). Briefly, 500 mg of frozen powder from tomato leaves were extracted with 5 mL of ice cold 0.1% 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 were added 500 µL phosphate buffer 10 mM (pH 7.0) and 1 mL of potassium iodide (1 M). The mixtures were then incubated in the dark for 40 min and measured at 525 nm by using a Nano Photometer TM (Implen, Munich, Germany). Three separated biological replicates for each sample and three technical assays for each biological repetition were measured. The concentration was expressed in mmol/g FW.

The first fully open leaf was taken for the determination of malondialdehyde (MDA). The MDA levels in leaf tissues indicate the levels of membrane lipid peroxidation. Briefly, 0.2 g of leaf sample was ground by adding 1 mL of ice cold 0.1% 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 1,250 mL reaction solution (TCA 20% + TBA 0.5%), water-bathed for 30 min at 95 °C and measured at 532 and 600 nm by using a Nano Photometer TM (Implen, Munich, Germany). Three separated biological replicates for each sample and three technical assays for each biological repetition were measured. The concentration was expressed as quantity of MDA-TBA complex (Zhang and Kirkham,1996)

Quantification of reduced ascorbic acid (AsA) and total ascorbic acid (AsA + dehydroascorbate − DHA) measurements were carried out by using a colorimetric method (Stevens et al., 2006) with modifications reported by Rigano et al. (2014) and Francesca et al. (2020). Briefly, 500 mg of frozen powder from tomato leaves were extracted with 600 µL of ice cold 6% trichloroacetic acid (TCA) and the mixture was then incubated for 15 min on ice and centrifuged at 14,000 rpm for 20 min. For reduced AsA evaluation, to 20 µL of supernatant were added 20 µL of 0.4 M phosphate buffer (pH 7.4), 10 µL of double distilled (dd) H_2O and 80 µL of color reagent solution. This solution was prepared by mixing solution A (31% (w/v) H3PO4, 4.6% (w/v) TCA and 0.6% (w/v) FeCl3) with solution B (4% (w/v) 2,20-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 (NEM; 0.5% (w/v) in water) were added and left for 1 min at room temperature. Eighty microliters of color reagent were added as previously described for reduced AsA. Both the final mixtures were incubated at 37 °C for 40 min and measured at 525 nm by using a Nano Photometer TM (Implen, Munich, Germany). Three separated biological replicates for each sample and three technical assays for each biological repetition were measured. The concentration was expressed in µmol/g of fresh weight (FW). For glutathione determination, 0.3 g of frozen powder

from tomato leaves was homogenized with cold 5% metaphosphoric acid at 4 °C in a 1:6 ratio (w/v) in order to obtain deproteinized extracts. After centrifugation at 20,000 g for 15 min, the supernatants were collected and used for the analysis of glutathione content and redox state according to De Pinto et al. (1999). The concentration of reduced and total glutathione was expressed in nmol/g of fresh weight (FW).

3.2.7. Enzymatic antioxidant activity assay

For the determination of enzyme activities, 0.5 g of frozen powder from tomato leaves was ground to a fine powder in a mortar in the presence of liquid nitrogen and mixed with an extraction buffer, consisting of 50 mM Tris–HCl (pH 7.5), 0.05% cysteine and 0.1% bovine serum albumin (BSA), 1 mM PMSF, 5% PVPP in a 1:4 ratio (w/v). Supernatants obtained after centrifugation at 22,000 g for 20 min were used for spectrophotometric analyses.

Cytosolic APX (L-ascorbate: hydrogen peroxide oxido-reductase, EC 1.11.1.11) activity was measured by following the H_2O_2-dependent oxidation of AsA at 290 nm in a reaction mixture containing 0.1 M Tris-acetate buffer, pH 6.4, 350 µM AsA, 170 µM H_2O_2, 50 µg of protein. Catalase (CAT, EC 1.11.1.6) activity assay was performed by following H_2O_2 dismutation at 240 nm in a reaction mixture consisting of 0.1 M phosphate buffer, pH 7.0, 50 µg protein and 18mM H_2O_2 (ε=39,6 $M^{-1}cm^{-1}$). Peroxidase (POD EC 1.11.1.7) activity was measured following the oxidation of 3,3´,5,5´-Tetramelbenzidine (TMB) at 652 nm (ε = 26,9 mM^{-1} cm^{-1}).

Protein content was determined according to Bradford (1976) using bovine serum albumin as standard. All enzyme activities were measured using a Beckman (Fullerton – CA) DU 7000 spectrophotometer.

3.2.8. Genotyping by sequencing (GBS) Analysis

In order to perform a highly robust analysis of the private loci in E42 and LA3120, 27 different genotypes were genotyped and compared to LA3120 and E42. The 29 tomato genotypes were selected from a wide tomato germplasm collection available at the University of Naples Federico II (Ruggieri et al., 2015). Genotype are hosted at LabArchive repository (http://dx.doi.org/10.6070/H4TT4NXN). Genomic DNA (gDNA) was extracted from 100 mg of young leaf tissue using the DNeasy plant mini kit (Qiagen). DNA concentration was determined by using Qubit fluorometer (Invitrogen, Carlsbad, CA). The estimation of 260/280 and 260/230 ratios were determined by using a NanoDrop spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). DNA sequencing was performed using 1 µg of DNA diluted in 30 µL of sterile Milli-Q water to constitute libraries for the ddRAD technology, as reported by Peterson et al., 2012. The double digestion reaction was performed

by using the restriction enzymes *MboI* and *SphI* and the fragments were sequenced using the V4 chemistry paired end 125 bp mode on a HiSeq2500 instrument (Illumina, San Diego, CA, USA). The demultiplexing step was performed using Stacks v2.0 (Catchen et al., 2013) clean raw Illumina reads and detect the variants. The filtered reads were aligned to the reference genome of *Solanum lycopersicum* (version SL4.0) by using BWA-MEM with default parameters through the software Samtools 1.6 (Li et al., 2009) selecting reads mapping one-time on genome. The raw variants were filtered and manipulated using VCFtools v.0.1.13 (http://vcftools.sourceforge.net) (Danecek et al., 2011) by setting the following parameters: minimum mean of Depth of Coverage (min-mean DP) = 5, max missing data (max-missing) = 0.5. The loci showing heterozygous conditions were manually discarded. The annotation and prediction of the possible effect of the SNP mutations were evaluated using SnpEff tool (http://snpeff.sourceforge.net/) (Cingolani et al., 2012), using iTAG4.1 annotation as references.

3.2.9. Statistical analyses

Data were subjected to analysis of variance using a two-way ANOVA. To separate means within each parameter, the Tukey'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.

3.3 Results

3.3.1. Effect of single and combined abiotic stresses on plant growth parameters

Water deficit, heat and combined stress had no effect on leaf number in LA3120 compared to the non-stressed control **(Fig.1a)**. In contrast, combined stress had a significant effect on leaf number in E42, which was lower in response to this treatment compared to control **(Fig.1a)**. The shoot fresh weight of LA3120 decreased under limited water availability and combined stress compared to control, in contrast, the shoot fresh weight of E42 decreased under both heat and combined stress **(Fig.1b)**. The root fresh weight of E42 was affected by heat and combined treatments, while no differences were evidenced in LA3120 compared to the control **(Fig.1c)**. The root/shoot ratio did not change in LA3120 in response to different treatments, whereas in E42 a tendency to increase under water-limiting condition and to decrease under heat and combined stress were detected **(Fig.1d)**. The leaf area was significantly lower under heat treatment only in the genotype E42 compared to the control **(Fig.1e)**. Moreover, the specific leaf area (SLA) of E42 increased under combined stress compared to control plants, while in LA3120 there was no change in response to the different

treatments **(Fig.1f)**. In both genotypes, plants under heat and combined stress showed a strong reduction in the viability of the pollen compared to the control treatment. In particular, in the genotype LA3120 the heat treatment decreased pollen viability by 61.61% while in E42 the combined treatment decreased pollen viability by 94.63% **(Table 1)**. Moreover, in E42 the fruit fresh weight also decreased under water stress (**Table 1**). Consistent with the pollen viability data, no fruits were present on plants of both genotypes subjected to heat and combined stress (**Table 1**).

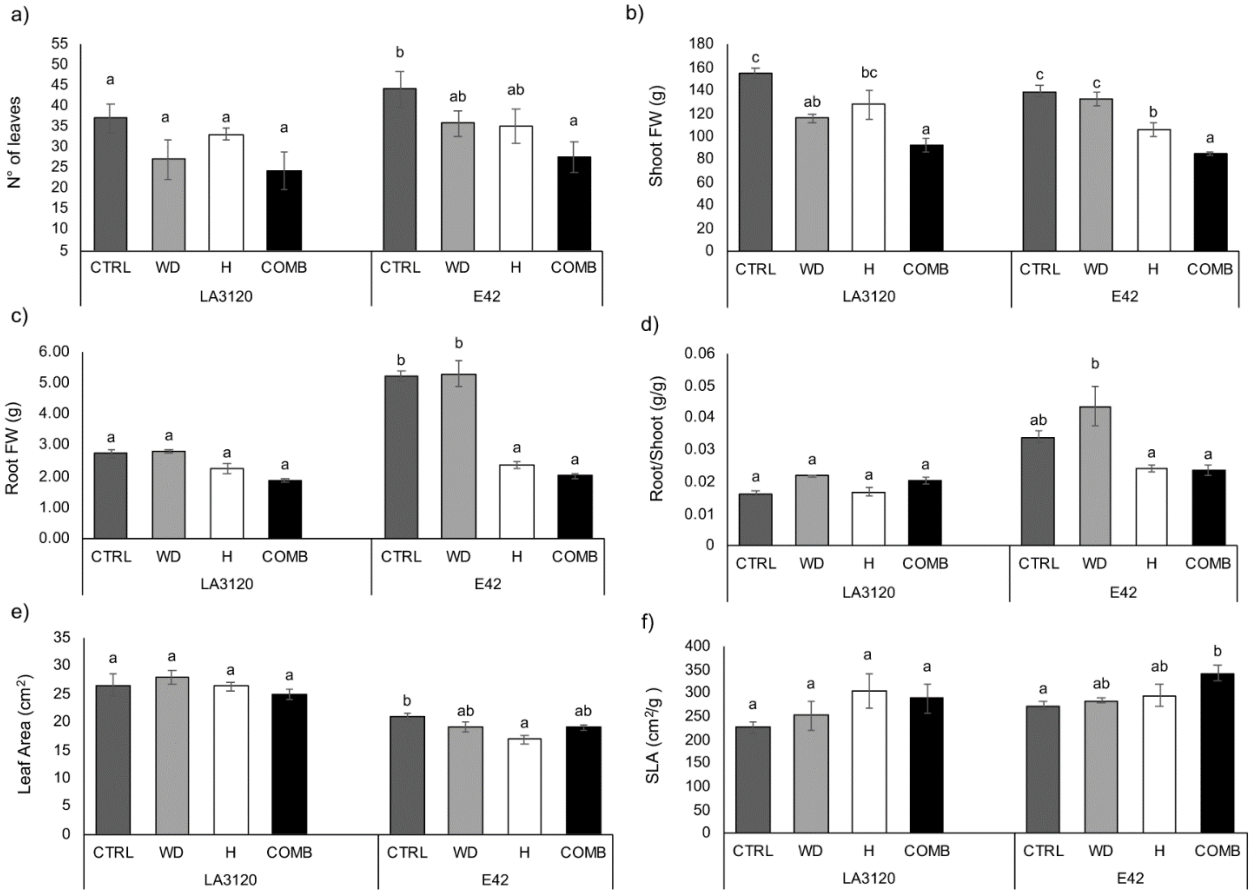


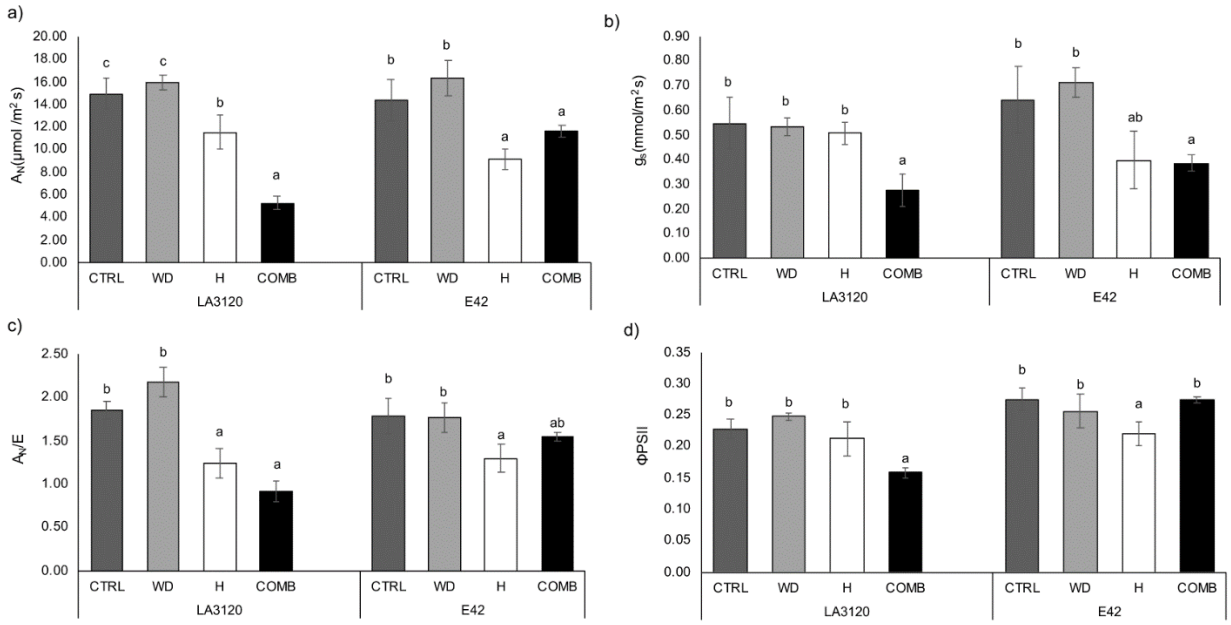
Figure 1: Plant growth parameters of two tomato genotypes under control (CTRL), water deficit (WD), heat (H) and combined stress (COMB). Different panels represent **a)** leaf number, **b)** shoot fresh weight, **c)** root fresh weight, **d)** root/shoot ratio, **e)** leaf area and **f**) specific leaf area (SLA). The data represent mean value± SE (n=5). Within each tomato line, different letters indicate significant differences among treatments (P<0.05).

Table 1. Pollen viability and fresh weight of fruits of two tomato genotypes under control (CTRL), water deficit (WD), heat (H) and combined stress (COMB). The data represent mean value± SE (n=3). Within each tomato line, different letters indicate, for each variable (pollen, Fruit FW and N° of fruits), significant differences among treatments (P<0.05).

Genotype	Treatment	Pollen (%)	Fruit FW (g)	N° of fruits
LA312O	CTRL	80.82±0.05 b	95.73±5.14 b	6.75±2.06 b
	WD	80.58±0.07 b	86.29±5.20 b	5.6±3.50 b
	H	31.02±0.20 a	0±0.00 a	0±0.00 a
	COMB	22.22±0.25 a	0±0.00 a	0±0.00 a
E42	CTRL	79.63±0.06 c	78.85±3.43 c	9.25±2.87 b
	WD	73.11±0.04 c	37.06±14.09 b	11±4.69 b
	H	33.77±0.20 b	0±0.00 a	0±0.00 a
	COMB	4.28±0.01 a	0±0.00 a	0±0.00 a

3.3.2. Effects of single and combined stresses on leaf gas exchange and fluorescence measurements

The imposed stresses negatively affected leaf gas exchange in both genotypes (**Fig. 2a, b, c**). Net photosynthesis (A_N) and stomatal conductance (g_s) were more affected when heat and water deficit were simultaneously applied than when applied as single stress (**Fig. 2a, b**). In particular, under combined stress, the genotype LA3120, showed a decrease in net photosynthesis of 64.75% compared to the control, while the genotype E42 had a 18.99% reduction for this parameter. In LA3120 the water use efficiency (A_N/E) significantly decreased both under heat and combined stress (-32.95% and -50.80% respectively) (**Fig. 2c)**, whereas in E42 it was significantly reduced only under heat stress (-27.71%) compared to control. The quantum yield of PSII (Φ_{PSII}) resulted negatively affected in LA3120 genotype only under combined stress conditions, whereas in E42 it was reduced only under heat conditions (**Fig. 2d)**.



307

308 **Figure 2:** Gas exchange and fluorescence measurements in the leaves of two tomato genotypes under control (CTRL),
309 water deficit (WD), heat (H) and combined stress (COMB). Different panels represent **a)** net photosynthesis (A_N), **b)**
310 stomatal conductance (g_s), **c)** water use efficiency (A_N/E), **d)** quantum yield of PSII (Φ_{PSII}). The data represent mean
311 value± SE (n=6). Within each tomato line, different letters indicate significant differences among treatments (P<0.05).

312

313 **3.3.3. Effects of single and combined stresses on pigments and antioxidants contents**

314 High temperatures and combined stress increased the content of carotenoids, and chlorophylls a and
315 b in both genotypes **(Table 2)**. Specifically, under elevated temperatures the LA3120 genotype
316 showed an increase of 109.54%, 85.21% and 145.60% of total carotenoids, chlorophyll a and
317 chlorophyll b, respectively, compared to the control. In LA3120, the highest content of photosynthetic
318 pigments compared to control was detected under heat stress conditions followed by the combined
319 stress treatment. In E42 the combined stress increased the carotenoid content of 47.07% and the
320 chlorophylls a and b content, of 38.50% and 61.07 %, respectively, compared to the control.
321 Conversely, under limited water availability the level of Chl a significantly decreased in this
322 genotype. Water stress in plants also causes the formation of ROS that can lead to oxidative stress
323 and induce lipid peroxidation (Sanchez-Rodriguez et al. 2016). To verify if stress conditions caused
324 oxidative damages in the two genotypes, the concentrations of H_2O_2 and MDA were determined (**Fig.**
325 **3**). Surprisingly the H_2O_2 content of LA3120 and E42 significantly decreased under heat and
326 combined stress compared to control. The highest concentration of H_2O_2 was found when plants grew
327 under water-limiting condition with 1.44 mmol/g recorded in LA3120 and 3.97 mmol/g recorded in
328 E42 **(Fig. 3a)**. Similarly, the MDA content in both genotypes significantly declined under high
329 temperatures compared to the control treatment **(Fig. 3b)**. The different stress conditions did not
330 change the content of reduced AsA **(Fig. 3c)**. However, total AsA decreased under heat and combined

treatments in both genotypes. Specifically, in the genotype LA3120 a 36.62% lower content of total AsA was registered in plants under combined stress **(Fig. 3d).** In both genotypes, reduced glutathione increased under heat stress and decreased with the combined stress (**Fig. 3e**). In the genotype LA3120, a decrease of the total glutathione pool occurred under water deficit and combined stress. Conversely, the content of total glutathione significantly increased in E42 subjected to all the stress conditions (**Fig. 3f**). However, a significant reduction of glutathione redox state was observed in both genotypes subjected to combined treatment.

Table 2. Pigments content in two tomato genotypes under control (CTRL), water deficit (WD), heat (H) and combined stress (COMB). The data represent mean value ± SE (n=3). Within each tomato line, different letters indicate, for each variable (Total carotenoids, chlorophyll a and chlorophyll b), significant differences among treatments ($P<0.05$).

Genotype	Treatment	Total carotenoids (mg/100 g FW)	Chlorophyll a (mg/100 g FW)	Chlorophyll b (mg/100 g FW)
LA312O	CTRL	26.58±3.31 a	78.18±4.67 a	30.42±4.24 a
	WD	27.11±2.61 a	76.41±5.78 a	33.15±5.37 a
	H	55.69±5.73 c	144.80±14.78 c	74.71±3.03 c
	COMB	42.97±4.82 b	107.64±8.50 b	50.95±6.94 b
E42	CTRL	32.99±4.31 a	95.76±5.06 b	37.15±4.22 a
	WD	40.67±7.44 b	82.71±8.42 a	56.38±7.75 b
	H	43.48±5.16 bc	106.70±3.08 c	52.86±6.39 b
	COMB	48.52±3.41 c	132.63±2.97 d	59.84±5.16 b

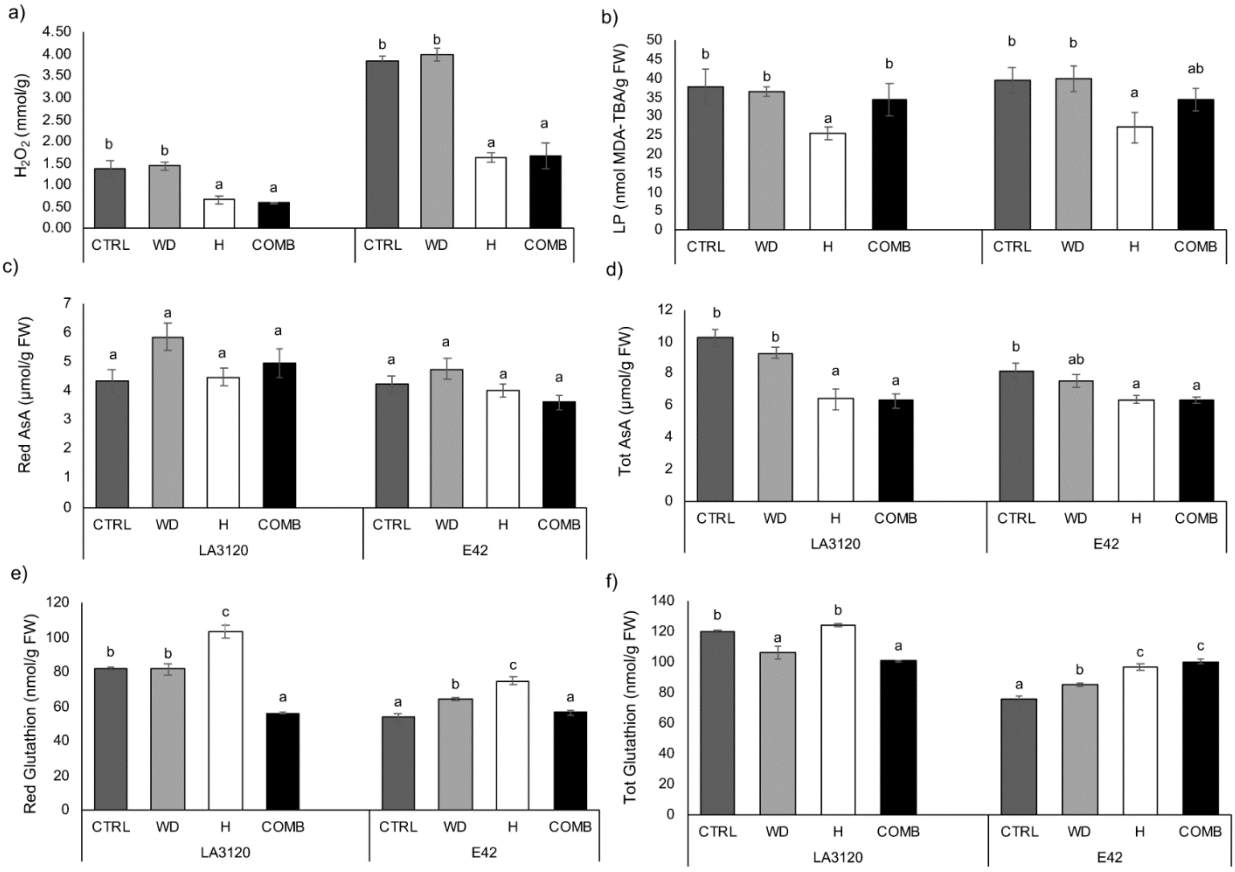


Figure 3: Oxidative markers and hydrophilic antioxidants of two tomato genotypes under control (CTRL), water deficit (WD), heat (H) and combined stress (COMB). Different panels represent **a)** hydrogen peroxide (H_2O_2) concentration, **b)** lipid peroxidation (LP), measured as malondialdehyde (mda-tba) content **c)** reduced AsA, **d)** total AsA, **e)** reduced, and **f)** total glutathione content. The data represent mean value± SE (n=3). Within each tomato line, different letters indicate significant differences among treatments ($P<0.05$).

3.3.4. Effects of single and combined stresses on antioxidant enzyme activities

The enzymes involved in ROS scavenging responded very differently in the two genotypes. In LA3120, ascorbate peroxidase (APX) was not significantly affected by water deficit and heat taken singularly; however, the combined treatment significantly increased enzyme activity. On the other hand, in E42 only heat stress was able to induce the activity of this enzyme, which remained at values comparable with control under water deficit and combined stress (**Fig. 4a**). The activity of catalase (CAT) after water deficit, heat and combined stress treatment did not significantly change in LA3120. Conversely, an increase in CAT occurred after heat and combined stress in the E42 genotype (**Fig. 4b**). In LA3120 subjected to all the stress conditions an increase in peroxidase (POD) activity was observed, even if the extent of the increase was higher after heat and combined treatment. In E42, POD activity did not change after heat and combined stress and significantly decreased in plants subjected to water deficit (**Fig. 4c**).

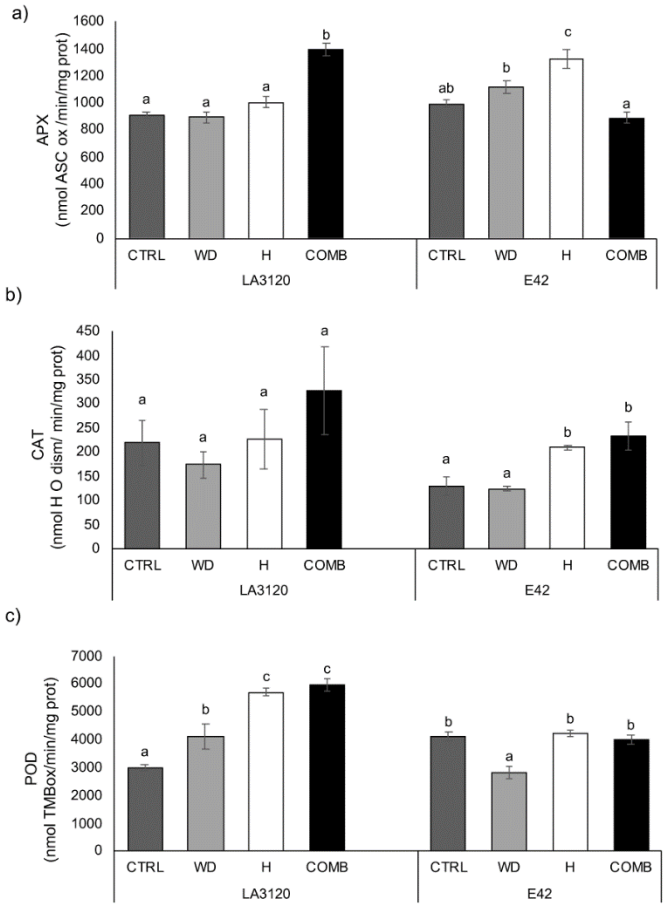


Figure 4: Activity of ROS removal enzymes in two tomato genotypes under control (CTRL), water deficit (WD), heat (H) and combined stress (COMB). Different panels represent **a)** ascorbate peroxidase (APX), **b)** Catalase (CAT), **c)** Peroxidases (POD). The data are the mean value± SE (n=3). Within each tomato line, different letters indicate significant differences among treatments ($P<0.05$).

3.5. Principal Component Analysis

To provide a broad overview of the different analyses conducted on the two tomato genotypes in response to the different stresses applied, a principal component analysis (PCA) was conducted. Based on our experimental data, seven principal components (PCs) were associated with Eigenvalues higher than one and accounted for 100% of the total variance, with PC1, PC2, PC3, PC4, PC5, PC6 and PC7 accounting for 50.69%, 21.55%, 10.98%, 6.96%, 5.12%, 2.71% and 1.98% (**Table S1**). Principal component 1 was the primary driver for differences between genotypes (**Fig. 5**) and the main parameters leading to this separation were fruit fresh weight, pollen viability, stomatal conductance, number of fruit and net photosynthesis (**Table S1**). There was also a treatment-dependent clustering with the primary differences driven by PC2 (**Fig.5**). The main parameters of PC2 were specific leaf area, root/shoot ratio and hydrogen peroxide content (**Table S1**).

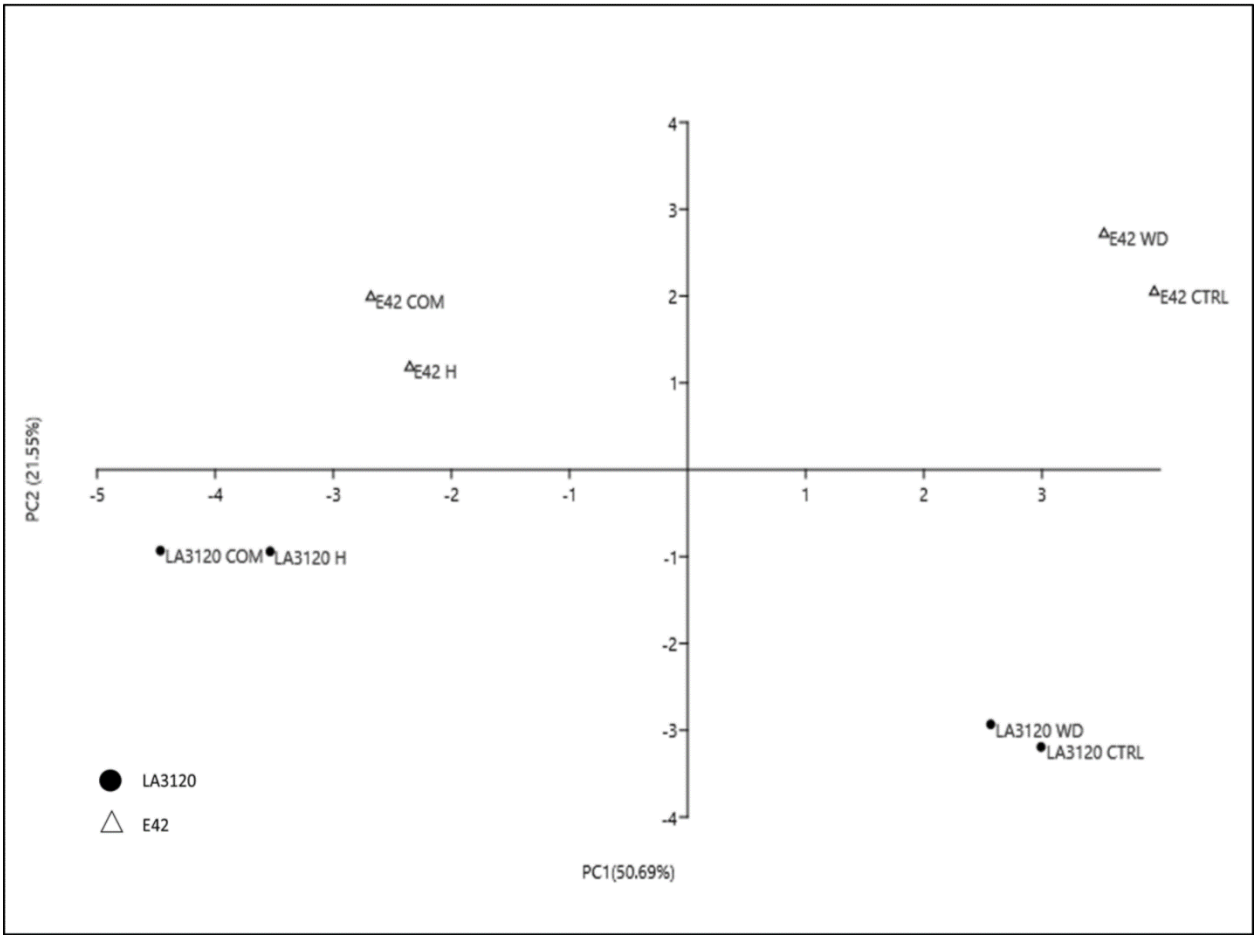


Figure 5: Principal component loading plot and scores of principal component analysis (PCA) in two tomato genotypes under control (CTRL), water deficit (WD), heat (H) and combined (COMB) stress.

3.6. Genotyping by sequencing (GBS) Analysis

In order to identify unique SNP variants in the two genotypes of the trials, a genotyping characterization using a GBS approach was carried on a larger group of genotypes. The SNP calling revealed a raw dataset of 108,936 different variants which were reduced to 17,283 variants applying filters, consisting in 16,328 SNPs and 955 InDels variants. Focusing on the two genotypes LA3120 and E42, the calculation of the private variants was carried out and revealed 251 private variants for LA3120, 6,086 for E42, and 4 common to both the genotypes, corresponding to the ~37 % of the total SNP dataset (**Table 3**). Moreover, snpEff analysis was carried out to identify variants with significant impact on the protein function. In particular, most of common and private variants of E42 and LA3120 showed a modifier impact (6,248 SNPs) whereas 36 showed a low impact. Therefore, regarding the variants with significant impact on the protein function, 54 SNPs showed moderate impact. In particular 53 were private of E42 and 1 of LA3120, whereas also 3 SNPs with high impact were found to be private of E42. Totally the variants affected a total of 1,402 genes. Among the affected genes, the annotation of the genes with variants showing high and moderate impacts was predicted (**Table S2**). Among the 47 genes affected by these variations we identified some that could

be involved in the abiotic stress response. Among these genes, we identified a gene coding for curvature thylakoid protein (*Solyc01g056890*), a gene coding for the large chain subunit of the rubisco (*Solyc07g021200*), a gene coding for a transcription factor GRAS (*Solyc01g079380*), a gene coding for Arabinogalactan protein (*Solyc07g053640*) and a gene coding for a Isocitrate dehydrogenase (*Solyc02g086610*), showing a high impact variant private of the genotype E42.

Table 3. Summary of the private loci detected in E42 and LA3120. The number of analyzed loci, the snpEff impact, the number of affected genes were also reported.

Genotype	Loci (n.)	High	Moderate	Low	Modifier	Affected genes
E42	6086	3	53	34	5996	1268
LA3120	251	0	1	1	249	130
Common	4	0	0	1	3	4
Total	6341	3	54	36	6248	1402

3.4 Discussion

Water stress and high temperatures, alone or in combination, can greatly affect world crop production. Previous studies have clearly demonstrated that the responses of crops to each individual stress do not necessarily reflect plant responses to combined abiotic stresses, a much more frequent condition in nature and agricultural contexts (Zhou et al., 2019). In order to study the different mechanisms engaged by tomato plants to withstand combined abiotic stresses, two putative heat stress tolerant genotypes were selected to test whether their genetic/physiological traits were also useful to overcome water shortage and combined stress (Moles et al., 2018). LA3120 genotype was an accession of *S. lycopersicum*, which is reported as heat-tolerant by the Tomato Genetic Resources Center whereas E42 genotype was a landrace from an arid and warm region of Southern Italy and selected in our laboratory as a high and stable yielding genotype when grown under high temperatures in open field (Olivieri et al., 2020). For these genotypes physiological responses to both limited water availability and elevated temperatures in response to long-term stress were analyzed. Using this system, clear differences, and also similarities, were found between the two genotypes.

3.4.1. Combined stress has severe impact on plant growth parameters

One of the more sensitive stage to heat stress is the reproductive phase of development. Exposure to high temperatures is known to affect pollen viability, fertilization and fruit formation (Balfagon et al., 2018). Although both genotypes were previously classified as heat stress tolerant lines under field

conditions (Arena et al. 2020), in our experimental conditions the prolonged high temperatures compromised the viability of the pollen and no fruits were formed in both genotypes under heat and combined heat-water stress. More dramatic effects were evidenced in E42 where pollen viability dramatically dropped under combined stress compared to heat stress alone.

In line with previous results (Zhou et al., 2017), drought caused more severe damages on plant growth parameters than individual stresses. Indeed, a drop in the number of leaves and in shoot and root fresh weight was only observed under combined stress (**Fig. 1**). Moreover, more severe effects on plant growth parameters were found in the genotype E42 and not in LA3120 when subjected to heat and combined stress. Nevertheless, the genotype E42 under moderate water stress was able to preserve the same shoot and root fresh weight, proving its ability to maintain high plant carbon gain under water stress (**Fig.1**). Moreover, in E42, a high root/shoot ratio was evidenced under water stress, a specific trait that has been previously reported for drought tolerant genotypes (Moles et al., 2018), which may have provided an advantage in nutrient and water uptake in E42.

3.4.2. Different genotypes had unique responses in response to combined stress

Based on the observed growth parameters, it can be hypothesized that the two genotypes E42 and LA3120 used different strategies to overcome individual and combined stresses. With respect to leaf gas exchange measurements, both genotypes were not significantly affected by water shortage so showing elevated ability to overcome periods with reduced water availability. Conversely, the sensitivity to heat, as well as to combined stress, varied significantly in the two genotypes. In both genotypes, heat stress impaired CO_2 fixation and reduced the instantaneous water use efficiency (A_N/E), indicative of biochemical limitations of photosynthesis, including of Rubisco activity (Vitale et al., 2008). However, contrary to E42 where the electron transport was down-regulated, heat stress alone did not determine in LA3120 a reduction in PSII efficiency (ΦPSII). When genotypes were exposed to combined stress, they showed a different response. Indeed, in LA3120 the combined stress induced a strong decline of net photosynthesis (**Fig. 2**), followed by a decrease in stomatal conductance and quantum yield of PSII electron transport, suggesting that the combined stress determined both stomatal and non-stomatal limitation to carbon assimilation. On the contrary, in E42 plants subjected to combined stress photosynthesis was preserved and the electron transport rate increased compared to plants subjected to heat stress. Under combined stress, that limited CO_2 assimilation, the photosynthetic electron flow was more departed towards pathways alternative to CO_2 fixation, so maintaining oxidised the electron transport chain and preventing irreversible oxidative damages. Considering the data on antioxidant enzyme activity, gas exchange and florescence measurements, we hypothesized a more important role of photorespiration in E42 than in

LA3120 in sustaining the electron transport under combined stress, as indicated by the observed increase in CAT activity. In contrast, the occurrence of the AsA-GSH cycle would seem the main alternative pathways to CO_2 assimilation under combined stress in LA3120 (Vitale et al. 2020a). Moreover, even if under heat stress the water use efficiency is lower in the two genotypes, E42 showed a better capability to utilise the limiting water resource when exposed to heat. The analyses of the functional leaf traits under combined stress in the two genotypes seem to confirm this hypothesis. Indeed, the E42 genotype exhibited higher SLA values compared to non-stressed samples, indicating a better state of leaf hydration and a higher photosynthesis capacity per unit of leaf dry biomass. The increased SLA under combined stress may represent a further adaptive strategy of leaf morphological traits to the changing environment in order to maximize the photosynthetic rate (Vitale et al. 2020b). Moreover, the decrease in leaf area in E42 under heat stress may implicate a reduction of whole plant water loss by transpiration, which could allow plants to maintain greater leaf water content and, in turn, higher tolerance to abiotic stresses.

3.4.3. Heat and combined stress activate effective antioxidant defense mechanisms

Despite the differences highlighted in the physiological responses of both genotypes to individual and combined abiotic stresses it is clear that both the heat-tolerant tomato genotypes LA3120 and E42 were able to activate effective antioxidant defense mechanisms mainly in response to heat end combined stress. Abiotic stresses, including drought and heat, cause the overproduction of highly reactive oxygen species (ROS), among which superoxide (O_2^-) and hydrogen peroxide H_2O_2. H_2O_2, due to its high permeability across membranes and long half-life, can work as molecular signal able to activate downstream pathways with protective effects (De Pinto et al., 2015). However, ROS production over a threshold value can lead to oxidative stress, due to protein oxidation and lipid peroxidation (Nouairi et al., 2009, Sanchez-Rodriguez et al., 2016). MDA, the final product of peroxidation of unsaturated fatty acids in phospholipids, is often used as an indicator of lipid peroxidation to evaluate damages to the cell membranes caused by stress. In this study the accumulation of MDA under heat and H_2O_2 under heat and combined stress decrease in both genotypes. In plants subjected to drought and heat stress low levels of H_2O_2 and MDA can be considered as markers of stress tolerance (De Pinto et al., 2015; Zhou et al., 2019). It has been previously shown that in wheat genotypes exposed to heat stress, low levels of membrane damage have a positive correlation with chlorophyll and antioxidant contents (Almeselmani et al., 2006; Hameed et al., 2012). Accordingly, in both tomato genotypes used in this study, heat and combined stress increased the content of chlorophylls and carotenoids, as already shown in other heat-tolerant tomato genotypes (Zhou et al., 2015). Moreover, an accumulation of glutathione occurs in LA3120 under heat stress and in E42 subjected to both heat and combined stress. The accumulation of

antioxidant compounds contributes to prevent oxidative damage and lipid peroxidation and thereby to protect cell membrane (Zhou et al., 2019; Zhou et al., 2020b). Many studies conducted on sensitive and thermotolerant genotypes of the same species highlight a strong relationship between thermotolerance and the capacity to rise one or more ROS-scavenging enzymes. For instance, a study conducted in different wheat genotypes show that heat tolerance is associated with the ability of APX and CAT to cooperate in the removal of H_2O_2 (Dash and Mohanty, 2002). Moreover, plant defense responses to combination of water deprivation and heat may be different from those observed when these stresses are taken separately (Rizhsky et al., 2004; Rampino et al., 2012). Ascorbate peroxidase plays a key role in the scavenging of H_2O_2 in heat stress response (De Pinto et al., 2015; Zhu et al., 2020a). Therefore, the activation of APX under heat stress in E42 and under combined stress in LA3120 may be partly responsible for the lower levels of H_2O_2 observed. Similar results were previously observed in tomato subjected to heat stress and drought (Zhou et al., 2019). The increase in CAT activity in E42 subjected to drought, in absence of APX activation, could be a compensatory mechanism of this genotype in maintaining low levels of MDA and oxidative stress (Gill and Tuteja, 2010). On the other hand, the tolerance to combined stress of LA3120 may be related to the contemporary induction of APX and POD activities. Indeed, POD also plays an important role in decreasing H_2O_2 content, contrasting membrane lipid peroxidation and maintaining cell membrane integrity (Jaleel et al., 2008), and its activity is related to the water retention of leaves (Zhou et al. 2020a). Thus, the activation of POD evidenced in LA3120 under all the studied stress conditions, may play a key role in the defense mechanisms activated by this heat-tolerant genotype.

3.4.4 High impact variant private genes of E42

Nowadays, the Next-Generation Sequencing (NGS) techniques allowed for the identification of thousands of allelic variants in several genes. In a previous work (Olivieri et al., 2020), a genotyping-by-sequencing approach was performed to explore the genetic variability of E42 and allowed us to demonstrate that this genotype has a high genetic variability compared to other genotypes, due likely to a different breeding history that likely included crossing events with tomato wild species. including *S. pimpinellifolium* (Wang et al. 2020). Herein, an additional genotyping-by-sequencing (GBS) experiment was carried out on 12 genotypes that allowed us to demonstrate that E42 harbors 53 private mutations within the coding regions of 47 genes that can have a significant impact on protein functions. Among them, one mutation mapped in the gene *Solyc01g056890*, which codes for a curvature thylakoid protein, that is known to be involved in the responses to different light intensities. In particular, Trotta et al. (2019) demonstrated that the dynamics of thylakoid protein complexes are crucial in the optimization of photosynthesis under fluctuating light intensities (Trotta et al. 2019). A

mutation was also detected in the gene *Solyc07g021200* coding for the large chain subunit of the Ribulose-1,5-bisphosphate carboxylase. It has been reported that under high temperatures Rubisco activation state decreases, an event correlated with changes in the rate of electron transport (Perdomo et al. 2017). This is particularly interesting considering that in our study E42 preserved photosynthesis and increased the electron transport rate when subjected to heat stress. Other two gene variations were scored within the transcription factor GRAS (*Solyc01g079380*) and the Isocitrate dehydrogenase (*Solyc02g086610*), both involved in plant development and responses to abiotic stresses (Olivieri et al. 2020). Lastly, a mutation was detected in the gene *Solyc07g053640* coding for the Arabinogalactan protein. (AGP). AGPs increase cells thickness and stiffness of plant organs, such as leaves, thus limiting water loss and maintaining turgor pressure and cell integrity (Mareri et al. 2018). Accordingly, as previously described, E42 showed a better capability to use the limiting water resources when exposed to high temperatures and exhibited a better state of leaf hydration and a higher photosynthesis capacity per unit of leaf dry biomass when subjected to combined stress.

3.5 Conclusion

Novel tomato genetic resources can be a valuable source of traits for adaptation to stressful environments such as water stress and high temperatures. In this study one novel tomato genotype resistant to high temperatures and one known heat-tolerant genotype were used in order to analyse the different strategies developed in response to single and combined abiotic stresses (high temperature and water shortage). Noteworthy, both lines seemed to be tolerant to the prolonged water shortage applied in our experiment, as evidenced also by the fact that control and water stressed samples were not clearly differentiated by the PCA analyses. Heat and combined abiotic stresses instead clearly distinguished the two genotypes (**Fig. 5**) that employed different physiological responses in order to counteract the applied stresses. That said, both genotypes were able to employ efficient antioxidant defence mechanisms in response to single and combined stress, a trait that could be the key to the tolerance observed in both genotypes also in open fields in other papers (Olivieri et al., 2020). The identification of candidate genes, obtained by combining the phenotypic and genotyping analyses carried out in this work, might help dissect this complex trait and could explain the different physiological response to stress observed in the E42 genotype compared to LA3120. In conclusion, this paper highlighted the presence of interesting stress resistance traits in a heat-tolerant genotypes (LA3120) and in a novel genotype (E42) selected from an arid and warm region of Southern Italy that should be further studied and that could be used in future breeding programs in order to improve resistance to abiotic stress in commercial varieties.

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Supplementary Materials

Table S1. Eigenvalues, relative and cumulative percentage of total variance, and correlation coefficients for each character.

Principal Components	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**
Eigen value	12.67	5.39	2.75	1.74	1.28	0.68	0.49
Relative variance (%)	50.69	21.55	10.98	6.96	5.12	2.71	1.98
Cumulative variance (%)	50.69	72.24	83.23	90.19	95.31	98.02	100.00
Eigen vectors							
Fruit FW	**0.247**	-0.181	-0.031	-0.070	-0.088	0.165	-0.133
Pollen viability	**0.261**	-0.112	-0.026	0.184	0.024	-0.022	-0.116
Stomatal conductance (gs)	**0.242**	0.089	0.207	0.193	0.147	0.041	0.116
N° Fruit	**0.267**	0.064	-0.045	0.124	0.090	0.092	0.231
Net photosynthesis (P_N)	**0.246**	-0.013	0.245	-0.108	0.172	-0.092	0.089
SLA	-0.187	**0.275**	0.121	-0.200	0.160	0.086	-0.008
Total AsA	0.226	**-0.244**	-0.003	-0.087	-0.123	-0.003	0.073
Root/Shoot	0.149	**0.326**	-0.105	0.123	0.237	-0.094	0.131
Hydrogen peroxide (H2O2)	0.204	**0.290**	-0.033	0.072	0.035	0.058	0.000
Chlorophyll B	-0.191	0.179	**0.276**	0.166	0.240	-0.018	0.265
Carotenoids	-0.218	0.186	**0.230**	0.121	0.138	0.064	0.165
Reduced glutathione	-0.033	-0.250	**0.413**	0.250	0.087	-0.292	-0.100
Shoot FW	0.205	-0.106	0.196	**0.340**	-0.234	0.139	0.189
Ascorbate peroxidase (APX)	-0.136	0.094	-0.361	**0.409**	-0.076	-0.299	-0.021
Quantum yield of PSII (Φ_{PSII})	0.176	0.192	0.235	**-0.362**	0.025	0.030	-0.225
Water use efficiency (A_N/E)	0.245	-0.073	0.110	**-0.267**	0.142	-0.141	-0.174
Root FW	0.222	0.217	-0.020	**0.223**	0.101	0.167	0.011
N° Leaf	0.172	0.143	0.176	0.288	**-0.429**	0.178	-0.235
Reduced AsA	0.070	-0.239	-0.192	0.153	**0.603**	-0.099	-0.185
Chlorophyll A	-0.223	0.113	0.284	-0.030	0.001	**0.331**	-0.070
Leaf Area	0.019	-0.384	0.041	0.067	0.238	**0.412**	0.070
Peroxidases (POD)	-0.219	-0.078	-0.061	0.205	0.177	**0.484**	-0.400
Lipid peroxidation	0.217	0.038	-0.256	-0.179	0.071	0.310	**0.423**
Total glutathione	-0.107	-0.327	0.278	-0.006	-0.045	-0.116	**0.336**
Catalase (CAT)	-0.231	-0.163	-0.184	-0.053	-0.127	0.153	**0.308**

Boldface number indicate the most relevant traits for each principal components

Table S2 List of the 60 genes with a different impact effect on the protein structure.

Gene	Private	Effect	Impact	Gene annotation
Solyc01g008170	E42	missense_variant	MODERATE	Zinc finger transcription factor 5
Solyc01g013910	E42	missense_variant	MODERATE	Endoribonuclease Dicer 2b
Solyc01g014520	E42	missense_variant	MODERATE	Receptor-like protein kinase
Solyc01g017640	E42	missense_variant&splice_region_variant	MODERATE	Unknown protein
Solyc01g028900	E42	missense_variant	MODERATE	2-oxoisovalerate dehydrogenase subunit beta, mitochondrial
Solyc01g038231	E42	stop_gained	HIGH	Unknown protein
Solyc01g038231	E42	missense_variant	MODERATE	Unknown protein
Solyc01g038231	E42	missense_variant	MODERATE	Unknown protein
Solyc01g038231	E42	missense_variant	MODERATE	Unknown protein
Solyc01g056570	E42	missense_variant	MODERATE	Regulator of chromosome condensation
Solyc01g056890	E42	missense_variant	MODERATE	protein CURVATURE THYLAKOID 1A, chloroplastic-like
Solyc01g056890	E42	missense_variant	MODERATE	protein CURVATURE THYLAKOID 1A, chloroplastic-like
Solyc01g057703	E42	missense_variant	MODERATE	ATP-dependent DNA helicase
Solyc01g067790	E42	missense_variant	MODERATE	BRCT domain-containing DNA repair protein
Solyc01g068640	E42	missense_variant	MODERATE	AarF domain-containing protein kinase 4
Solyc01g079380	E42	missense_variant	MODERATE	Transcription factor GRAS
Solyc01g079380	E42	missense_variant	MODERATE	Transcription factor GRAS
Solyc01g079380	E42	missense_variant	MODERATE	Transcription factor GRAS

Solyc01g079380	E42	missense_variant	MODERATE	Transcription factor GRAS
Solyc01g079380	E42	missense_variant	MODERATE	Transcription factor GRAS
Solyc01g080360	E42	missense_variant	MODERATE	AP-5 complex subunit mu
Solyc01g088640	E42	missense_variant	MODERATE	E3 ubiquitin ligase BIG BROTHER-related-like
Solyc01g161160	E42	missense_variant	MODERATE	Copia-type polyprotein
Solyc01g161350	E42	missense_variant&splice_region_variant	MODERATE	Tetratricopeptide repeat
Solyc01g163060	E42	missense_variant	MODERATE	cysteine-rich RECEPTOR-like kinase
Solyc01g163080	E42	missense_variant	MODERATE	Gag/pol polyprotein
Solyc02g086610	E42	splice_acceptor_variant&intron_variant	HIGH	Isocitrate dehydrogenase [NADP]
Solyc03g161560	E42	missense_variant	MODERATE	Protein REVEILLE 1
Solyc04g024720	E42	missense_variant	MODERATE	Xyloglucan galactosyltransferase KATAMAR
Solyc05g013280	LA3120	missense_variant	MODERATE	Pseudomonas resistance
Solyc05g053650	E42	missense_variant	MODERATE	26S proteasome non-ATPase regulatory subunit
Solyc07g005250	E42	missense_variant	MODERATE	Unknown protein
Solyc07g005530	E42	missense_variant	MODERATE	Ubiquitin carboxyl-terminal hydrolase 23
Solyc07g017510	E42	missense_variant	MODERATE	1-phosphatidylinositol-3-phosphate 5-kinase FAB1B
Solyc07g020735	E42	missense_variant	MODERATE	Unknown protein
Solyc07g021200	E42	missense_variant	MODERATE	Ribulose bisphosphate carboxylase large chain
Solyc07g021370	E42	stop_lost&splice_region_variant	HIGH	Unknown protein
Solyc07g021370	E42	missense_variant	MODERATE	Unknown protein
Solyc07g021540	E42	missense_variant	MODERATE	GRAM domain-containing protein
Solyc07g041100	E42	missense_variant	MODERATE	Unknown protein

Solyc07g053300	E42	missense_variant	MODERATE	ABC transporter G family member 10
Solyc07g053340	E42	missense_variant	MODERATE	F-box protein At3g07870-like
Solyc07g053640	E42	disruptive_inframe_insertion	MODERATE	Arabinogalactan-protein
Solyc07g056020	E42	missense_variant	MODERATE	Translation initiation factor IF-2
Solyc07g062930	E42	missense_variant	MODERATE	Ribosomal L
Solyc07g064400	E42	missense_variant	MODERATE	Aminotransferase-like, plant mobile domain-containing protein
Solyc07g065870	E42	missense_variant	MODERATE	Regulatory protein RecX
Solyc07g150121	E42	missense_variant	MODERATE	Serine/threonine-protein phosphatase 7 long form-like protein
Solyc11g005100	E42	missense_variant	MODERATE	NAD kinase
Solyc11g006420	E42	missense_variant	MODERATE	Pyrimidine 5'-nucleotidase
Solyc11g007010	E42	missense_variant	MODERATE	Proline-, glutamic acid-and leucine-rich protein
Solyc11g007280	E42	missense_variant	MODERATE	Pleiotropic drug resistance protein 2
Solyc11g007370	E42	missense_variant	MODERATE	Glycosyltransferase
Solyc11g007370	E42	missense_variant	MODERATE	Glycosyltransferase
Solyc11g007700	E42	missense_variant	MODERATE	mRNA cap guanine-N7 methyltransferase 2
Solyc11g007780	E42	missense_variant	MODERATE	SEC12-like protein
Solyc12g011400	E42	missense_variant	MODERATE	Pentatricopeptide repeat-containing protein, mitochondrial

Chapter 4. The use of a plant-based biostimulant improves plant performances and fruit quality in tomato plants grown at elevated temperatures.

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Chapter 4. The use of a plant-based biostimulant improves plant performances and fruit quality in tomato plants grown at elevated temperatures.

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

4.1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most consumed vegetables worldwide also owing to the development of products such as soups, juices, purees and sauces (Del Giudice et al.2016). Tomato is an essential component of the Mediterranean diet and of other traditional diets. However, heat can negatively affect vegetative and reproductive growth phases in tomato resulting in up to 70% harvest losses (Rigano et al. 2016, Ruggieri et al. 2019). Indeed, in tomato, when temperatures exceed 35 °C different physiological functions result adversely affected including seed germination, seedling and vegetative growth, flowering and fruit set and ripening (Ruggieri et al. 2019). High temperature stress leads also to inhibition of chlorophyll biosynthesis and of photosystem II activity (Wen et al. 2019). Indeed, photosynthesis is one of the processes most affected by elevated temperatures (Szymanska et al. 2017). Considering the importance of this crop, the development of new management practices to enhance tolerance to abiotic stresses could contribute to global food production. The use of biostimulants is proposed as an innovative solution to address the novel challenge to improve the sustainability of agricultural systems and reduce the use of chemical fertilizers (Povero et al. 2016, Di Stasio et al. 2018). The most accepted and complete definition of a biostimulant is the one from Du Jardin that defines a plant biostimulant as any substance or

microorganism that applied to plants, regardless of its nutrients content, is able to enhance nutrition efficiency and also abiotic stress tolerance and quality traits (Du Jardin 2015). Du Jardin allocated the biostimulants into 8 class: humic substances, complex organic materials, beneficial chemical elements, inorganic salts, seaweed extracts, chitin and chitosan derivatives, anti-transpirant and free amino acids and considered other N-containing substances with microorganism a potential ninth category. The mechanisms activated in plants by the different biostimulants are still not known as they can act directly on plant metabolism and physiology or indirectly on soil conditions (Di Mola et al. 2019). The effects of biostimulants compounds include stimulation of enzyme activities of glycolysis, Krebs cycle, nitrate assimilation and of hormonal activities (Colla et al. 2017). It has been also demonstrated that biostimulants application is able to enhance tolerance to different abiotic stresses, such as drought (Feitosa de Vasconcelos et al. 2009, Petrozza et al. 2014), salinity (Ertani et al. 2013, Lucini et al. 2015, Di Stasio et al. 2018) and thermal stresses (Botta et al. 2013). For example, it has been demonstrated that applications of algal extracts are able to promote tolerance to drought, salinity and heat, while extracts rich in amino acids can help increasing tolerance to thermal stresses (Verkleij et al. 1992, Battacharyya et al. 2015). Lettuce plants (*Lactuca sativa*) treated with a mixture derived from enzymatic hydrolysis of proteins and subjected to cold showed higher fresh weights and better stomatal conductance compared to non-treated plants (Van Oosten et al. 2017). In another work, perennial ryegrass (*Lolium perenne* L.) treated with hydrolyzed amino acids had improved photosynthetic efficiency compared to non-treated plants at high temperatures (36°C) (Botta et al. 2013). In general, the application of amino acids was found to exert positive effect on plant growth due to their use for the biosynthesis of a large number of non-proteinic nitrogenous compounds (pigments, vitamins, coenzymes, purine and pyrimidine bases). Therefore, amino acids applications could directly influence the physiological activity in plant growth and yield also under abiotic stress (Hammad et al. 2014). Protein hydrolysates can also improve soil respiration, microbial biomass and activity and impact on plant nutrition by forming complexes and chelates between amino acids and soil nutrients (De Pascale et al. 2017). To improve the tolerance to high temperatures the use of biostimulants has been previously investigated, even if it is presently unclear to what extent these compounds are able to improve the physiological performance of tomato plants under elevated temperatures (Di Stasio et al. 2018). We hypothesize that the use of an amino acid-based biostimulant could stimulate natural processes to enhance plant performances also at elevated temperatures. Indeed, the use of protein hydrolysates could directly stimulate carbon and nitrogen metabolism and indirectly enhance nutrient availability, nutrient uptake and nutrient use-efficiency in plants. To verify this hypothesis, we used a novel plant-based biostimulant named CycoFlow and we have performed physiological and biochemical analyses on four different tomato landraces grown at elevated

temperatures and treated or not with this biostimulant. We reasoned that treatments with CycoFlow could facilitate stress adaptation because of its putative cytokinin-like action and its high concentration of glycine betaine known to mitigate the effect of heat stress (Sorwong et al. 2015, Di Stasio et al.2018). Considering climate changes and the expected rise of temperatures in the next few years, to understand the contribution of biostimulants to ensure good plant performances at high temperatures may become increasingly important.

4.2. Materials and Methods

4.2.1 Plant growth, experimental design and treatments

One-month-old tomato seedlings (landraces E17, E36, E107, PDVIT, described in Table 1) were transplanted in May 2018 under walk-in plastic tunnel (22 x 8 m^2) in Battipaglia in the Campania Region in Southern Italy (40°57'68''N 14°95'97''E). The tunnel was covered in polyethylene sheet and was open on both sides. Microclimatic conditions and temperatures were not regulated but were recorded during all the growing season. All four genotypes have an indeterminate growth habit. The genotype E17 is characterized by large fruits (200-500 g), the genotype E107 is characterized by medium-sized fruits (70-100 g) and the E36 and the PDVIT genotypes are characterized by small cherry fruits (Table 1). Only the mature fruits of the E107 genotype are yellow while the fruits of the other genotypes are red. Tomato plants were grown following the standard cultural practices of the area. The experimental design consisted of a completely randomized design with three replicates *per* treatment and ten plant *per* each biological replication. There were two different treatment groups: one control, which did not receive any biostimulant, and one that was treated with the biostimulant. The biostimulant tested 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. The aminogram of the Biostimulant Cyco Flow is reported in Supplementary Table S1. The product contains also Boron (0.2%), Manganese (1%) and Zinc (1.2%). The biostimulant has a pH of 5.0 and a density of 1200 kg/m3. The Biostimulant, in liquid formulation, was initially applied directly to the soil (400 mL *per* plant) at the moment of transplanting, and thereafter every 15 days, until the end of the cultivation cycle for a total of 4 total applications. CycoFlow was applied by fertigation at a final concentration of 3 g/l. The control and the treatment group received the same amount of water. No fertilizer has been applied. During the whole growing period climatic data (Figure S1) were recorded using the weather station VantagePro2 from Davis Instrument Corp. At the end of the cultivation cycle, plants were harvested and separated into leaves, stems, roots and fully ripe fruits. Plant height, numbers of leaves per plant, fresh weight of biomass, total number of fruits, weight of fruit and final yield were recorded. Dry biomass (in grams) was determined by drying plant tissues to constant weight in a forced-air-oven at 80°C for 72

hours. Measurements were done on three randomly selected plants *per* each biological replication *per* genotypes for each treatment.

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

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

4.2.2 Pollen viability

Pollen viability was analysed, using five flowers *per* plant sampled from three different plants *per* replicate. In the laboratory, pollen grains were spread on separate microscope slides. One droplet of DAB solution (SIGMA) was added on each pollen sample; slides were gently warmed with a gas lighter and mounted with a cover slip (Dafni, 1992). Scoring was made using an LEITZ Laborlux12 microscope.

4.2.3 Ascorbic acid quantification

Reduced Ascorbic Acid and total Ascorbic Acid (AsA + dehydroascorbate - DHA) measurements were carried out by using a colorimetric method (Stevens et al. 2006) with modifications reported by Rigano et al. 2014. Briefly, 500 mg of frozen powder from tomato fruits or leaves were extracted with 300 µl of ice cold 6% trichloroacetic acid (TCA) and the mixture was than incubated for 15 min on ice and centrifuged at 14,000 rpm for 20 min. For reduced AsA evaluation to 20 µl of supernatant were added 20 µl of 0.4 M phosphate buffer (pH 7.4), 10 µl of double distilled (dd) H_2O and 80 µl of colour reagent solution. This solution was prepared by mixing solution A (31% (w/v) H_3PO_4, 4.6% (w/v) TCA and 0.6% (w/v) $FeCl_3$) with solution B (4% (w/v) 2,2'-Dipyridyl). For total AsA, to 20 µl of sample, 20µl of 5 mM dithiotreitol, in 0.4 M phosphate buffer (pH 7.4), were added and the mixture was incubated for 20 min at 37°C. Ten microliters of N-ethyl maleimide (NEM; 0.5% (w/v) in water) were added and left for 1 min at room temperature. Eighty microliters of colour reagent were added as previously described for reduced AsA. Both the final mixtures were incubated at 37°C for 40 min and measured at 525 nm by using a Nano Photometer TM (Implen, Munich, Germany). Three separated biological replicates for each sample and three technical assays for each biological repetition were measured. The concentration was expressed in mg/100 g of fresh weight (FW).

4.2.4 Total carotenoids and chlorophylls content.

The evaluation of total carotenoids and chlorophylls was carried out according to the method reported by Wellburn 1994 and by Zouari et al.2014 as modified by Rigano et al. 2016. To obtain the lipophilic extract, 0.25 gram of sample was extracted with 24 mL of acetone/hexane (40/60, v/v). The mixture was centrifuged at 15000 rpm for 5 min at 4 °C. Supernatants were collected and stored at -20°C until analyses. For carotenoids and chlorophylls a and b levels determination, absorbance of lipophilic extracts was read at 470, 663 and 645 nm, respectively. For lycopene and β-carotene levels absorbance was read at 505 and 453 nm, respectively. Results were converted into mg/100 g FW. Three separated biological replicates for each sample and three technical assays for each biological repetition were measured.

4.2.5 Antioxidant activity determination

Hydrophilic antioxidant activity (HAA) was evaluated in the water-soluble fraction, obtained by adding to 2 g of frozen powder 25 mL of 80% methanol, using the ferric reducing/antioxidant power (FRAP) method (Benzie et al.1996) with slight modifications. The FRAP assay was carried out by adding in a vial 2.5 mL of acetate buffer, pH 3.6, 0.25 mL of TPTZ solution (10 mM) in 40 mM HCl, 0.25 mL of $FeCl_3 \cdot 6H_2O$ solution (12 mM), and 150 μL of methanolic extract. The mixture was incubated for 30 min in the dark, and then readings of the colored products (ferrous tripyridyltriazine complex) were taken at 593 nm using a spectrophotometer. Results were expressed as micromoles of Trolox equivalents (TE) per 100 g FW. Lipophilic antioxidant activity (LAA) determination was carried out according to the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method, using the lipophilic extract obtained as described in the previous paragraph [30]. The ABTS assay was based on the reduction of the ABTS•+ radical action by the antioxidants present in the sample. A solution constituted by 7.4 mM ABTS•+ (5 mL) mixed with 140 mM $K_2S_2O_8$ (88 μL) was prepared and stabilized for 12 h. This mixture was then diluted by mixing ABTS•+ solution with ethanol (1:88) to obtain an absorbance of 0.70 ± 0.10 unit at 734 nm using a spectrophotometer. Methanolic extracts (100 μL) were allowed to react with 1 mL of diluted ABTS•+ solution for 2.5 min, and then the absorbance was taken at 734 nm using a spectrophotometer. All biological replicates of samples were analyzed in triplicate. Results were expressed as micromoles of TE per 100 g FW.

4.2.6 Fluorescence emission measurements

Fluorescence emission measurements were performed on five replicates per each treatment, coming from five different plants. A portable FluorPen FP100max fluorometer, equipped with a light sensor (Photon System Instruments, Brno, Czech) was used for measurements, following the procedure reported in Figlioli et al.2019. The ground fluorescence signal, Fo, was induced on 40′ dark adapted leaves, by a blue LED internal light of about 1–2 μmol m^{-2} s^{-1}. The maximal fluorescence level in

the dark, Fm, was induced by a 1s saturating light pulse of 3000 μmol m^{-2} s^{-1}. The maximum quantum efficiency of PSII photochemistry, Fv/Fm, was calculated as (Fm − Fo)/Fm, according to Kitajima and Butler.

4.2.7 Leaf functional traits determination

Fully expanded leaves, without apparent damages, were collected to determine the functional leaf traits following Arena et al. 2013. Leaf area (LA) was measured by the program Image J 1.45 (Image Analysis Software) and expressed in *per* square centimeter, Specific Leaf Area (SLA) was measured as the ratio of leaf area to leaf dry mass and expressed as square centimeter per gram dry weight (DW). For dry mass determination, leaves were dried at 70 °C for 48 h. Leaf dry matter content (LDMC) was measured as the oven-dry mass of a leaf divided by its water-saturated fresh mass and expressed as gram *per* gram of water saturated leaf mass (WSLM). Relative Water Content in leaves (RWC) was calculated by dividing the amount of water in the fresh leaf tissue by the water in the leaf tissue after rehydration multiplied by 100 (Hossain et al.2010).

4.2.8 Statistical analysis

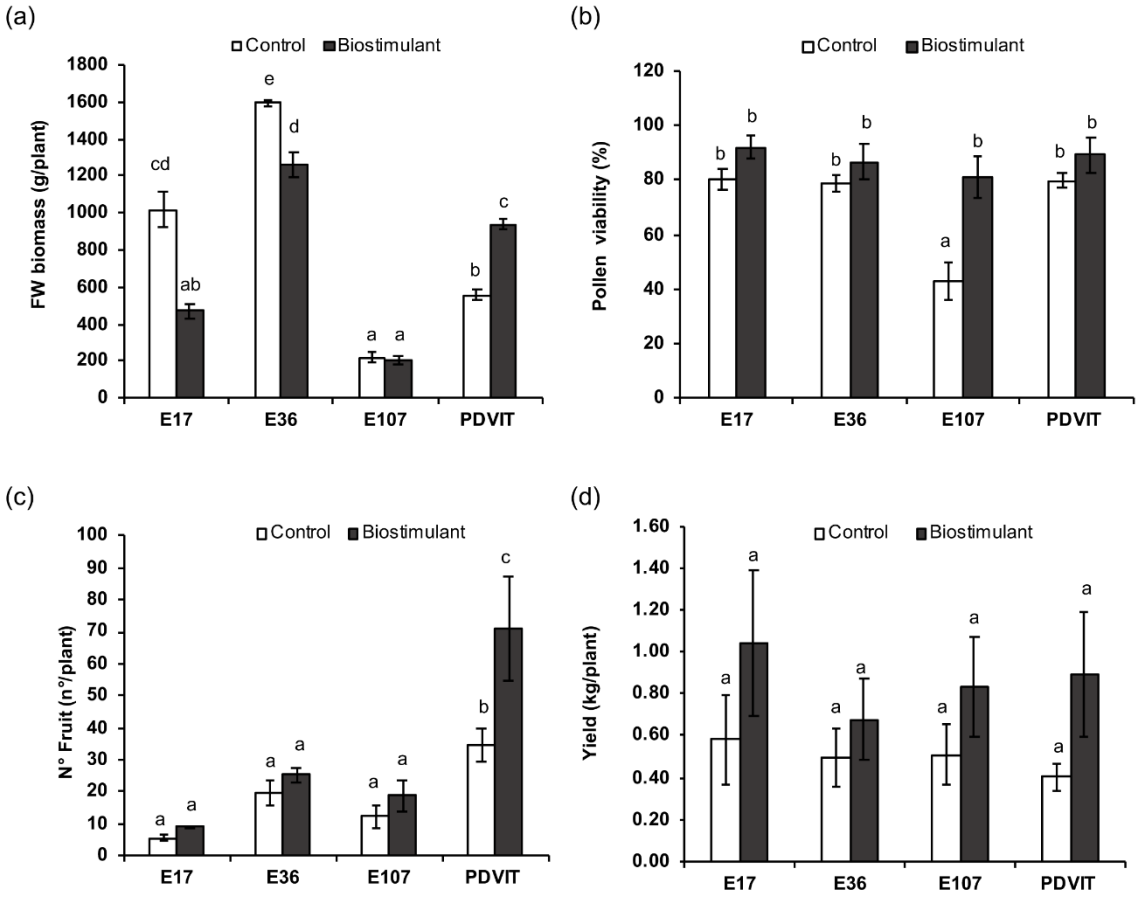
Data were subjected to analysis of variance using ANOVA two-way. To separate means within each parameter, the Tukey-HSD's test was performed. Differences at $P < 0.05$ were considered to be significant. ANOVA was performed by using SPSS (Statistical Package for Social Sciences) Package 6, version 23.0. To explore the overall data, we used the R environment for statistical computing and graphics R Core Team (2018). We first selected variables of interest for each genotype, treatment and plant part (4x2x2) then calculated the arithmetic mean (n=3), and finally used the scale function to center the data around the mean and scale it using the standard deviation. The transformed data were visualized using a heatmap (heatmap function). To aid interpretation of the data, we also performed a SVD-based Principal Component Analysis over the multivariate matrix (function prcomp in base R) after normalization.

4.3. Results

4.3.1. Phenotypic and physiological analyses

In this study four different tomato genotypes were transplanted under a plastic walk-in tunnel with a delay of one month compared to the usual transplanting period (tomato plants in the South of Italy are usually transplanted in April) thus imposing a high-temperature condition during flowering and fruit setting. Indeed, the maximum temperature of 32 °C during the day, that represent a critical threshold in the sensitive stages of reproductive development, was frequently exceeded in this trial (Ruggieri et al.2019) (Figure S2). The four different tomato landraces were treated with a plant-based

biostimulant named CycoFlow. According to ANOVA analyses, the treatment with the biostimulant increased the height of genotypes E107 and PDVIT by 48.5% and 30.1%, respectively (Supplementary Table S2). Generally, the number of leaves was lower in the biostimulant treated group compared to the control, independently from the genotype it was applied to (no significant interacton G x T). For the fresh biomass parameter, in PDVIT the treatment with CycoFlow increased the above ground fresh biomass by 68.4 % (Figure 1). Genotypes E17 and E36 showed, instead, lower values in treated plants compared to non-treated ones (-53.8% and -21.1%, respectively) (Figure 1). A slightly higher pollen viability was also observed in the genotypes treated with the biostimulant compared to the respective controls (Figure 1). In particular, in the genotype E107 the treatment with the biostimulant increased pollen viability by 125%. Generally, the treatment with the biostimulant increased the number of fruits, independently from the genotype (no significant interaction G X T). In particular, the treatment with the biostimulant increased the number of fruits in the genotype PDVIT by 105.3% (Figure 1). The parameter medium fruit weight was significantly affected only by the factor genotype (Supplementary Table S2). Generally, the final yield (kg per plant) showed a tendency to be higher in all the samples from the treated genotypes, even though these differences were not significant. Interestingly, the yield was significantly affected only by the factor treatment (Supplementary Table S2). The treatment with the biostimulant CycoFlow also increased the maximal PSII photochemical efficiency (Fv/Fm) in the E107 and PDVIT genotypes (Figure 2). The monitoring of leaf functional traits evidenced that biostimulant application did not affects these traits significantly (Supplementary Table S3).



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

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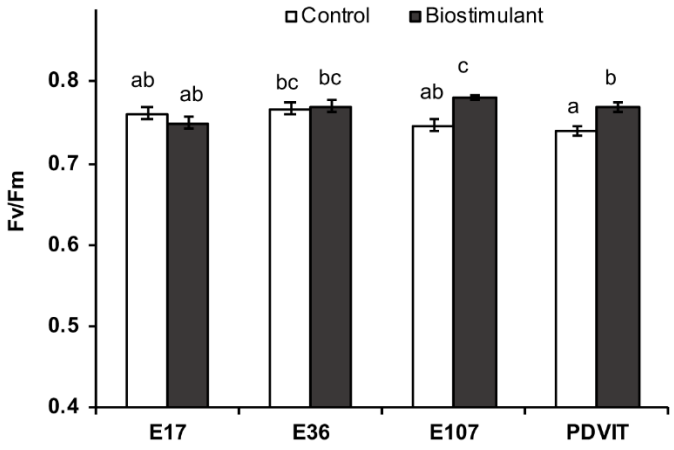

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248 **Figure 2.** Maximal photochemical efficiency, F_v/F_m, in leaves of four tomato genotypes. Data are mean ± SE (n=5).
249 Different letters indicate significant differences based on Tukey-HSD test ($p \leq 0.05$).

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251

4.3.2. Leaf and fruit antioxidant content

The main interaction effects of the biostimulant Cyco Flow on the content of antioxidants in leaves from treated and non-treated plants is reported in Table 2. For the hydrophylic antioxidants, the treatment with the biostimulant increased the content of reduced AsA in the genotypes E17 and E36 and of total ascorbic acid in the leaves of the genotypes E36 and PDVIT. In particular, in the genotype E107 a 60.8% higher content of total AsA was registered in leaves treated with the biostimulant. As for the content of phenolic compounds, two genotypes (E17 and E107) showed lower contents of total phenols in the leaf after treatment with the biostimulant. In particular, in the E17 genotype a 41.6% decrease in the treated compared to the non treated samples was demonstrated. Only in the PDVIT genotype the treatment with the biostimulant increased phenols content. It has been reported that phenolics compounds are the most important contributors to HAA [35]. Accordingly, in the leaves of the treated plants, HAA was lower in E17 compared to the respective non-treated control. For the lipophilic antioxidants, the treatment with the biostimulant increased the content of carotenoids in the genotypes E36 and E107 and the content of chlorophylls a and b only in the genotype E36. Particularly, the E36 genotype shows a 15.8% higher content of carotenoids in the treated leaves compared to the non-treated one, and 17.35% and 48% higher levels of chlorophyll a and b, respectively. The treatment with the biostimulant also increased total liphophilic antioxidant activities in E107 and surprisingly also in PDVIT, suggesting that other compounds outside of carotenoids, contribute to this paramenter. In Table 3 is reported the content of hydrophilic antioxidants determined in red ripe fruit from genotypes treated or non-treated with the biostimulant Cyco Flow. In general, the content of hydrophilic antioxidants in the fruits is higher in almost all the genotypes treated with biostimulants compared to the non-treated ones. The treatment with the biostimulant increased the content of reduced AsA independently from the genotype it was applied on (not significant interaction G X T). The content of reduced ascorbic acid was 28.7-58.7% higher in fruit from treated genotypes compared to non-treated genotypes. Moreover, a content 112.8% higher of total AsA was registered in fruit from PDVIT treated with the biostimulant compared to the respective non-treated control. Contrary to what seen in the leaf, the content of total phenols in berries of treated E17 and E36 genotypes was higher compared to the non-treated control. In particular in the E17 genotype 72.8% higher values were registered. Moreover, a significantly higher antioxidant activity HAA was demonstrated in fruits from E36 plants treated with CycoFlow, according to ANOVA analyses. Assessing the content of lipophilic antioxidants, the treatment with the biostimulant had no effects on the content of carotenoids and chlorophylls but only on the total lipophilic antioxidant activity as reported in Supplementary Table S4. In particular, LAA was higher in fruit from the treated genotypes E17, E36 and E107.

Table 2. Analyses of variance and mean comparison for reduced and total Ascorbic acid (AsA), total phenols, carotenoids, chlorophylls a and b and total liphophilic and hydrophilic antioxidant activities (LAA and HAA, respectively) in leaves of different tomato cultivars treated with the biostimulant Cyco Flow applied by fertirrigation 4 times. Means ± SD within a rows and columns followed by the different letter are significantly different based on Tukey-HSD test ($p \leq 0.05$).

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

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

Table 3. Analyses of variance and mean comparison for reduced and total Ascorbic acid (AsA), total phenols, hydrophilic antioxidant activities (HAA) in fruit of different tomato cultivars treated with the biostimulant Cyco Flow applied by fertirrigation 4 times. Means ± SD within a rows and columns followed by the different letter are significantly different based on Tukey-HSD test ($p \leq 0.05$).

		E17	E36	E107	PDVIT	SIGNIFICANCE	
AsA reduced (mg/100 g FW)	**control**	33.31 ± 2.99 a	39.56 ± 2.30 ab	47.14 ± 1.66 bc	50.36 ± 1.84 bc	G	***
	treated	47.3 6± 1.60 bc	59.87 ± 4.34 cd	74.79 ± 3.25 e	64.83 ± 2.34 de	T	***
						GxT	ns
AsA (mg/100 g FW)	**control**	61.97 ± 0.57 ab	78.03 ± 3.29 bc	85.40 ± 3.75 c	52.87 ± 4.24 a	G	**
	treated	79.34 ± 4.44 bc	87.15 ± 2.35 c	93.36 ± 6.19 cd	112.53 ± 4.08 d	T	***
						GxT	***
Phenols (mg/100 g FW)	**control**	9.62 ± 0.46 a	13.70 ± 0.68 b	16.17 ± 0.58 c	22.35 ± 0.37 e	G	***
	treated	16.62 ± 0.46 c	18.92 ± 0.76 d	16.88 ± 0.44 c	22.55 ± 0.19 e	T	***
						GxT	***
HAA (mg/100 g FW)	**control**	129.28±33.95 a	189.22 ± 49.66 b	179.38 ± 20.62 bc	309.06 ± 39.51 d	G	***
	treated	151.57±8.71c	304.38 ± 30.92 c	212.47 ± 7.08 c	333.03 ± 46.91 d	T	***
						GxT	***

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

4.3.4. Heat map analysis

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

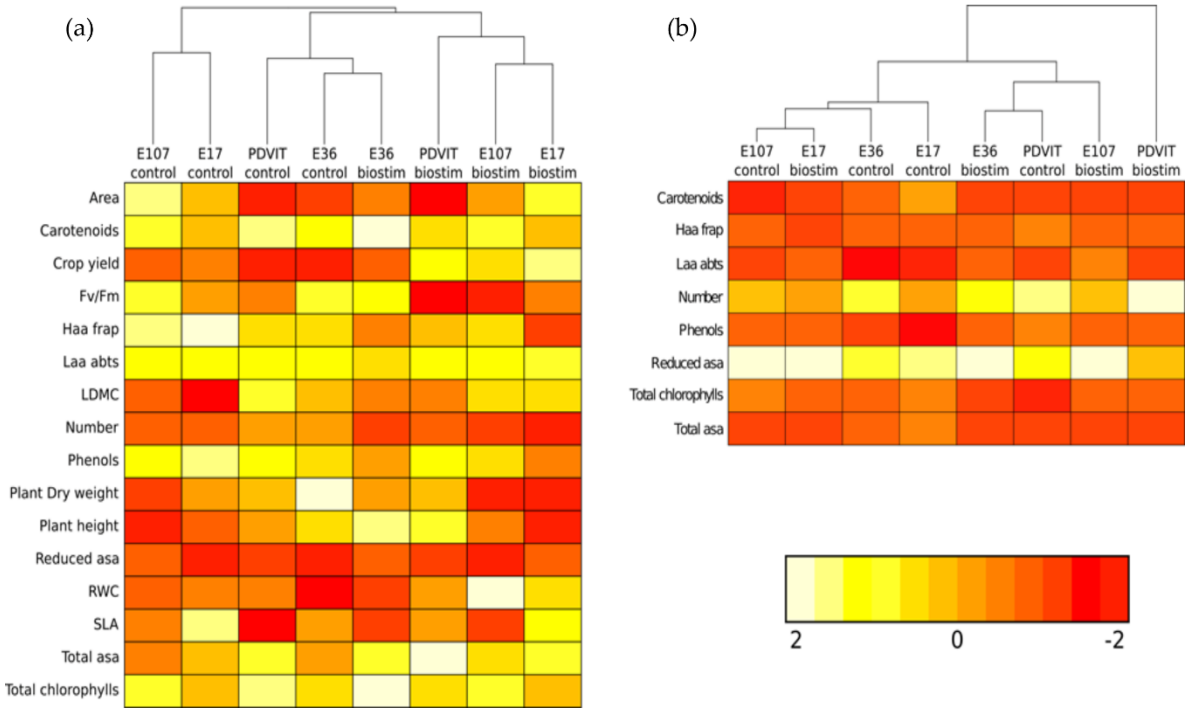

Figure 3. Cluster heat map analysis summarizing the behavior of the different tomato genotypes E36, E17, E107, PDVIT treated or not treated with the biostimulant Cyco Flow in leaf (panel a) and in fruit (panel b). The heat map was generated using the R environment for statistical computing and graphics https://www.R-project.org/ online program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage.

4.4. Discussion

In this paper four different tomato landraces were grown at elevated temperatures under a plastic walk-in tunnel and were treated or not with a plant-based biostimulant named Cyco Flow. The higher heights demonstrated in the majority of the tomato plants treated with Cyco Flow compared to non-treated plants is in agreements with previous studies on different plant species and biostimulants (Chedade et al. 2017, Kocira et al. 2018, Cristiano et al. 2018, Polo, J. and Mata, P.2018). Probably, the presence of signaling molecules in the biostimulant, such as free amino acids, could have promoted endogenous phytohormonal biosynthesis thus stimulating growth and also fruit setting (Rouphael et al. 2017). Indeed, several authors demonstrated that the application of plant-based biostimulants exhibited cytokinin-like activity promoting cell division (Matsuo et al. 2012). Moreover, cytokinins mitigate stresses induced by free radicals by direct scavenging and also by preventing ROS formation inhibiting xanthine oxidation (Polo, J. and Mata, P. 2018). Also, the treatment with Cyco Flow overall increased the number of fruits, as previously demonstrated also in tomatoes treated with other biostimulants (Colla et al.2017, Chedade et al. 2017, Polo, J. and Mata, P. 2018, Rouphael et al. 2017). For example, Rouphael et al. 2017 demonstrated that application of a protein hydrolysate in tomato increased in one cultivar the fruit mean weight and in another cultivar the number of fruits. In this study, in the genotype E107, the higher number of fruits observed was

also linked to a higher pollen vitality observed after Cyco Flow treatment. This result could be due to a combination of multiple effects. While the cytokinin-like activity could have favored cell division, the high level of proline present in the biostimulant, an amino acid whose natural content in the flower organs is ten times higher than that in the leaves, may have played an important role (Figlioli et al. 2019). Indeed, it is known that also the amino acid proline promotes the translocation of nutrients towards developing flowers (sink) (Sato et al. 2006). The positive effects of biostimulants based on amino acid on growth and yield is also due to the fact that the amino acids present in plant-based biostimulants stimulate plant defenses, participate in the synthesis of organic compounds (such as amines, purines, pyrimidines, vitamins) and affect the uptake of macro and micronutrients (Kocira et al. 2018). The CycoFlow effects observed in this study on yield and yield components are even more remarkable considering the elevated temperatures (up to 43°C) reached under the plastic walk-in tunnel in Battipaglia. Indeed, this temperature normally impair fertilization and reduce pollen viability (Colla et al. 2017). It can be hypothesized that the presence of glycine betaine in the CycoFlow may have enhanced the tolerance of tomato plants to elevated temperatures. Indeed, it has been previously demonstrated that during tomato germination glycine betaine applied exogenously improved tolerance to high temperatures and enhanced the expression of heat shock genes (Li et al. 2011). At elevated temperatures, the glycine betaine compound may have also a crucial role in the repair of photodamaged PSII, in maintaining the activity of Rubisco and in alleviating the inhibition of gas exchanges (Sorwong et al. 2015). Accordingly, a higher maximal photochemical efficiency was observed in the genotypes E107 and PDVIT treated with the biostimulant. These results are consistent with other papers that demonstrated that applications of plant- and animal- based biostimulants are able to enhance photosynthetic rates and ensure a higher carbon assimilation efficiency (Cristiano et al. 2017, Xu et al. 2017). For example, under drought stress conditions, Arabidopsis plants treated with an *Ascophyllum nodosum*-extract maintained a better photosynthetic performance compared to non-treated plants during the dehydration period, showing a higher capacity to dissipate thermally the excess of energy in the PSII reaction centers (Santaniello et al., 2017). These results were linked to the fact that pre-treatment with the Ascophyllum-extracts induced partial stomatal closures and also modifications of the expression levels of genes involved in ABA-responsive and antioxidant system pathways (Santaniello et al. 2017). Accordingly, our data indicate that biostimulant treatment induced the activation of the antioxidant defence system, as demonstrated by the higher content of reduced and total AsA in treated leaves. Although the precise reasons for these increases are not explained, it is known that biostimulants components, including glycine betaine, can promote the activity of specific enzymes involved in antioxidant homeostasis (Sorwong et al. 2015, Rouphael et al.2017, Parrado et al. 2008). The ability to maintain an optimal chlorophyll

content during heat stress is another key heat tolerance trait in tomato (Parrado et al. 2008). Interestingly, herein we observed higher contents of carotenoids and chlorophylls in two genotypes (E36 and E107) treated with the biostimulants compared to the non-treated samples. The higher chlorophylls content detected in these genotypes could be linked to limited chlorophyll degradation and leaf senescence (Di Mola et al. 2019). In particular, this could be the case for the genotype E107 that demonstrated a higher maximal photochemical efficiency after treatment with the biostimulant. The biostimulant-mediated effects on photosynthesis and secondary metabolism could also enhance fruit quality (Colla et al. 2017). Indeed, one interesting finding of this study is the positive effect of the biostimulant CycoFlow on the quality of the tomato fruits. In general, the content of hydrophilic antioxidants in the fruits, including AsA, was higher in almost all the genotypes treated with biostimulants compared to the non-treated ones. Higher content of reduced AsA was observed in all the genotypes and of total AsA in the genotypes E17 and PDVIT. This result confirm data previously obtained in other studies that demonstrated an increase in AsA content in tomato, in kiwi fruits and in peppers after the application of plant-based biostimulants (Chehade et al. 2017, Rouphel et al. 2017). Contrary to what seen in the leaf, the content of total phenols in berries of treated E17 and E36 genotypes was higher compared to the non-treated control. Moreover, a significantly higher antioxidant activity HAA was demonstrated in fruits from E36 plants treated with CycoFlow. These results are in agreement with results previously obtained in other crops (soybean seeds, common bean, tomato, corn), even if the reported effects depended on the type of biostimulants, their concentrations and the number of applications (Kocira et al. 2018). Assessing the content of lipophilic antioxidants, the treatment with the biostimulant had no effects on the content of carotenoids and chlorophylls but only on the total lipophilic antioxidant activity. Similar results were obtained by Chehade et al. 2017 in tomato. On the contrary, Rouphael et al. 2017 demonstrated that in tomato foliar applications of a legume-derived protein hydrolysate had an effect also on lycopene content. Also, Colla et al. 2017 demonstrated that foliar applications of protein hydrolysate, plant and seaweed extract affected lycopene content in greenhouse tomato. In the future, foliar application of Cyco Flow will be also tested in order to see if the results obtained in this study are also linked to the used application regimen. Altogether, the genotypic factors remain decisive in the response obtained in the different tomato lines to the biostimulant. Indeed, for almost all the traits considered the effect of the biostimulant depended on the cultivar it was applied on, as seen by the interaction between the effect of the biostimulant and cultivars in most of the studied parameters. These variations observed in this study can be explained by the differences in the genetic background between the different cultivars that were used in this study (Arena et al. 2013). Indeed, the four genotypes here tested differed in terms of fruit shape and dimension and also in terms of fruit colour (e.g. fruit of E107 is yellow). Also

the geographical origin is different with the E107 genotype coming from Spain and the other coming from Italy. These further highlight the fact that one biostimulant should be tested on a certain number of cultivars in order to assess its mechanisms of action.

4.5. Conclusions

In this paper we investigated the effects of the application of one plant-based biostimulant named Cyco Flow on the nutritional quality and yield of tomatoes grown in walk-in tunnel under elevated temperatures. In this study, the application of the Cyco Flow biostimulant based on algae, plant and yeast extracts, rich in amino acids and that contains microelements such as boron, zinc and manganese had a clear effect on plant growth and final crop quality and yields. Indeed, Cyco Flow application had a significant effect on the content of hydrophilic antioxidants in both tomato leaves and fruits. In particular, the content of ascorbic acid increased after treatments with Cyco Flow. These results are particularly interesting considering that, in plants ascorbic acid is active in the removal of ROS, has an important role as an enzymatic cofactor, and participates in plant development, senescence, defense, division, electron transfer and also in fruit ripening (Arena et al. 2013, Xu et al. 2017, Smirnoff et al. 2000). Moreover, in humans, AsA shows significant ability as electron donor and antioxidant, it protects against oxidation of LDL (low-density lipoprotein) by different types of oxidative stress and inhibits LDL oxidation by vascular endothelial cells (Xu et al. 2017, Calafiore et al. 2016, Raiola et al. 2014). Herein, biostimulants application improved plant performances and fruit quality mostly in the genotypes E107 and PDVIT. In particular, in the genotype PDVIT application with Cyco Flow determined a higher plant height, a higher number of fruits, a higher pollen vitality, a higher photochemical efficiency, a higher accumulation of ascorbic acid and a higher antioxidant activity. Additional studies are now planned in order to investigate if different applications regimen, such as foliar application, can also influence the observed effects.

4.6 References

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Supplementary Materials

Supplementary Table S1: Amino acid composition expressed in g / 100 g of the biostimulant CycoFlow

Glycine betaine	3.62
Total amino acid	
Aspartic acid (including asparagine)	2.22
Glutamic acid (including glutamine)	5.04
Alanine	1.36
Arginine	1.06
Phenylalanine	0.83
Glycine	1.02
isoleucine	1.06
Histidine	0.4
Leucine	1.48
Lysine	1.68
Proline	0.81
Serine	1.04
Tyrosine	0.76
threonine	0.98
Valine	1.23
Total cysteine and cystine	0.21
Total tryptophan	0.27
Methionine	0.32
TOTAL	21.77
Free Amino Acid	
Lysine	0.62
Aspartic acid	0.55
Glutamic acid	0.91
Alanine	0.79
Arginine	0.49
Phenylalanine	0.56
Glycine	0.24
isoleucine	0.58
Histidine	0.13
Leucine	0.95
Methionine	0.22
Proline	0.26
Serine	0.43
Tyrosine	0.37
threonine	0.43
Valine	0.72
TOTAL	8.25

Supplementary Table S2: Analyses of variance and mean comparison for height, number of leaves, fresh weight (FW) and dry weight (DW) biomass, number of fruits, medium fruit weight , yields and pollen viability (%) *per* plants of different tomato cultivars treated with the biostimulant CycoFlow applied by fertirrigation 4 times. Means ± SD within a rows and columns followed by the different letter are significantly different based on Tukey-HSD test ($p \leq 0.05$).

		E17	E36	E107	PDVIT	SIGNIFICANCE	
Height (cm)	**control**	129 ± 14 ab	186 ± 7 cd	101 ± 16 a	156 ± 9 bc	G	***
	treated	119 ± 9 ab	203 ± 13 d	150 ± 30 bc	203 ± 5 d	T	**
						GxT	**
Number of leaves	**control**	66 ± 3 ab	243 ± 60 c	34 ± 2 a	131 ± 30 ab	G	***
	treated	43 ± 7 a	145 ± 49 bc	33 ± 2 a	83 ± 12 ab	T	**
						GxT	ns
FW biomass (g/plant)	**control**	1018 ± 158 cd	1596 ± 28 e	219 ± 44 a	557 ± 47 b	G	***
	treated	470 ± 67 ab	1259 ± 121 d	206 ± 40 a	938 ± 48 c	T	**
						GxT	***
DW biomass (g/plant)	**control**	151 ± 34 bc	312 ± 44 de	42 ± 5 a	162 ± 16 bcd	G	***
	treated	89 ± 11 ab	240 ± 26 e	43 ±8 a	179 ± 21 cd	T	*
						GxT	*
Number of fruits	**control**	5.53 ± 1.38 a	19.86± 6.71 a	12.26 ± 6.00 a	34.56 ± 9.50 b	G	***
	treated	9.03 ± 0.77 a	25.26± 4.28 a	18.83 ± 8.43 a	70.96 ± 28.38 c	T	*
						GxT	ns
Medium fruit weight (g/plant)	**control**	98 ± 54 bc	24 ± 7 ab	43 ± 8 abc	12 ± 1 a	G	***
	treated	113 ± 57 c	26 ± 9 ab	45 ± 8 abc	12 ± 4 a	T	ns
						GxT	ns
Yield (kg/pt)	**control**	0.50±0.25 a	0.58±0.36 a	0.49±0.24 a	0.40±0.11 a	G	ns
	treated	0.83±0.41 a	1.03±0.60 a	0.67±0.33 a	0.89±0.52 a	T	*
						GxT	ns
Pollen viability (%)	**control**	80.25 ± 6.33 b	78.54 ± 5.27 b	42.92 ± 12.24 a	79.80 ± 4.47 b	G	***
	treated	92.03 ± 6.99 b	86.57 ± 11.43 b	81.09 ± 12.72 b	89.12 ± 10.90 b	T	***
						GxT	**

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

Supplementary Table S3: Analyses of variance and mean comparison for maximal PSII photochemical efficiency (Fv/Fm), leaf area (LA), Specific leaf area (SLA), Leaf dry matter content (LDMC) and Relative water content (RWC) *per* plants of different tomato cultivars treated with the biostimulant Cyco Flow applied by fertirrigation 4 times. Means ± SD within a rows and columns followed by the different letter are significantly different based on based on Tukey-HSD test ($p \leq 0.05$).

		E17	E36	E107	PDVIT	SIGNIFICANCE	
Fv/Fm	**control**	0.76 ± 0.02 ab	0.77 ± 0.02 bc	0.75 ± 0.02 ab	0.74 ± 0.01 a	G	ns
	treated	0.75 ± 0.02 ab	0.77 ± 0.02 bc	0.78 ± 0.01 c	0.77 ± 0.01 b	T	**
						GxT	***
LA (cm^2)	**control**	29.89 ± 2.03 c	21.47 ± 5.48 ab	29.93 ± 4.03 c	19.49 ± 1.86 a	G	***
	treated	30.43 ± 7.40 c	25.17 ± 3.61 abc	27.90 ± 2.43 bc	19.02 ± 2.31 a	T	ns
						GxT	ns
SLA (cm^2/g)	**control**	227.28 ± 25.58 c	195.51 ± 42.34 bc	180.45 ± 12.55 ab	146.65 ± 18.27 a	G	***
	treated	203.40 ± 16.33 bc	187.07 ± 17.73 abc	212.42 ± 9.42 bc	183.56 ± 18.48 abc	T	ns
						GxT	**
LDMC (g/g)	**control**	0.09 ± 0.01 a	0.10 ± 0.02 ab	0.10 ± 0.01 ab	0.12 ± 0.01 b	G	ns
	treated	0.10 ± 0.01 ab	0.10 ± 0.00 ab	0.09 ± 0.00 ab	0.10 ± 0.01 ab	T	ns
						GxT	*
RWC (%)	**control**	62.01 ± 12.79 a	59.61 ± 17.97 a	68.94 ± 21.50 a	60.82 ± 8.36 a	G	*
	treated	71.47 ± 1.77 a	63.99 ± 11.21 a	87.27 ± 17.11 a	62.54 ± 8.98 a	T	ns
						GxT	ns

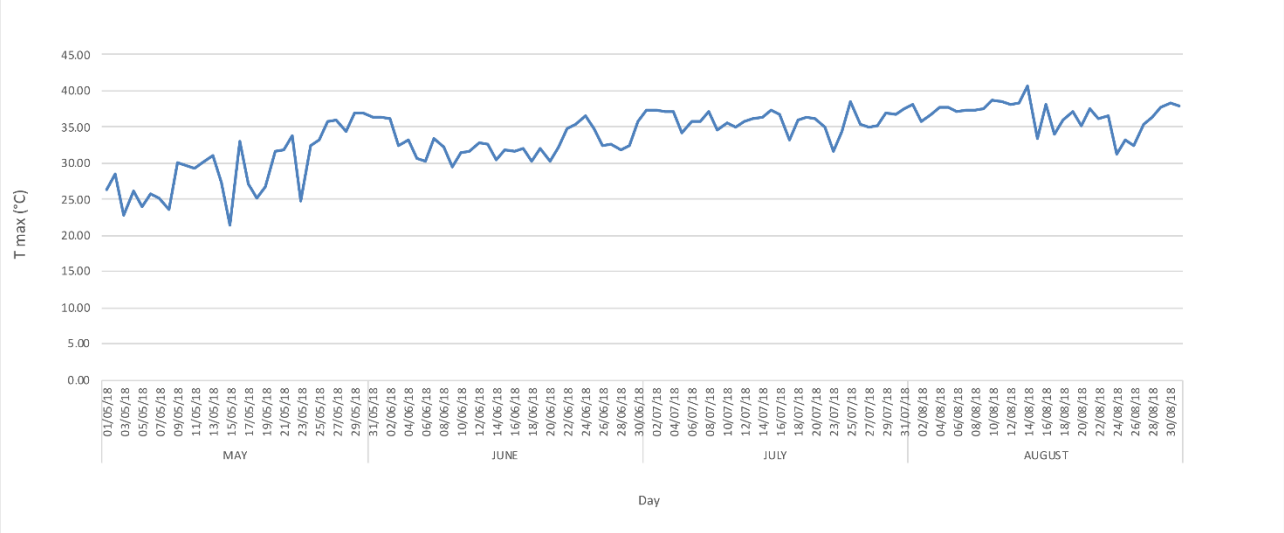
G = genotype; T = treatment; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$

Supplementary table S4: Analyses of variance and mean comparison for total lipophilic antioxidant activities (LAA), carotenoids, chlorophylls a and b (Chl A and Chl B, respectively) content in fruit of different tomato cultivars treated with the biostimulant Cyco Flow applied by fertirrigation 4 times. Means ± SD within a rows and columns followed by the different letter are significantly different based on Tukey-HSD test ($p \leq 0.05$).

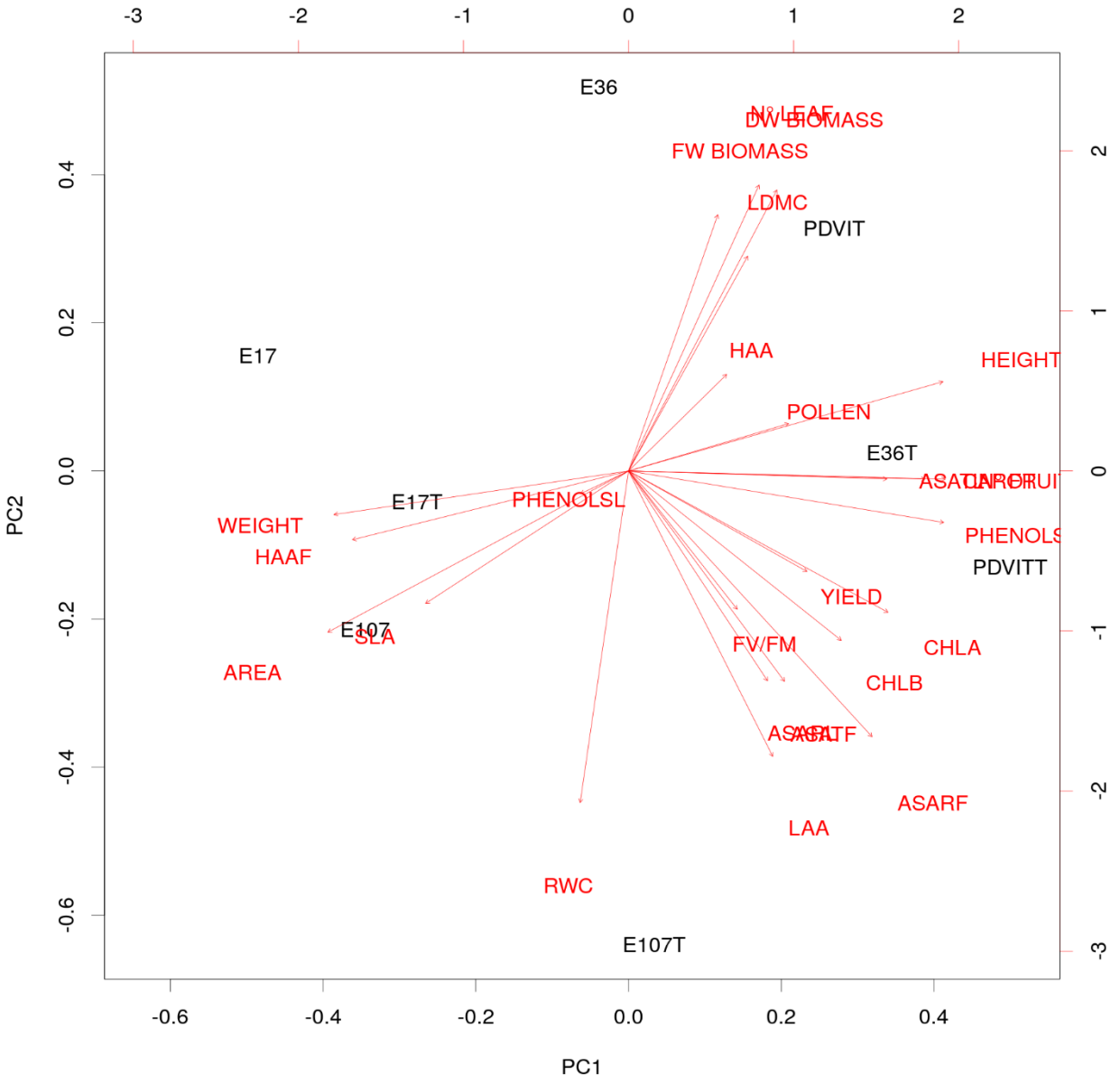
		E17	E36	E107	PDVIT	SIGNIFICANCE	
Carotenoids (mg/100 g FW)	**control**	5.01 ± 0.36 c	6.09 ± 0.09 cd	0.94 ± 0.04 a	6.90 ± 0.40 d	G	***
	treated	3.17 ± 0.30 b	5.26 ± 0.23 c	0.99 ± 0.03 a	8.43 ± 0.59 e	T	ns
						GxT	***
Chl A (mg/100 g FW)	**control**	2.94 ± 0.39 b	1.53 ± 0.19 ab	2.45 ± 0.46 ab	1.14 ± 0.21 a	G	**
	treated	2.30 ± 0.33 ab	1.48 ± 0.16 ab	2.07 ± 0.35 ab	2.04 ± 0.32 ab	T	ns
						GxT	ns
Chl B (mg/100 g FW)	**control**	3.84 ± 0.50 a	2.24 ± 0.25 a	2.43 ± 0.30 a	2.59 ± 0.50 a	G	*
	treated	3.38 ± 0.33 a	2.24 ± 0.15 a	2.48 ± 0.36 a	2.85 ± 0.56 a	T	ns
						GxT	ns
LAA (mg/100 g FW)	**control**	18.26 ± 0.31 a	18.88 ± 0.30 ab	19.32 ± 0.04 bc	19.67 ± 0.11 bc	G	***
	treated	19.57 ± 0.25 bc	19.74 ± 0.05 c	19.89 ± 0.02 c	19.85 ± 0.03 c	T	***
						GxT	**

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

Figure S1: Maximum temperatures recorded in the experimental field located in Battipaglia during the day from May to August 2018**.**



Supplemetary Figure 2: Principal Component Analysis (PCA) of phenotypic and physiological traits in tomato plants treated or not with the biostimulant Cyco Flow. The treated genotypes are indicated by the letter T after the name.



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

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

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

1. Introduction

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

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

Chapter 5. Plant phenotyping to understand the biostimulant action of a protein hydrolysate in tomato plants under combined abiotic stresses.

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Chapter 5. Plant phenotyping to understand the biostimulant action of a protein hydrolysate in tomato plants under combined abiotic stress.

Abstract: Adverse environmental conditions due to climate change today require an accurate identification of the effects of abiotic stresses critical to crop growth. Plant phenotyping can play a pivotal role for the selection of resilient genotypes and, in general, for the identification of the best strategies to increase plant tolerance. In the present study, performed in the framework of an EPPN2020 plant phenotyping program, we investigated the effects of one protein hydrolysate-based biostimulant (CycoFlow-Agriges) on the physiological responses of two tomato genotypes (E42 and LA3120) subjected to heat stress, drought and combined stress. Biostimulants could be the ideal strategy to improve plant resilience to abiotic stress regulating physiological processes in plants and promoting growth. In order to understand the complexity of plant responses to abiotic stresses and to the applied biostimulant, we analyzed biometric traits, physiological responses and the amount of key metabolites. Interestingly, the application of the biostimulant increased plant height (up to 11.86% higher), number of leaves (up to 29.89%), shoot fresh weight (up to 28.12 %) and chlorophyll content (up to 12.03 %) in treated plants subjected to combined stress. An increase in net photosynthetic rate (P_N) and in the maximum quantum efficiency of photosystem II (F_v/F_m) under osmotic stress was also registered. Under heat stress, biostimulant application increased also stomatal conductance and transpiration rate. Under combined stress the amount of proline, H_2O_2 and malodialdehyde content in plants treated with the biostimulant significantly decreased, while the ascorbic acid content increased in treated plant compared to non-treated plants. Altogether, these results provide evidence that the application of one plant-based biostimulant increased plant performances, highlighting the great potential of this strategy to increase plant resilience to abiotic stress.

5.1 Introduction

Plants are continuously subjected to a multitude of stresses, from seed germination through to the whole life cycle. These stresses are commonly divided into two categories, biotic and abiotic stresses, depending on the nature of the trigger factor (Deryng, D. et al. 2014). Results from a variety of biotic and abiotic stresses as well as their interactions show that the impact of climate changes on crop production are complex and diverse. Drought and heat stress are reported to be major abiotic stresses limiting crop yield worldwide (Deryng, D. et al. 2014). The occurrence of high temperature or soil water depletion can result in a range of morphological, anatomical, physiological and biochemical changes in plants. It can directly induce alterations in existing physiological processes, or indirectly promote alterations in the pattern of the plant's development (Chaves, M.M et al.2009). Moreover,

abiotic stress caused by adverse environmental conditions, such as drought, heat, heavy metal toxicity, and high light, do not directly affect photoinhibition but rather facilitate the inhibition of photosystem II (PSII) damage repair (Murata, N. et al. 2007). Several studies in plants have suggested that the extent of photodamage to PSII is directly proportional to the intensity of incident light and that this proportionality remains unaffected by various environmental stresses (Gombos, Z. et al.1994; Nishiyama, Y et al. 2004; Takahashi, S. and Murata, N. 2008). In particular, tomato (*Solanum lycopersicum*), one important vegetable crop widely grown worldwide, is regarded as a heat sensitive crop, although this sensitivity varies among genotypes (Zhou,R. et al. 2016; Zhou, R. et al.2018a; Zhou, R. et al.2018b). Considering also global climate change, it is likely that crops will face a higher incidence of combined heat and drought stresses in the future years. Despite this fact, a limited number of papers focused on the response of crops to several abiotic stresses simultaneously occurring during plant growth (Zhou, R. et al.2018a; Zhou, R. et al.2018b; Poudyal, D. et al. 2018; Zhou, R. et al.2019a, Zhou, R. et al.2019b). In addition, up to date, the possible strategies and management practices, including the application of specific biostimulants, that can be used to alleviate plant abiotic stresses are still unclear. Application of algal extracts, protein hydrolysates, humic and fulvic acids, and other compounded mixtures are known to be able to improve nutrient use efficiency and enhance tolerance to abiotic stresses (Van Oosten, M.J. et al. 2017). Treatments with specific biostimulants may also be able to enhance both leaf and fruit pigments and antioxidants (phenolic acids, carotenoids) contents (Paradikovic, N. et al. 2011; Chehade, L.A. et al. 2018). Beneficial effect of protein hydrolysates applications on plant growth, development and final yield is reported in different work (Parrado et al., 2007; Kowalczyk et al., 2008; Ertani et al., 2009; Gurav and Jadhav, 2013). For instance, Ertani et al. (2009) observed that two protein hydrolysates increased nitrate reductase (NR) and glutamine synthetase (GS) activities in maize seedlings, suggesting a beneficial effect in the induction of nitrate conversion into organic nitrogen. Colla et al. (2014) demonstrated the effects of the plant-derived protein hydrolysate on 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 of hydroponically grown maize plants (Nardi, S. et al. 2016). Protein hydrolysates can also improve crop tolerance to abiotic stresses as reported by Ertani et al. (2013) who tested the efficacy of these compounds to increase salinity tolerance in *Zea mays*. In a previous study, we investigated the response to high temperatures in tomato plants grown in open fields and treated with a novel protein hydrolysate-based biostimulant (Cycoflow, Agriges). It was demonstrated that this product had a clear effect on plant growth and on plant yield and yield component (Francesca, S. et al. 2020). In order to further understand the physiological response of tomato plants to this protein hydrolysate-based biostimulant, in the present

study analyses were carried out by using a phenotyping platform in plants grown under controlled conditions and subjected to combined abiotic stresses. Our hypothesis was that the use of an amino acid-based biostimulant could enhance plant performances under combined abiotic stress because of its putative cytokinin-like action and its high concentration of glycine betaine known to mitigate the effect of abiotic stress (Di Stasio, E. et al.2018; Sorwong, A. and Sakhonwansee, S. 2015). The outcomes of this work will be important for the selection and breeding of tomato genotypes tolerant to abiotic stresses and will suggest the useful management practise to be used to improve plant performances and final yields.

5.2 Materials and methods

5.2.1 Plant material/ Experimental design/ Growth conditions

Two different genotypes selected based on previous studies carried out at the Department of Agricultural Sciences of the University of Naples, 'E42' and 'LA3120', were used. 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, air temperature was 24±3°C during the day and 18±3°C during the night. The seedlings were irrigated by flooding the benches every morning for 10 min with a full nutrient solution. The 26-day-old seedlings were randomly divided into eight groups with 10 plants per cultivar for each treatment.

The experiment 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 three days when the tomatoes under single drought and combined stresses showed significant phenotypical changes. The biostimulant, in liquid formulation, was initially applied directly to the pots (50 mL per plant) at 20 DAS (days after sowing) and 26 DAS (the day before starting stress). The biostimulant was applied by syringe at a final concentration of 3 g/L, according to previous study. The treatments without biostimulant received the same amount of water. The biostimulant tested 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. Its composition was previously reported [Francesca, S. et al. 2020]

5.2.2 Biometric measurements

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 hours and dry weight (DW) of shoot was measured. On day 3, one leaflet from the first fully expanded leaf of three plants per cultivar and per treatment was cut to calculate relative water content (RWC in %) = [(Fresh Weight-Dry Weight) / (Turgid Weight-Dry Weight)] * 100. Shoot water content (SWC in %) was calculated using formula = [(Fresh Weight-Dry Weight) / (Fresh Weight)] *100.

5.2.3 Gas exchange and chlorophyll fluorescence content

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 and light intensity setting of the cuvette during the measurements corresponded to the respective growth conditions of plants at each treatment. The leaf was placed in the cuvette and the measurements were recorded when P_N and g_s reached a steady state. To maintain the 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. Five replications for each sample were recorded. Chlorophyll fluorescence measurement was performed on the first fully expanded leaf of three plants per cultivar per treatments on day 3 using a MINI-PAM (Walz, Effeltrich, Germany). Prior to measurement of F_v/F_m, leaves were dark adapted with a dark leaf clip for 30 min. For each leaf, three random spots were measured.

5.2.4 Dualex measurements

Leaf chlorophyll (chl) content was non-destructively monitored using a Dualex 4 Scientific (Dx 4) (FORCE-A, Orsay,France) [Chen, L. et al.2012]. Three plants *per* cultivar and *per* treatment were measured. Three random spots from both adaxial and abaxial side of each leaf were monitored, and the six values were averaged. For each treatments the measurements were performed the same day of gas exchange. In the same way chlorophyll, anthocyanins, flavonols content and nitrogen balance index (NBI) were measured.

5.2.5 Ascorbic acid content

Quantification of reduced ascorbic acid (AsA) and total ascorbic acid (AsA + dehydroascorbate − DHA) measurements were carried out by using a colorimetric method (Stevens et al., 2006) with

modifications reported by Rigano et al. (2014). Briefly, 500 mg of frozen powder from tomato leaves were extracted with 600 µL of ice cold 6% trichloroacetic acid (TCA) and the mixture was then incubated for 15 min on ice and centrifuged at 14,000 rpm for 20 min. For reduced AsA evaluation, to 20 µL of supernatant were added 20 µL of 0.4 M phosphate buffer (pH 7.4), 10 µL of double distilled (dd) H_2O and 80 µL of color reagent solution. This solution was prepared by mixing solution A (31% (w/v) H3PO4, 4.6% (w/v) TCA and 0.6% (w/v) FeCl3) with solution B (4% (w/v) 2,20-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 (NEM; 0.5% (w/v) in water) were added and left for 1 min at room temperature. Eighty microliters of color reagent were added as previously described for reduced AsA. Both the final mixtures were incubated at 37 °C for 40 min and measured at 525 nm by using a Nano Photometer TM (Implen, Munich, Germany). Three separated biological replicates for each sample and three technical assays for each biological repetition were measured. The concentration was expressed in µmol/g of fresh weight (FW).

5.2.6 Hydrogen peroxide and malondialdehyde determination

Quantification of H_2O_2 content was carried out by using a colorimetric method (Sergiev et al.,1997). Briefly, 500 mg of frozen powder from tomato leaves were extracted with 5 mL of ice cold 0.1% 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 were added 500 µL phosphate buffer 10 mM (pH 7.0) and 1 mL of potassium iodide (1 M). 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 biological replicates for each sample and three technical assays for each biological repetition were measured. The concentration was expressed in mmol/g FW. The first fully open leaf was taken for the determination of malondialdehyde (MDA). The MDA levels in leaf tissues indicate the levels of membrane lipid peroxidation. Briefly, 0.2 g of leaf sample was ground by adding 1 mL of ice cold 0.1% 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 1,250 mL reaction solution (TCA 20% + TBA 0.5%), water-bathed for 30 min at 95 °C and measured at 532 and 600 nm by using a Nano Photometer TM (Implen, Munich, Germany). Three separated biological replicates for each sample and three technical assays for each biological repetition were measured. The concentration was expressed as quantity of MDA-TBA complex [Zhang and Kirkham,1996].

5.2.7 Proline content measurement and soluble sugar determination

Proline content was determined according to the method of Claussen (2005), 250 mg of frozen powder from tomato leaves were suspended in 3 mL of 3% 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 of a 6:3:1 solution of glacial acetic acid, distilled water and 85% 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 for 5 min. Readings were taken immediately at a wavelength of 546 nm. The proline concentration was determined by comparison with a standard curve. Fresh leaf was extracted in 80% 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, ATP, NADP and G6PDH. The absorption was recorded at 340 nm before adding 1 µl Hexokinase, then when stabilised measure OD and add1 µl Phosphoglucose isomerase, then when stabilised measure OD and add 1 µl Invertase then when stabilised measure OD. The reading from each sample were espressed as µmol eq. g FW^{-1}.

5.2.8 Stomatal anatomy

Abaxial (lower) and adaxial (upper) surfaces of the mid-region primary flag leaf 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 of each genotype in each treatment were sampled on the last day of stress treatment and three pictures were taken per leaflet using a magnification of 20x (Leica DM R microscope equipped with a DFC 425 C camera (Leica Microsystems, Germany)). Stomatal number was accessed on 9 fields per treatment and stomatal characteristics (length, width) were determined.

5.2.9 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 heat map, generated by using the http://biit.cs.ut.ee/clustvis (accessed on 1 April 2021) program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage heatmap.

5.3 Results

5.3.1 Effect of abiotic stresses and biostimulant treatment on plant growth parameters and metabolite content

In this study two different tomato genotypes (E42 and LA3120) were treated with a protein hydrolysate-based biostimulant and were subjected to single and/or combined abiotic stresses. Drought and combined stress induced a significant reduction in plant height in both genotypes (Table1), In particular, under drought condition, plant height decreased by 17% in E42 and 13% in LA3120 in treated plants compared to control non-treated plants. The treatment with the biostimulant increased the plant height of 'LA3120' under control condition and 'E42' under combined stress (Table 1). When the E42 and LA3120 plants were subjected to combined stress, the leaf number of both genotypes significantly increased after the treatment with the biostimulant (+30 % and +40%, respectively) (Table 1). For the genotypes 'E42' the leaf area was significantly lower under drought and combined stress compared to control and heat stress (Table 1). In 'LA3120' treatment with the biostimulant increased leaf area when plants were subjected to drought (Table 1). For the shoot fresh weight, in 'E42' the treatment with the protein hydrolysate increased this trait by 28% under combined stress (Table 1). According to ANOVA, the interaction of genotypes, stresses and biostimulant treatment did not induce significant differences on biometric parameters (Table S1). Both genotypes maintained a steady level of RWC and SWC under all treatments. Only in LA3120, drought treatment significant reduced shoot water content (SWC) compared to plants under control conditions, however, the treatment with the protein hydrolysate led to a 5% increase compared to non-treated plants under drought stress. In both genotypes, plant under drought and combined stress showed wilted stem but the plants treated with the biostimulant under drought appeared less stressed than non-treated plants (Fig.1). On the contrary, plants under heat stress did not show clear differences compared to the control (Fig.1 a,b). The chlorophyll content measured non-destructively in both genotypes treated with the protein hydrolysate increased by 12.03% in 'E42' under combined stress compared to non-treated plants (Table 2). The treatment withthe biostimulant had no effects on the content of flavonols, anthocyanins and NBI, but it is possible to note the differences due to the different stresses applied (Table 2).

Table 1. Plant height, leaf number, leaf area, shoot fresh weight (FW), shoot dry weight (DW), relative water content (RWC) and shoot water content (SWC) of two tomato genotypes after three day of control (C), drought (D), heat (H) and combined (H+D) 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							
		C	**C_B**	**H**	**H_B**	**D**	**D_B**	**H+D**	**H+D_B**
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
RWC (%)	**E42**	61.57±0.62 a	64.59±4.18 a	64.86±5.97 a	63.77±10.57 a	63.19±5.88 a	59.19±8.31 a	59.33±3.54 a	59.09±1.80 a
	LA3120	56.31±6.80 ab	54.28±8.58 ab	67.91±6.48 bc	73.50±1.86 c	46.71±8.80 a	57.04±7.26 ab	55.69±9.91 ab	59.51±3.00 ab
SWC (%)	**E42**	92.94±0.20 a	92.95±0.37 a	91.64±1.36 a	87.15±4.65 a	91.86±2.98 a	86.41±8.51 a	88.24±2.03 a	86.82±3.93 a
	LA3120	92.61±1.17 b	91.58±0.36 b	90.69±2.03 b	92.20±0.94 b	85.61±4.69 a	89.53±1.97 ab	89.43±1.69 ab	90.78±1.54 b

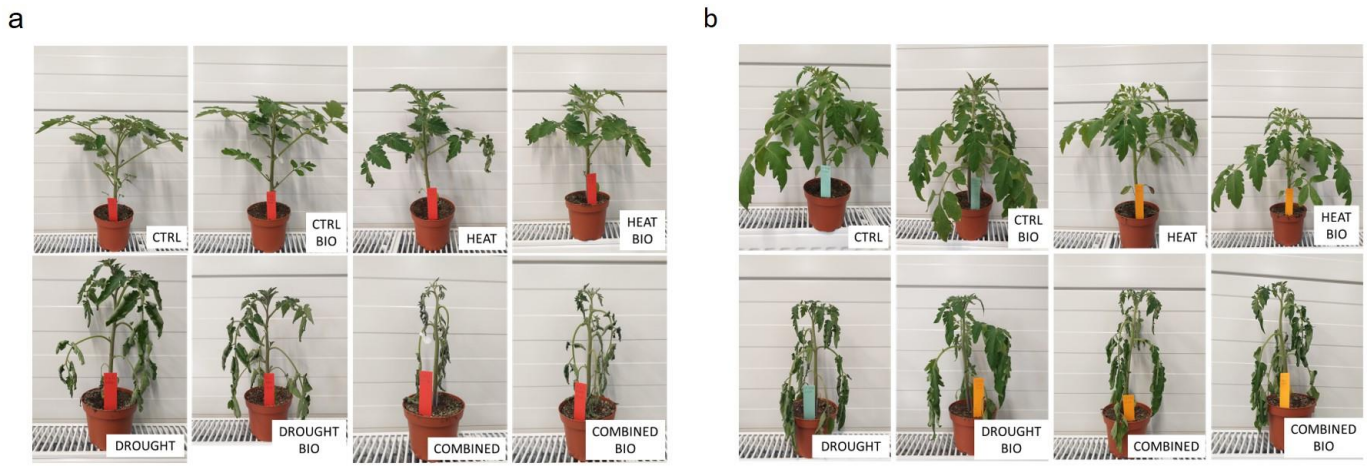

Figure 1. The effect of heat, drought and combined stress (heat+drought) on phenotypes of a 'E42' and b 'LA3120'.

Table 2. Dualex measurements: chlorophyll content (Chl), flavonols content, anthocyanins content and nitrogen balanced index (NBI) in the leaves of two tomato genotypes after three day of control (CTRL), drought (D), heat (H) and combined (H+D) 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							
		C	C_B	H	H_B	D	D_B	H+D	H+D_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
Flavonols (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
Anthocyanins (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

5.3.2 Effect of abiotic stresses and biostimulant treatment on leaf gas exchange and chlorophyll fluorescence

The imposed drought and combined stress negatively affected transpiration rate (E), stomatal conductance (g_s) and net photosynthetic rate (P_N) in both genotypes. Biostimulant induced higher E values in 'LA3120'plants under heat stress and 'E42' control plants; further, biostimulant treatments induced high g_s in both genotypes subjected to heat stress (Fig.2a, b). Whereas, under drought, 'E42' plants treated with the protein hydrolysate, showed a greater net photosynthesis level compared to non-treated plants (Fig.2c).The Fv/Fm value determined in 'E42' and 'LA3120' was significantly lower in plants subjected to drought stress compared to the control. Only in the genotype 'E42' biostimulant treatments significantly increased Fv/Fm levels under drought (Fig. 3).

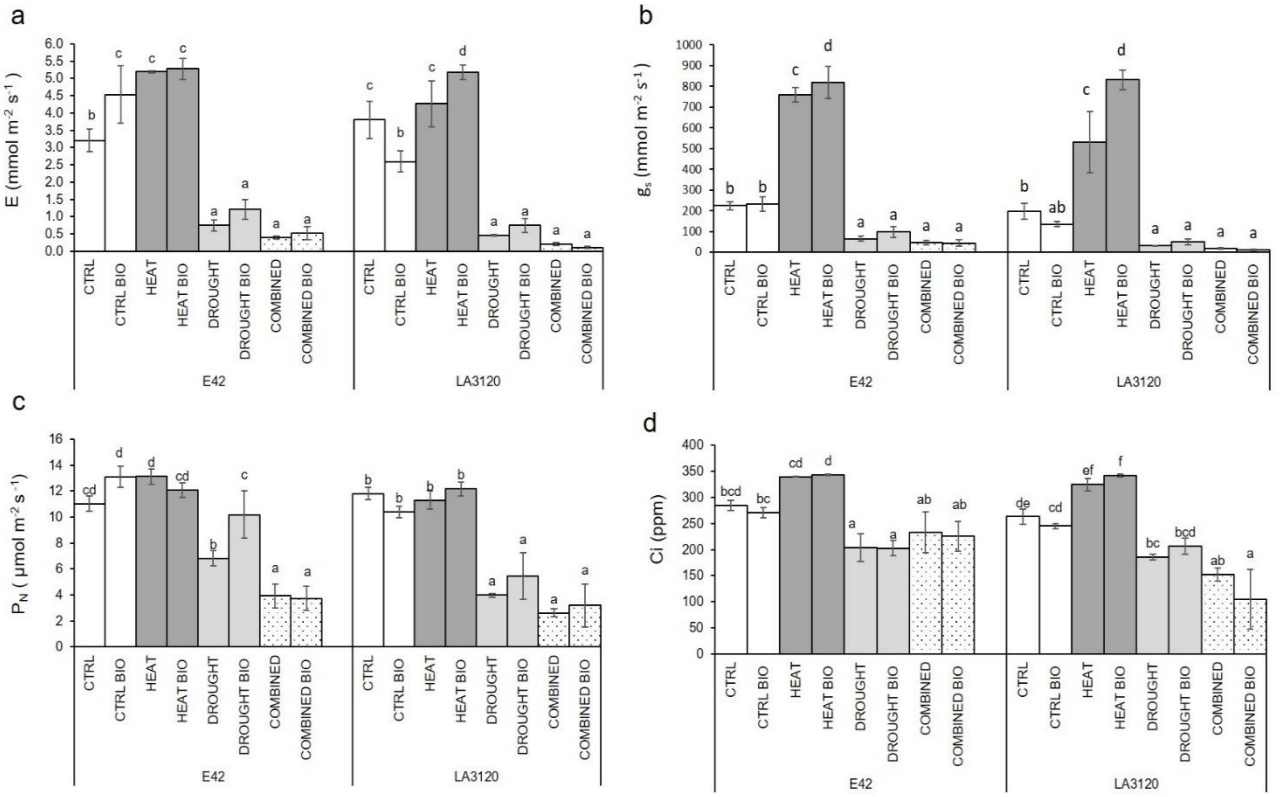

Figure 2. Gas exchange measurements in the leaves of two tomato genotypes under control, drought, heat and combined stress, with and without biostimulant. Different sub-graphs represent a traspiration rate (E), b stomatal conductance (gs), c net photosynthetic rate (Pn) and d intracellular CO_2 concentration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

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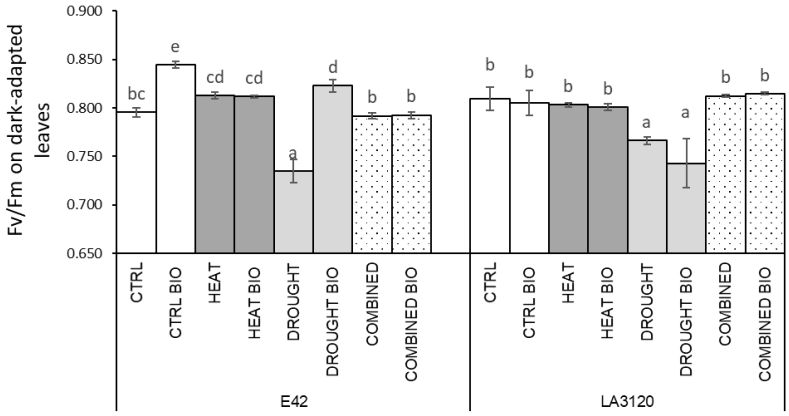

Figure 3. Fv/Fm on dark adapted leaves of two tomato genotypes under control, drought, heat and combined stress, with and without biostimulant. The data represent mean value± SE (n=3). Different letters above the bars indicate significant differences (P<0.05).

5.3.3 Effect of abiotic stresses and biostimulant treatment on antioxidants content

Total ascorbic acid content increased under drought in both genotypes. Under combined stress in 'LA3120' a decrease of total ascorbic acid was registered, while, on the contrary, in 'E42' plants an increase of total ascorbic acid content was demonstrated. The use of biostimulant leads to an increase in the total ascorbic acid content in all treatments in 'E42' (Fig.4a). In the 'E42' non treated plants, the content of reduced ascorbic acid does not change under drought and combined stress. On the contrary, in the 'LA3120' genotype, the content of reduced ascorbic acid increased under all applied stresses (Fig.4b). The H_2O_2 content of 'LA3120' and 'E42' significantly increased under drought and combined stress compared to control. The biostimulant application leads to a decrease in the accumulation of H_20_2 content in both genotypes under all stresses, except for treated 'E42' plants under heat stress (Fig.4c). Similarly, the MDA content in both genotypes significantly increased under combined stress compared to the control treatment (Fig.4d). Plants treated with the protein hydrolysate under drought in 'LA3120' and combined stress in 'E42' showed a decrease in MDA content compared to non-treated plants.

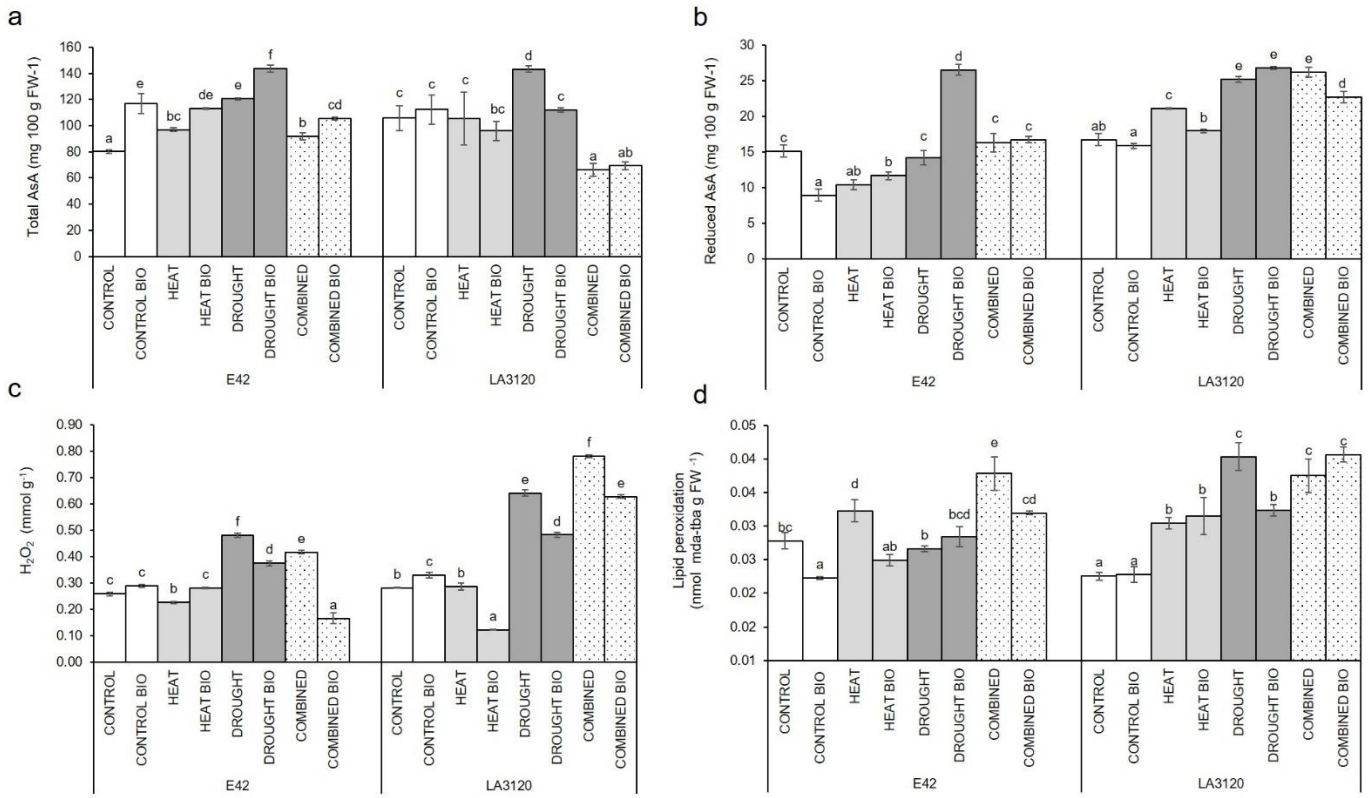

Figure 4. Hydrophilic antioxidants and oxidative markers in the leaves of two tomato genotypes under control, drought, heat and combined stress, with and without biostimulant. Different sub-graphs represent **a** Total ascorbic acid (AsA), **b** reduced ascorbic acid (AsA), **c** hydrogen peroxide (H2O2) concentration, **d** lipid peroxidation (LP), measured as malondialdehyde (mda-tba) content. The data represent mean value± SE (n=3). Different letters above the bars indicate significant differences (P<0.05).

5.3.4 Effect of abiotic stresses and biostimulant treatment on proline and soluble sugars content

Metabolic alterations induced by drought and combined stress generally include leaf accumulation of the osmolyte proline. Proline amount in stressed plants decreased in response to biostimulant application only in genotype ‘E42’ (Fig. 5a). Drought and combined stress increased the soluble sugars concentration in both genotypes (Fig.5b, c, d). In particular, the content of glucose in the leaves of LA3120 plants was significantly reduced by protein hydrolysate treatment under drought and combined stress (Fig.5d).

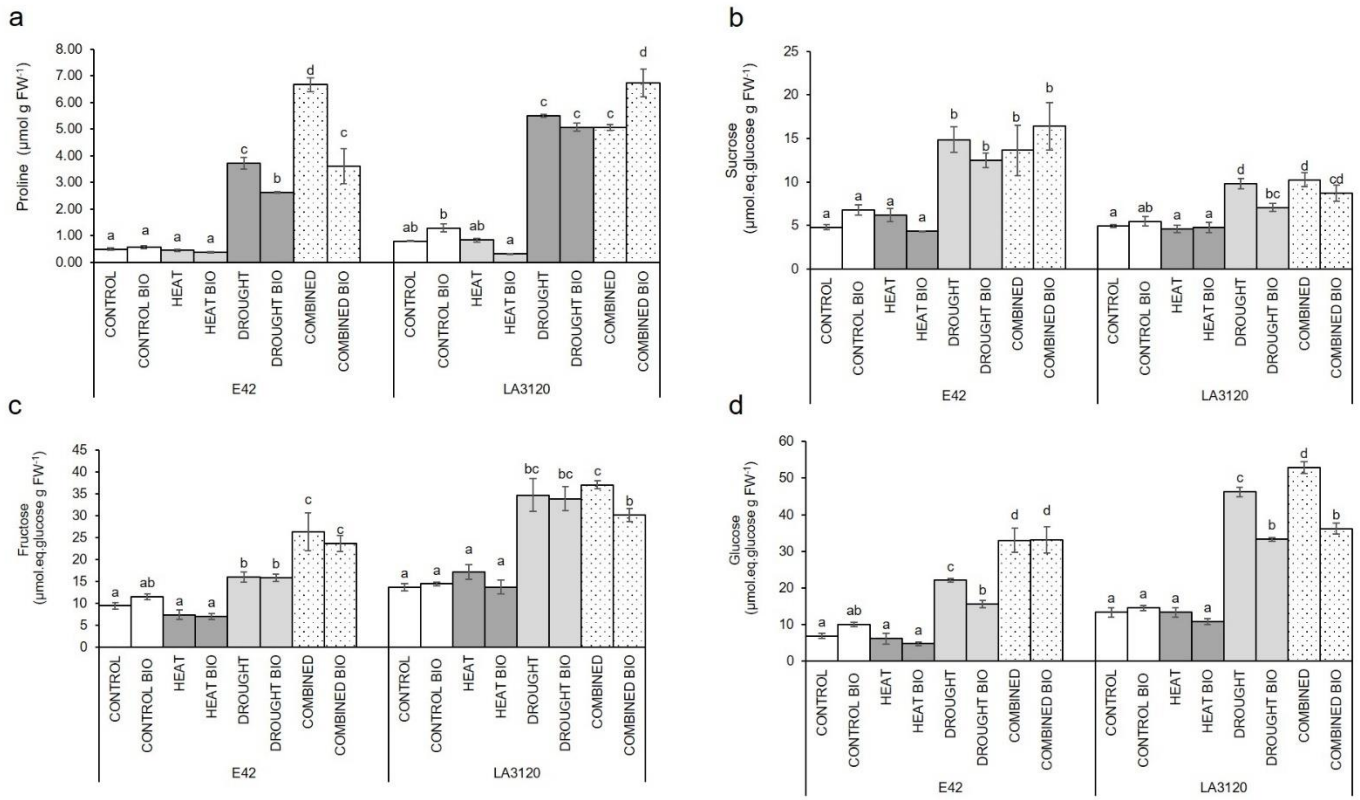

Figure 5. Proline and soluble sugars content in the leaves of two tomato genotypes under control, drought, heat and combined stress, with and without biostimulant. Different sub-graphs represent **a** proline content, **b** sucrose, **c** fructose, **d** glucose content. The data represent mean value±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

5.3.5 Effect of abiotic stresses and biostimulant treatment on stomatal anatomy

The two genotypes showed a significant difference in stomatal anatomy under different stress applied (Fig. 6). The stomatal number of the leaflets significantly increased in E42 under all the stresses applied and only under combined stress in LA3120. After the application of the biostimulant under heat stress in E42 and under drought in LA3120 there was a decrease in stomatal number (Fig.6a). Stomatal length in E42 genotype decreased under all stress applied compared to control plants, while the LA3120 genotype exhibited an opposite behavior (Fig.6b). For stomatal width, in E42 it is possible to note the same trend evidenced for stomatal length under all stresses applied (Fig.6c).

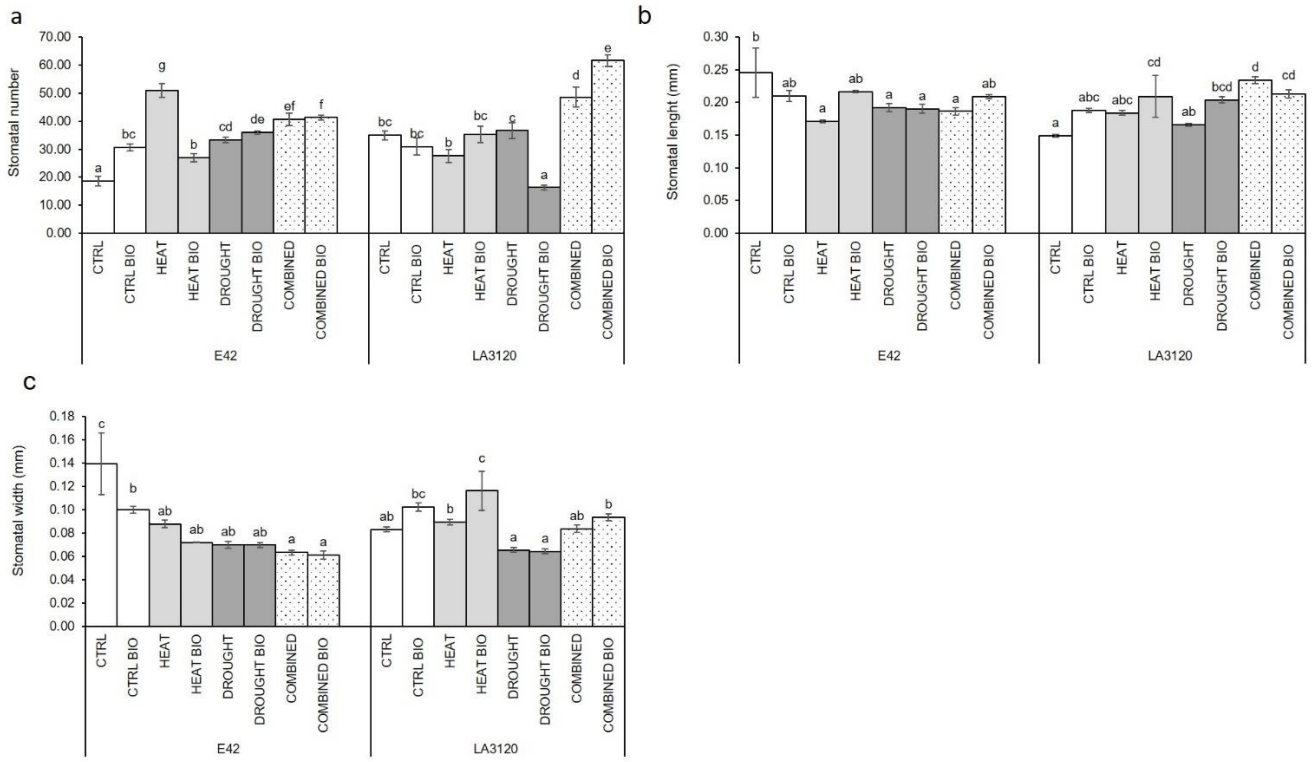

Figure 6. Stomatal characterization of the second fully expanded leaf of two tomato genotypes under control, drought, heat and combined stress, with and without biostimulant. Different sub-graphs represent **a** stomatal number, **b** stomatal lenght and **c** stomatal width. The data represent mean value± SE. Different letters above the bars indicate significant differences (P<0.05).

5.3.6 Principal component and Cluster heat map analysis

A comprehensive overview of biostimulant application and abiotic stress effects on all parameters studied in the two tomato genotypes was obtained through Principal Component Analysis (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 46.44% and 18.02%, respectively (Table S2). The loading plot (Fig 7a) revealed that variables clustered into four main groups, based on the type of stress applied. Control samples with and without biostimulant treatment clustered on the lower right side of the 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 condition is displayed in Figure 7b. The heat-map identified two main clusters which divided the analyzed samples differently. 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. The second cluster associated the samples under combined stress in a sub-group and drought samples in another sub-group. In this last this sub-group the samples "E42 drought plus biostimulant" were further separated (Fig.7b). Considering all the analysed traits in both genotypes, photosynthetic traits (P_N, C_i, g_s, E) and biometric parameters (height, n° leaves, leaf area) were positively correlated with the biomass (Shoot FW), and were negatively correlated with the soluble sugar analysed (Fig. S1).

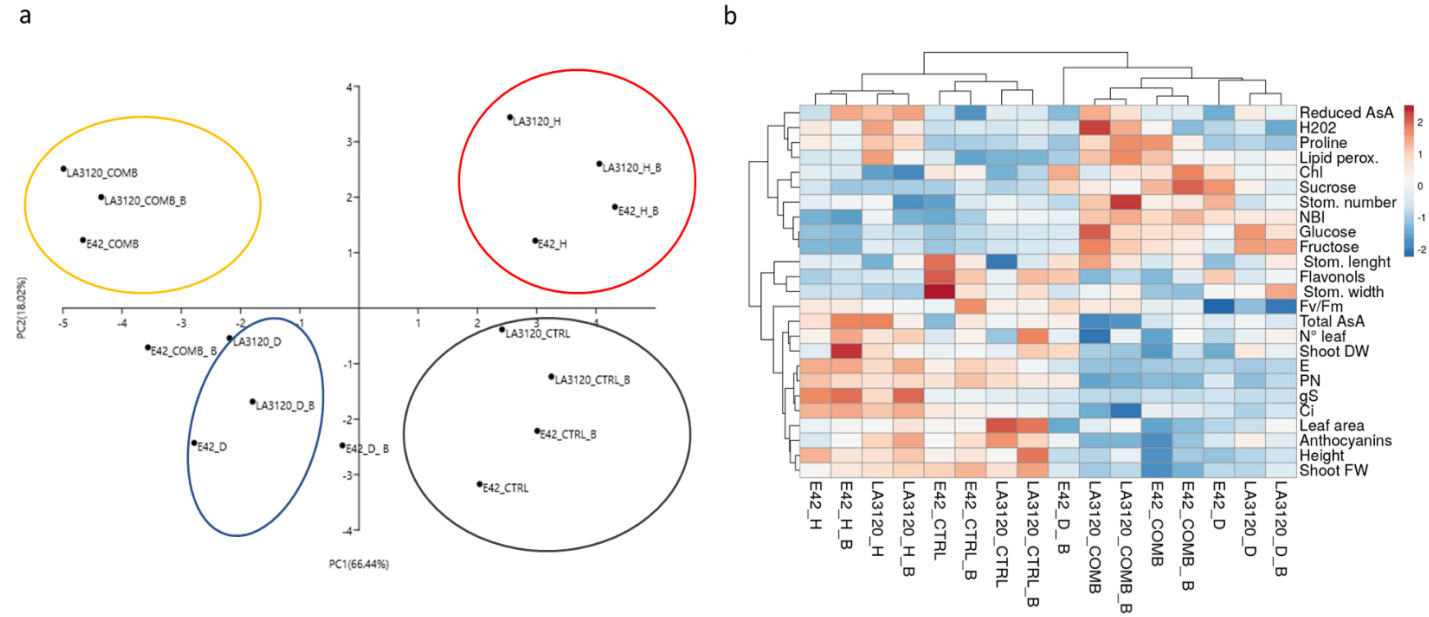

Figure 7. Principal component loading plot and scores of principal component analysis (**a**) and Cluster heat map analysis (**b**) of two tomato genotypes under control (C), drought (D), heat (H) and combined (COMB) stress, without and with biostimulant (_B).

5.4 Discussion

The use of amino acid-based biostimulant could enhance crop performances under combined abiotic stress because of their putative cytokinin-like action and high concentration of glycine betaine known to mitigate the effect of abiotic stress. Recent studies have identified the target metabolic pathways and some of the mechanisms through which protein hydrolysate exert their effects on plants, however, the mode of action of these compounds in treated plants is mostly unknown (Francesca et al. 2021). Moreover, despite the response of tomato plants to combined abiotic stress have been widely described in the literature (Zhou, R. et al. 2018b; Zhou, R. et al. 2017; Camejo, D. et al. 2005), almost nothing is known on the combined action of biostimulant application and combined abiotic stresses. Here, a phenotyping platform was used in order to understand the biostimulant action of a novel protein hydrolysate-based biostimulant (CycoFlow/Agriges) in two different tomato genotypes grown under single and combined abiotic stress. Moreover, physiological and biochemical analysis

were used to further investigate biostimulant-induced tolerance mechanisms. The single and combined stress had a clear effect in both genotypes, as evidenced by the Principal Component Analysis in Fig. 7 dividing the samples into four subgroups based on the different stress applied. The effect of the biostimulant was instead evident mostly for the genotype E42 subjected to drought and combined conditions and treated with the protein hydrolysate. These results are consistent with our previous work, which demonstrated that treatment with this biostimulant on the same genotype (E42) under water deficit, induced better performances, in terms of growth, yield and yield components in plants grown in open field (Francesca et al., 2021).

The different stress applied had a clear effect on plant growth in both genotypes, regardless of the biostimulant application (Table 1). Indeed, in response to single drought and heat stress, a decrease in shoot fresh weight was evidenced without significant differences between treated and non-treated plants. Accordingly, early works showed that drought and heat stress (Zhou, R. et al. 2019a) greatly influenced plant size in tomato. Notably, the combination of drought and heat stress, applied by growing plants to high temperature (30 °C) without water for 3 days, resulted in the most severe reduction in plants biomass, although a higher biomass and height was evidenced in E42 stressed plants treated with protein hydrolysate compared to non-treated ones. This suggest that the presence of free amino acids in the biostimulant, promoted endogenous phytohormonal biosynthesis stimulating growth in E42 treated plants, as demonstrated by Rouphael et al. (2017). The reason for this could be also a cytokinin-like activity promoting cell division triggered by the biostimulant used in this study (Matsuo et al.,2012).

Interestingly, in our study plant biomass was positively correlated with photosynthetic traits (P_N, Ci), stomatal conductance (g_s) and the transpiration rate (E) (Fig S1). According to our expectations, the value of stomatal conductance and transpiration rate increased under heat stress compared to control plants. However, in the same growth conditions, biostimulant application increased even more these two parameters. In contrast, drought and combined stresses led to a strongly decrease in g_s and E, and the protein hydrolysate did not mitigate the adverse effect of drought stress on the transpiration measurements. Generally, stomatal closure is the first reaction of plants to water depletion in order to reduced water loss, accordingly we found that drought stress affected the morphological behaviour of stomata in E42 showing an increase in stomatal number combined with a decrease in stomatal size (length and width). On the contrary, biostimulant application decrease stomatal number in LA3120 genotype under drought condition, contributing to a lower loss of the CO_2 assimilated. In fact, in our experiment treated plants of LA3120 under drought condition had less stomata (no change in stomatal size) and a higher intracellular CO_2 concentration (Ci) compared to non-treated plants. Heat stress had no effect on Fv/Fm values suggesting that in both genotypes a photoprotective mechanism was able to avoid photoinhibition events, according with Zhou et al. (2017). On the contrary, drought and combined treatments caused a strong decrease in P_N (net photosynthesis rate) in both genotypes.

However, in E42 plants under drought stress biostimulant treatment caused an increase in net photosynthesis level and maximal efficiency of PSII photochemistry (Fv/Fm). The higher photosynthetic activity in treated E42 plants under osmotic and combined stress could be related to the presence of glycine betaine and aspartic acid in the biostimulant. Indeed, it is known that, at elevated temperatures, glycine betaine has a crucial role in the repair of photodamaged PSII, in maintaining the activity of Rubisco and in alleviating the inhibition of gas exchanges (Francesca et al., 2020). Different studies have reported the positive effect of this kind of molecules under stressful conditions. For example, glycine betaine accumulation enhanced the net photosynthetic rate 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).

During the water deficit, the overproduction of ROS mainly targets membrane lipids which leads to oxidative damage. Hydrogen peroxide (H_2O_2) is considered to be the main compound involved in the lipid peroxidation of the membrane (Miller et al., 2010). According to Sanchez-Rodriguez et al. (2010), our data showed an increase in H_2O_2 content and lipid peroxidation in both genotypes under drought and combined stress. In response to a redox unbalance and damages from ROS, plant activates their own antioxidant defences. Indeed, both genotypes increased total and reduced ascorbic acid content under drought and combined stress, while H_20_2 and antioxidants content were unchanged under high temperature, suggesting a partial heat tolerance of the two genotypes. Furthermore, biostimulant application increased antioxidant content, including total and reduced ascorbic acid, and decrease hydrogen peroxide (H_2O_2) content in E42 treated plants under water and combined stress conditions. This aspect could be related with specific metabolites that are found in high concentrations in the formulation of the target biostimulant: the glutammine. Recent studies have established a interlink between glutamine and oxidative stress responses (Skopelitis et al.,2006; Liu et al., 2010). For instance, Ji et al. (2019) revealed that this amino acid is involved in the regulation of cellular redox state under abiotic stress in mutants of Arabidopsis (Ji et al.,2019). These results are consistent with a previous work, which demonstrated that biostimulant treatment induced the activation of the antioxidant defense system, increasing content of reduced and total AsA in leaves (Francesca et al.,2020).

Osmotic adjustment is a physiological adaptation of plants associated with water stress tolerance. Proline can act as an osmolyte an protect also enzymes and other cellular macromolecules from damages induced by drought stress (Hare et al., 1998). The current study observed higher levels of proline content in tomato leaves under drought and combined stresses. Interestingly, the proline content decrease in treated plants under water stress conditions compared to non-treated ones, demonstrating the improved tolerance to drought stress of plants treated with the protein hydrolysate. Moreover, our findings revealed that the concentration of soluble sugars of tomato leaves subjected to combined stress increased in both genotypes Sugar accumulation is considered to have an

important role in osmotic adjustment under drought stress and other papers have demonstrated an increase in sucrose content under drought and combined stress (Hare et al., 1998; Martinez et al., 2004). Rizhsky et al., 2004). 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 the activity of sucrose-synthesizing enzymes (Osorio et al., 2014; Keller and Ludlow,1993). Interestingly, sugar accumulation significantly decrease after biostimulant applications, further demonstrating that protein hydolysate application mitigated the effect of abiotic stress. Indeed, the presence of the protective metabolites, such as glycine betaine and proline, in the protein hydrolysate may have enhanced the tolerance of the tomato plants to water deficit, according to previous findings demonstrating that these compounds applied exogenously increased drought tolerance in plants grown under hyper-osmotic conditions, thanks to different mechanisms such as osmotic adjustment, membrane and proteins stabilization, and antioxidant activity (Francesca et al, 2021).

5.5 Conclusions

Altogether in this paper we demonstrated that under water and combined stress, biostimulant treatment provided protection to treated plants. The treatment with the biostimulant had effect dependently of the genotype it was applied on, with E42 showing a stronger response to protein hydrolysate application compared to LA3120. In the future additional studies will be necessary in order to fully understand the mechanisms of action of this class of biostimulants.

5.6 References

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Supplementary material

Supplementary Table 1 Analysis of variance (ANOVA) showing the significance level of the main factors genotypes (G), stress (S), treatment with biostimulant (B) and their interactions on different traits.

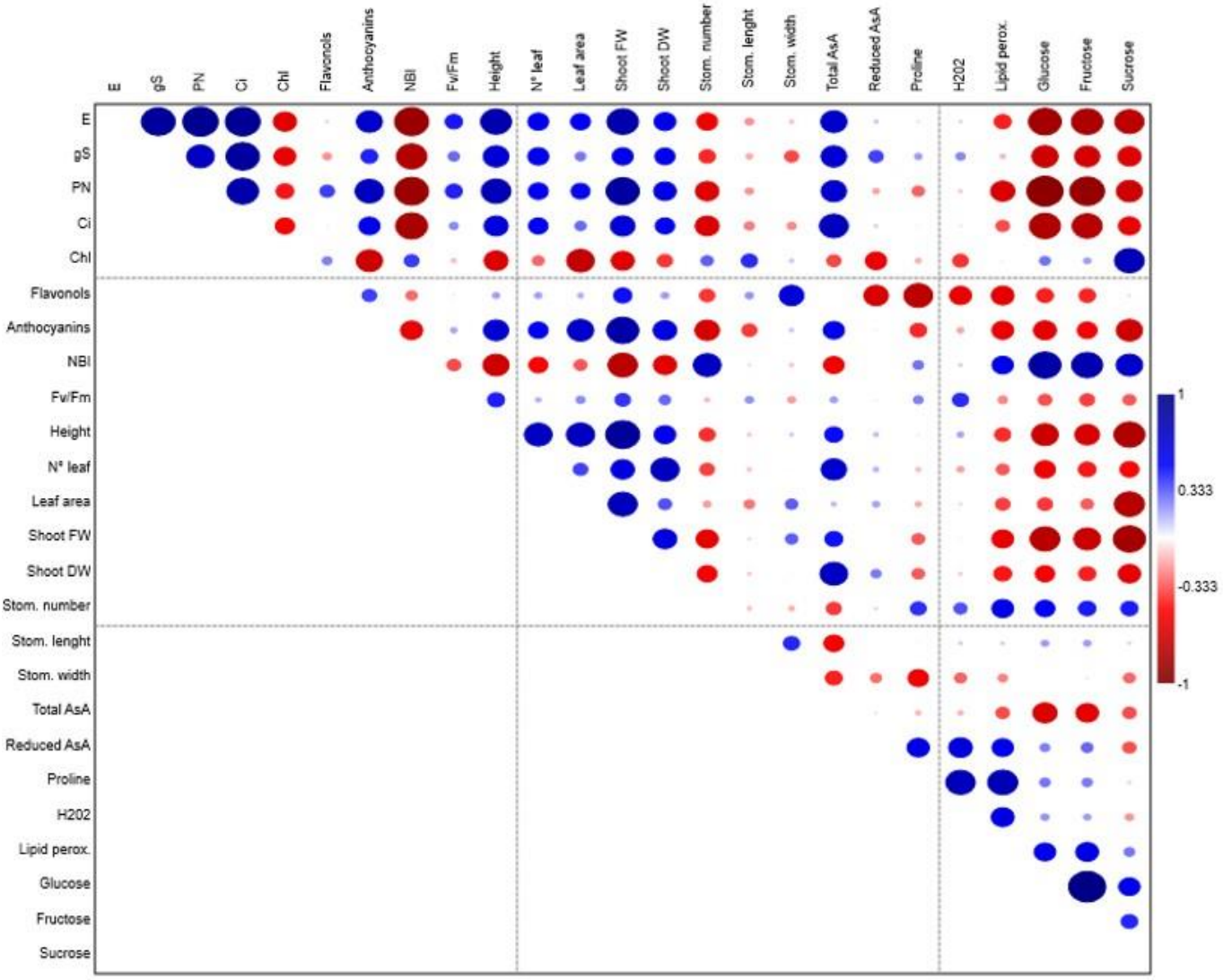
	Main factors			Interactions			
Parameters	**G**	**S**	**B**	**G ×S×B**	**G×S**	**G×B**	**S×B**
Height	**	***	***	ns	*	**	**
N° leaf	ns	***	***	ns	ns	ns	*
Leaf Area	***	***	ns	ns	***	ns	ns
Shoot FW	**	***	***	ns	**	ns	ns
Shoot DW	ns	**	*	ns	ns	*	ns
RWC (%)	ns	**	ns	ns	*	ns	ns
SWC (%)	ns	*	ns	ns	ns	*	ns
Chl	***	***	*	ns	ns	ns	ns
Flavonols	***	*	ns	ns	*	ns	ns
Anthocyanin	***	***	ns	ns	*	*	ns
NBI	ns	***	ns	ns	ns	ns	ns
E	*	***	ns	**	ns	ns	ns
g_s	**	***	**	**	ns	ns	***
P_N	**	***	ns	*	ns	ns	ns
C_i	**	***	ns	ns	**	ns	ns
F_v/F_m	ns	***	**	***	**	***	*
Total AsA	*	**	*	ns	**	***	ns
Reduced AsA	***	***	ns	***	***	***	***
Lipidi perox.	***	***	**	***	***	*	ns
H_20_2	***	***	***	***	***	***	***
Proline	***	***	**	***	***	***	*
Glucose	***	***	***	**	***	***	***
Fructose	***	***	ns	ns	***	ns	ns
Sucrose	***	***	ns	ns	**	ns	ns
Stom. Number	ns	***	ns	***	***	ns	***
Stom. Width	ns	***	ns	ns	***	**	ns
Stom. Lenght	ns	ns	*	**	**	ns	ns

Supplementary Table 2 Eigenvalues, relative and cumulative percentage of total variance, and correlation coefficients for each character.

Principal components	**PC 1**	**PC 2**	**PC 3**	**PC 4**	**PC 5**	**PC 6**	**PC 7**	**PC 8**	**PC 9**	**PC 10**	**PC 11**	**PC 12**	**PC 13**	**PC 14**	**PC 15**
Eigen value	11.61	4.51	2.39	1.85	1.30	1.01	0.80	0.42	0.41	0.22	0.21	0.11	0.09	0.05	0.02
Relative variance (%)	46.44	18.02	9.55	7.39	5.21	4.05	3.20	1.68	1.63	0.88	0.84	0.44	0.38	0.19	0.09
Cumulative variance (%)	46.44	64.46	74.02	81.41	86.61	90.66	93.87	95.55	97.18	98.06	98.90	99.34	99.72	99.91	100.00
Eigen vectors															
E	0.275	0.100	-0.069	0.091	-0.083	-0.125	-0.001	-0.104	-0.076	0.062	0.039	0.365	0.008	0.218	-0.018
gS	0.224	0.223	-0.170	0.091	0.117	-0.199	0.125	-0.163	-0.085	-0.124	-0.261	0.138	-0.231	-0.021	0.041
PN	0.280	-0.040	-0.079	0.092	-0.151	-0.051	-0.043	0.112	-0.086	0.065	-0.058	0.073	-0.159	-0.380	0.074
Ci	0.247	0.088	-0.218	0.031	0.046	-0.299	0.038	-0.053	0.088	0.074	0.188	0.051	0.589	0.064	0.035
Chl	-0.173	-0.254	-0.242	0.265	0.074	0.186	0.177	-0.079	-0.073	-0.058	0.184	0.020	-0.129	0.249	0.489
Flavonols	0.092	-0.397	0.105	0.082	-0.019	0.043	0.134	0.536	0.079	-0.193	-0.070	-0.028	0.123	0.055	-0.136
Anthocyanins	0.241	-0.043	0.136	-0.272	-0.040	-0.001	-0.255	0.308	0.179	-0.136	0.010	0.351	-0.342	0.178	0.213
NBI	-0.260	0.027	0.053	-0.215	-0.137	0.233	0.040	0.123	0.193	-0.026	0.102	0.108	0.255	-0.277	0.324
Fv/Fm	0.113	0.118	0.021	0.437	-0.205	0.474	-0.401	-0.026	0.021	0.059	0.329	0.092	-0.046	0.137	-0.014
Height	0.252	0.083	0.167	0.065	-0.081	0.173	0.225	-0.130	0.264	0.147	-0.369	-0.156	0.122	0.085	0.499
N° leaf	0.199	0.023	-0.032	-0.112	0.322	0.398	0.436	-0.101	0.355	0.082	-0.046	0.032	-0.216	0.059	-0.252
Leaf area	0.179	0.041	0.400	-0.186	-0.274	0.084	0.050	-0.288	0.088	-0.300	0.152	-0.033	0.206	-0.188	0.035
Shoot FW	0.270	-0.043	0.212	0.036	-0.073	0.055	0.056	0.162	0.084	0.050	0.008	0.225	0.152	0.110	-0.263
Shoot DW	0.212	0.016	-0.007	-0.113	0.405	0.383	-0.107	0.117	-0.389	-0.097	0.038	-0.313	0.110	-0.119	-0.057
Stom. number	-0.195	0.126	0.011	-0.039	-0.346	0.277	0.420	-0.082	-0.457	-0.108	-0.092	0.379	0.033	0.001	-0.175
Stom. lenght	-0.065	-0.091	0.199	0.559	0.398	-0.004	-0.013	-0.067	0.096	-0.116	-0.123	0.361	0.178	-0.367	0.006
Stom. width	0.013	-0.284	0.449	0.091	0.081	-0.163	0.250	-0.010	-0.228	0.285	0.202	-0.070	0.043	0.294	0.005
Total AsA	0.208	0.075	-0.336	-0.217	0.115	0.202	0.017	0.159	-0.141	0.351	0.100	0.189	0.265	-0.033	0.089

Reduced AsA	0.004	0.385	0.182	-0.091	0.353	-0.032	-0.026	0.036	-0.136	-0.514	0.129	0.052	0.072	0.219	0.154
Proline	-0.087	0.402	-0.044	0.196	-0.087	-0.002	0.151	0.070	0.360	0.014	0.304	-0.226	-0.056	0.071	-0.259
H202	-0.029	0.386	0.168	0.234	-0.145	0.060	-0.079	0.346	-0.164	0.164	-0.452	-0.205	0.081	0.076	0.030
Lipid perox.	-0.180	0.296	0.039	0.033	0.089	-0.173	0.315	0.446	0.013	0.095	0.254	0.132	-0.091	-0.098	0.193
Glucose	-0.259	0.071	0.154	-0.133	0.155	0.061	-0.216	-0.120	0.052	0.179	-0.210	0.163	0.171	0.409	-0.028
Fructose	-0.242	0.079	0.208	-0.174	0.220	0.042	-0.172	-0.072	0.084	0.372	0.003	0.229	-0.088	-0.225	-0.067
Sucrose	-0.231	-0.114	-0.318	0.003	-0.034	0.088	-0.041	0.108	0.233	-0.279	-0.273	0.113	0.233	0.174	-0.158

Figure S1 Linear correlation between all studied variables**.**



Chapter 6. A Novel Protein Hydrolysate-Based Biostimulant Improves Tomato Performances under Drought Stress

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Chapter 6. A Novel Protein Hydrolysate-Based Biostimulant Improves Tomato Performances under Drought Stress

Abstract: Abiotic stresses adversely affect crop production causing yield reductions in important crops, including tomato (*Solanum lycopersicum* L.). Among the different abiotic stresses, drought is considered to be the most critical one, since limited water availability negatively impacts plant growth and development, especially in arid and semi-arid areas. The aim of this study was to understand how biostimulants may interact with critical physiological response mechanisms in tomato under limited water availability and to define strategies to improve tomato performances under drought stress. We investigated the physiological responses of the tomato genotype 'E42' grown in open fields under optimal conditions (100% irrigation) and limited water availability (50% irrigation) treated or not with a novel protein hydrolysate-based biostimulant (CycoFlow, Agriges, BN, Italy). Plants treated with the protein hydrolysate showed a better water status and pollen viability, which also resulted in higher yield under drought stress compared to untreated plants. The treatment with the biostimulant had also an effect on antioxidant contents and activity in leaves and fruits depending on the level of irrigation provided. Altogether, these results indicate that the application of protein hydrolysates on tomato improved plant performances under limited water availability and in different experimental fields.

6.1. Introduction

Tomato is one of the most important crops, with more than five million hectares cultivated, worldwide (Russell et al. 1955). Being a summer crop, transient or extended drought periods are common during its cultivation cycle, especially in the most sensitive periods of fruit set and enlargement (Di Stasio et al. 2020). These are the two phases in which even a transitory drought stress can lead to heavy yield losses (Cui et al. 2020). Climate change will make these events more frequent in arid and semi-arid environments, with detrimental consequences for tomato productivity (Barnabas et al. 2008, Costa et al. 2007). Generally, plants respond to drought with a series of physiological mechanisms including stomatal closure, repression of cell growth and photosynthesis, and activation of stress hormones and antioxidant mechanisms, which overall lead to a reduction in plant growth and productivity (Tardieu et al. 2018). The forecasted lack of water and the consequent increase in competition for water resources between agriculture and other sectors require the exploration of alternative and sustainable crop management strategies that can save water for irrigation and, at the same time, still maintain satisfactory levels of crop production (Paradikovic et al. 2019). One of the most promising strategies that can be used to improve plant response to drought stress is the use of biostimulants. These are substances or micro-organisms whose application is beneficial for plant

growth and productivity (Calvo et al. 2014). The application of biostimulants can also induce enhanced tolerance to different abiotic stresses (Van Oosten et al. 2017, Van Oosten et al. 2018). Among biostimulants, protein hydrolysates seem to be promising, since they contain high amounts of molecules such as amino acids, small peptides and osmoactive compounds (proline, glycine betaine) which are beneficial for plant productivity under unfavorable environmental conditions (Van Oosten et al. 2017). Plant-based biostimulants are also effective in enhancing growth, yield, quality and bioactive compounds' content in various crops. For example, the application of an extract from moringa (*Moringa oleifera* Lam.) increased yield and growth in tomato, basil, cabbage and pepper, as well as the quality of tomato, lettuce, radish, spinach, rocket and pepper (Zulfiqar et al. 2020). These extracts also improved plant tolerance to different abiotic stresses such as drought (Paul et al. 2019), salinity (Lucini et al. 2015), heat (Francesca et al. 2020) and heavy metal contamination (Elrys et al. 2018). Ertani et al. 2014, found that foliar application of alfalfa (*Medicago sativa* L.) and red grape (*Vitis vinifera* L.) extracts improved growth and yield in *Capsicum chinensis* L.,and triggered the accumulation of secondary metabolites in leaves. On rocket, the use of two vegetal-based biostimulants enhanced productivity under both optimal and sub-optimal nitrogen fertilization rates (Mola et al. 2019). Despite the high number of scientific papers in which protein hydrolysates and plant-based biostimulants have been proven to increase crop productivity and abiotic stress tolerance, the functional cause–effect relationship and physiological basis that determine the growth stimulant and/or protective action of these products is still unclear (Di Stasio et al. 2020). The aim of this study was to link the physiological responses and agronomic performances of tomato plants treated with a plant-derived protein hydrolysate. This biostimulant (CycoFlow, Agriges, Benevento, Italy) was previously found to be effective in heat stress protection on different tomato varieties grown in open field. In this study, we tested whether the application of this biostimulant could be beneficial for tomato productivity and fruit quality under two different water regimes (optimal and water limited) and in different environmental fields. The physiological bases of these responses are discussed.

6.2. Results

6.2.1. Biomass and Yield Components

We investigated the performances of one tomato genotype ('E42') grown in the year 2019 in an open field located in Battipaglia in the Campania Region (Italy) under optimal conditions (100% irrigation) and limited water availability (50% irrigation) and treated or not with a protein hydrolysate-based biostimulant. Pollen viability decreased by 27% under water deficit in non-treated plants. On the contrary, plants treated with the protein hydrolysate and subjected to water deficit showed an increase of 51% in pollen viability compared to non-treated plants (Figure 1a). Water deficit significantly reduced the number of fruits per plant. The biostimulant treatment partially compensated the effect

of water deficit (50% water regimen), as demonstrated by the 70% higher number of fruits per plant upon biostimulant treatment vs. non-treated plants (Figure 1b). The treatment with the biostimulant increased the average weight of a single fruit under reduced water regimen by 95% (Figure 1c). Under the 50% water regimen, biostimulant treatment increased the final yield six-fold (Figure 1d). Moreover, treatment with the protein hydrolysate increased the fruits' water content under reduced water regimen (98% vs. 91%—Table S1). According to ANOVA, the combined effect of water stress and biostimulant did not induce significant differences in shoot fresh weight. Conversely, the single effect of the water stress induced a significant reduction in this parameter, while the biostimulant treatment had an opposite effect (Table S1). In order to further confirm the effect of the protein hydrolysate on plant growth and on final yield and yield components, an additional experiment was carried out in the year 2020 in another experimental field located in Benevento (Campania Region, Italy) on plants grown in optimal conditions (Table 1). These analyses evidenced the positive effect of the protein hydrolysate on pollen viability, number of fruits and final yield (+112% in treated plant compared to non-treated ones). Moreover, considering the fresh biomass accumulation, the biostimulant treatment induced a higher shoot fresh weight in this experimental field (Table 1).

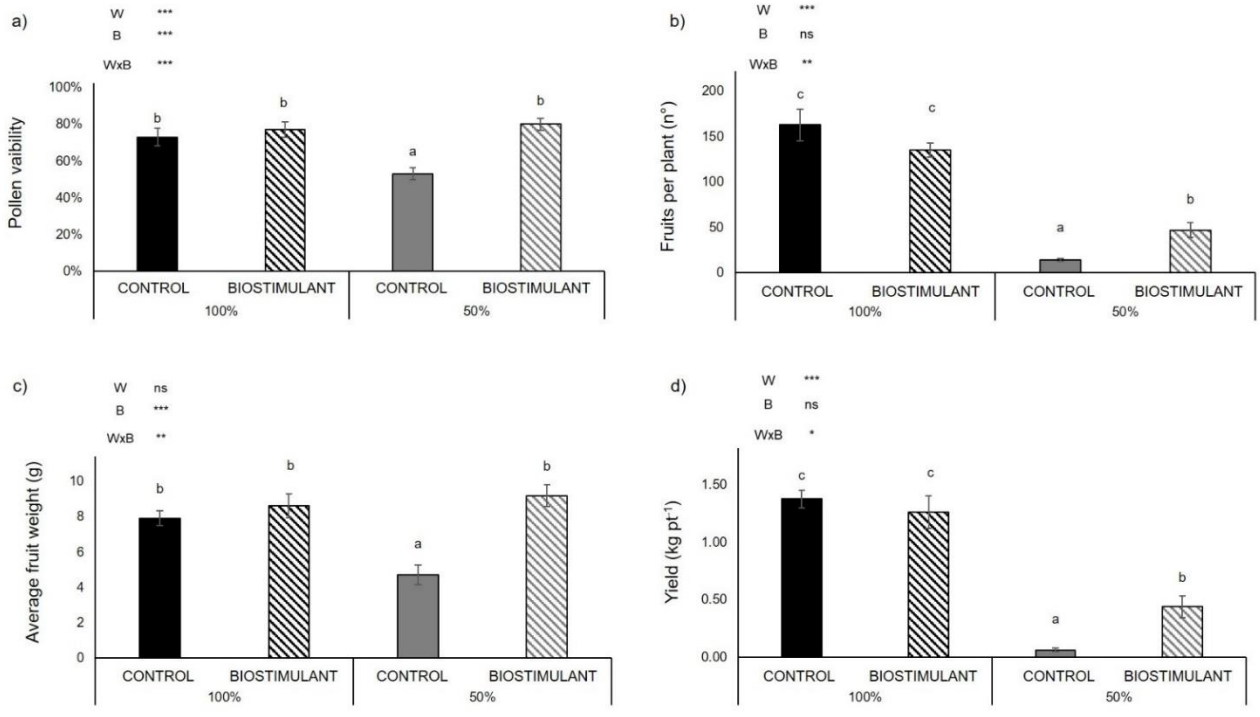

Figure 1. Pollen viability (**a**); number of fruits per plant (**b**); average fruit weight (**c**) and yield *per* plant (**d**) in the tomato genotype 'E42' grown in open field in Battipaglia under optimal (100% irrigation) and limited water availability (50% irrigation) and treated (biostimulant) or not (control) with the biostimulant. Values are mean ± SE. Asterisks indicate significant effect of limited water availability (W), biostimulant treatment (B) and their interaction (W × B) according to ANOVA (ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Different letters indicate significant differences based on Duncan's test ($p \leq 0.05$).

Table 1. Pollen viability, average fruit weight, number of fruits per plant, yield per plant and shoot fresh weight (mean ± SD) in the tomato genotype 'E42' treated (biostimulant) or not (control) with the protein hydrolysate and grown in Benevento in the year 2020. Asterisks indicate significant differences according to Student's *t*-test (** = $p < 0.01$; *** = $p < 0.001$).

	Control	Biostimulant	Significance
Pollen viability (%)	48 ± 0.30	53 ± 0.15	***
Average fruit weight (g)	9.69 ± 0.0017	9.08 ± 0.0003	***
Number of fruits *per* plant	61.87 ± 18.29	139.53 ± 25.77	***
Yield (kg pt^{-1})	0.60 ± 0.18	1.27 ± 0.23	***
Shoot FW (g)	578.33 ± 160.68	966.67 ± 208.77	**

6.2.2. Physiological Traits

In the plants grown in the open field located in Battipaglia under optimal conditions (100% irrigation) and limited water availability (50% irrigation), treatment with the biostimulant had a significant effect on stomatal conductance, which, under full irrigation, increased by 84% after treatment (Figure 2a). The 50% water regimen significantly reduced the leaf water potential compared to plants under full irrigation; however, the treatment with the protein hydrolysate led to a 27% increase in the leaf water potential compared to non-treated plants under water deficit, confirming beneficial effects in terms of plant water status (Figure 2b).

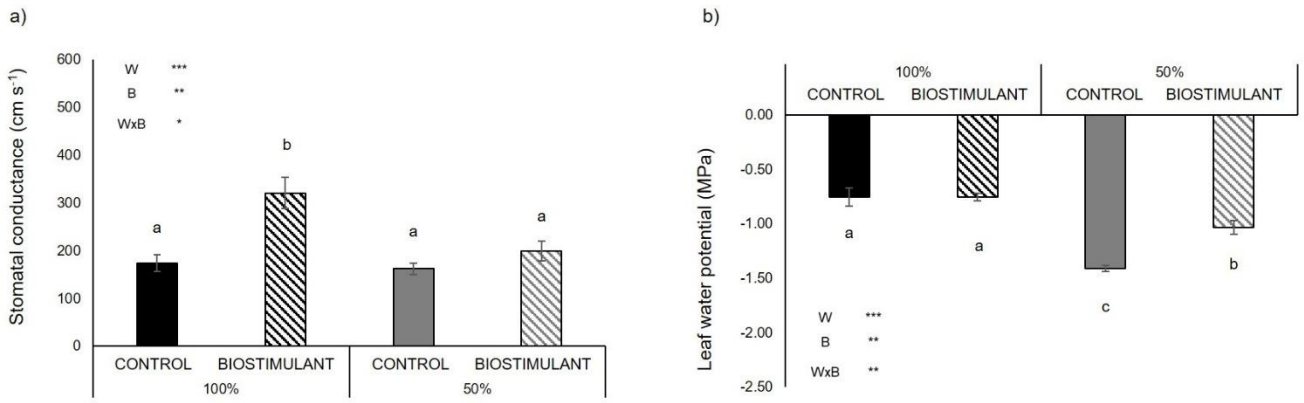

Figure 2. Stomatal conductance (**a**); and leaf water potential (MPa) (**b**); in the tomato genotype 'E42' grown in open field in Battipaglia under optimal (100% irrigation) and limited water availability (50% irrigation) and treated (biostimulant) or not (control) with the biostimulant. Values are mean ± SE. Asterisks indicate significant effect of limited water availability (W), biostimulant treatment (B) and their interaction (W × B) according to ANOVA (ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Different letters indicate significant differences based on Duncan's test ($p \leq 0.05$).

6.2.3. Leaf Antioxidant Activity

The content of total and reduced ascorbic acid (AsA) in the leaves of the plants grown in the open field located in Battipaglia was significantly reduced by biostimulant treatment only under 100% irrigation (−29% for both reduced and total AsA) (Figure 3a,b). Under limited water availability, the total antioxidant activity (FRAP) in the leaves increased by 98% after treatment with the biostimulant, while no change was reported under full irrigation (Figure 3c). Overall, the 50% water regimen increased carotenoid content. The interaction between water regimen and biostimulant treatment significantly reduced the content of chlorophylls a and b by 14% under full irrigation (Table S2).

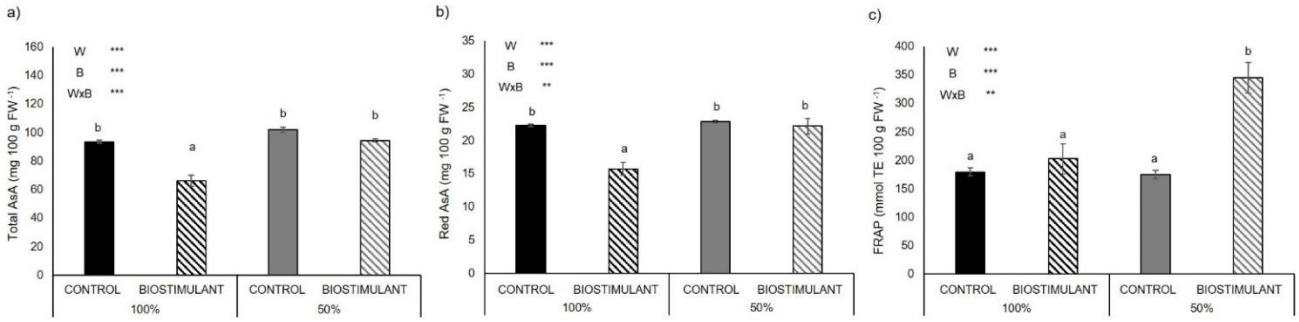

Figure 3. Total (**a**) and reduced (Red) (**b**) ascorbic acid (AsA) content and total antioxidant activity (FRAP)(**c**) in leaves of tomato genotype 'E42' grown in open field in Battipaglia under optimal (100% irrigation) and limited water availability (50% irrigation) and treated (biostimulant) or not (control) with the biostimulant. Values are mean ± SE. Asterisks indicate significant effect of limited water availability (W), biostimulant treatment (B) and their interaction (W × B) according to ANOVA (ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Different letters indicate significant differences based on Duncan's test ($p \leq 0.05$).

6.2.4. Fruit Antioxidant Activity

In fruits of the plants grown in the open field located in Battipaglia carotenoid and lycopene contents were significantly affected by the interaction between biostimulant treatments and water regimen (Figure 4). In particular, under the 100% water regimen, both carotenoid and lycopene contents in fruits were significantly increased by biostimulant treatment (+33% and +31%, respectively). Under the 50% water regimen, this trend was inverted, with 20% and 15% lower carotenoids and lycopene accumulation in the fruits of treated plants compared to non-treated ones (Figure 4). Comparing non-treated plants, water stress caused an accumulation of carotenoids and lycopene (+43% and +34%, respectively) (Figure 4). The treatment with the biostimulant reduced the content of total ascorbic acid both under full irrigated (−12%) and water limited (−8%) conditions (Table S2). The content of reduced ascorbic acid was significantly affected by the interaction between water regimen and biostimulant treatments, with 10% reduction in plants treated with the biostimulant under full irrigation (Table S2). Finally, only the effect of the reduced water regimen induced significant changes in β-carotene accumulation and total antioxidant activity (Table S2).

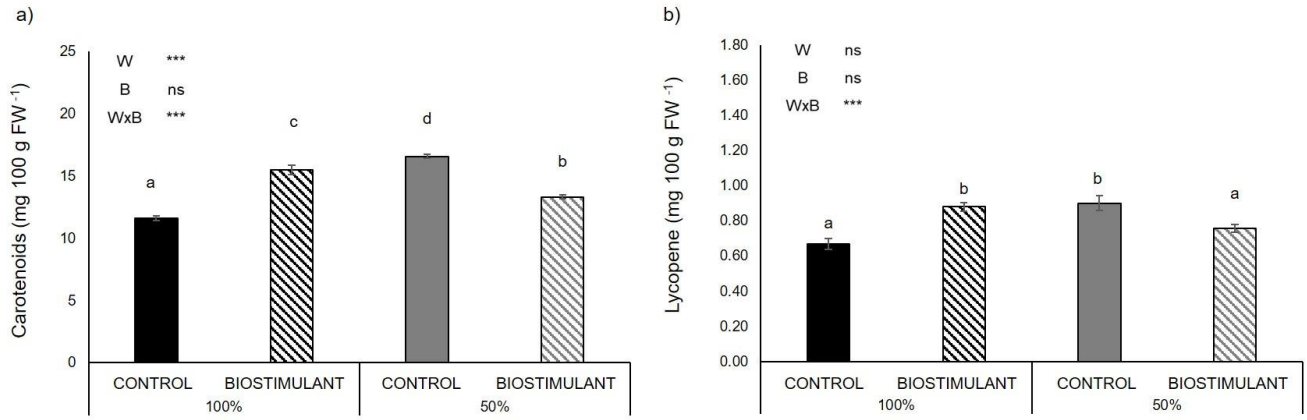

Figure 4. Content of (**a**) carotenoids and (**b**) lycopene in fruit of the tomato genotype 'E42' grown in open field in Battipaglia under optimal (100% irrigation) and limited water availability (50% irrigation) and treated (biostimulant) or not (control) with the biostimulant. Values are mean ± SE. Asterisks indicate significant effect of limited water availability (W), biostimulant treatment (B) and their interaction (W × B) according to ANOVA (ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Different letters indicate significant differences based on Duncan's test ($p \leq 0.05$).

6.2.5. Heat Map Analysis

The aggregated data heat-map analysis (Figure 5), summarizing plant responses to biostimulant application and water deficit in plants grown in Battipaglia, identified a first cluster corresponding to the different water regimen applied. Two separate sub-clusters could be defined under each single water treatment, which basically depended on the treatment with the biostimulant.

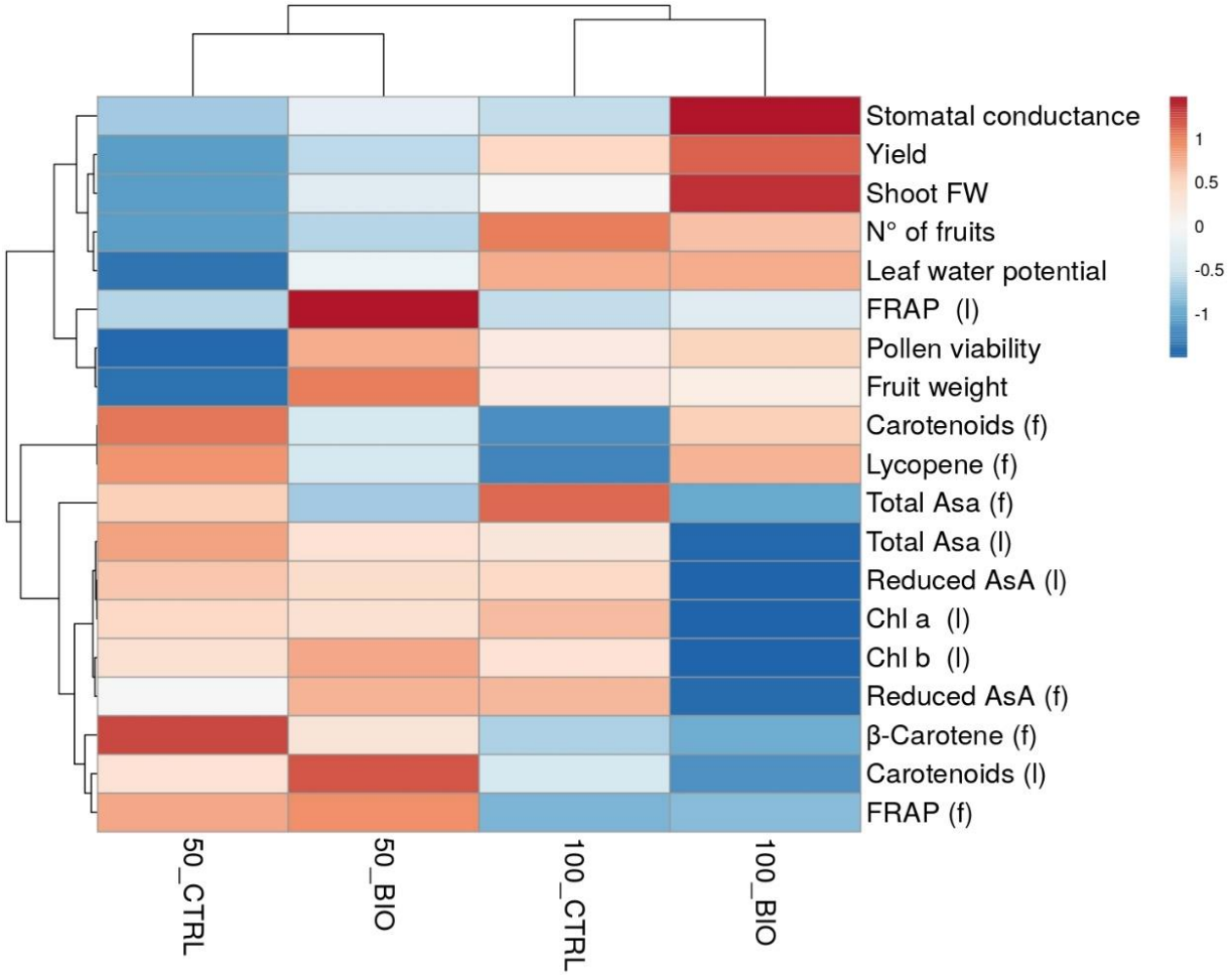

Figure 5. Heat map analysis summarizing plant responses to biostimulant application and water deficit in plants grown in Battipaglia under optimal (100) and limited water availability (50) and treated (BIO) or not (CTRL) with the protein hydrolysate. The letters in brackets indicate measurements taken from leaves (l) and fruits (f). The Figure was generated using the http://biit.cs.ut.ee/clustvis program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage.

6.3. Discussion

6.3.1. A Protein Hydrolysate-Based-Biostimulant Protects Pollen Viability from Drought-Induced Desiccation

In this study, we investigated the performances of one tomato genotype ('E42') grown in open fields located in Battipaglia and in Benevento in the Campania Region (Italy), treated with a protein hydrolysate rich in glutamic acid, glycine betaine and micronutrients as boron, manganese and zinc. Biostimulants have demonstrated beneficial effects in promoting growth and alleviating the effects of abiotic stresses in horticultural crops (Du Jardin. 2015). Herein, we demonstrated, for two years and in different experimental fields, the positive effect of the protein hydrolysate-based biostimulant on plant growth, final yield and yield components (Figure 1, Table 1), in agreement with previous results (Francesca et al. 2020). Additionally, we tested the effect of the protein hydrolysate in plants grown under optimal conditions (100% irrigation) and limited water availability (50% irrigation).

Both the water regimen and the biostimulant treatment had an effect on tomato plants, as shown by the heat map of Figure 5. Plant growth and yield were reduced in water-stressed plants compared to well-irrigated ones, but after treatment with the protein hydrolysate, both well-watered and water-stressed plants showed better performance in the field. Altogether, plants treated with the protein hydrolysate showed higher shoot biomass under both well-watered and water shortage conditions and in both experimental fields (Table 1 and S1), pointing out at the double effect of this biostimulant as a growth-promoting and stress-protective effector on plants. More interestingly, we found that treated plants showed higher pollen viability compared to non-treated ones under drought in 2019 and also under optimal conditions in 2020 (Figure 1a, Table 1). Pollen viability has been recently used to identify heat-tolerant tomato genotypes, indicating pollen thermo-tolerance as an important parameter to consider for future breeding programs. Shen et al. 2014 indicated that the β-alanine plays a role in pollen germination under high temperatures. It is possible that the high concentration of this molecule, which is the third most representative free amino acid contained in the biostimulant (Table S3), was responsible for the higher pollen viability found in the biostimulant-treated plants. Other than heat stress, pollen viability is highly sensitive to drought and salinity (Pacini et al. 2019, Fang et al. 2010, Gusmao et al. 2012, Saragih et al. 2013). Under several abiotic stresses, indeed, the concentration of reactive oxygen species (ROS) increases and leads to oxidative stress, which irreversibly damages pollen, thus reducing its viability and development (Rao et al.2019, Meng et al. 2020, Santiago et al. 2019, Xu et al. 2017). The reduction in pollen viability and germination is generally correlated with yield losses, since it is tightly correlated with plant productivity and contributes to determining the level of fruit/seed set (Prasad et al. 2006, Dong et al. 2017, Paupiere et al. 2017).

6.3.2. Plant Yield Improves upon Biostimulant Treatment

In this study, we found a higher number of fruits in treated plants compared to non-treated ones under drought in 2019, and also under optimal conditions in 2020 (Figure 1b, Table 1). This can be the direct consequence of the higher pollen viability induced by biostimulant treatment, and thus one of the reasons for the higher yield of treated plants compared to non-treated ones (Figure 1a,d, Table 1). Moreover, the biostimulant treatment increased the average weight of single tomato fruits compared to untreated plants under drought (Figure 1c). Therefore, other than the enhancement of fruit set, the protein hydrolysate reduced the drought-induced fruit shrinkage. This can be the result of the better water status of the treated plants under drought compared to the non-treated ones, as indicated by their higher leaf water potential (Figure 2b) and the different fruit water content, which was significantly higher in the former compared to the latter (98% vs. 91%—Table S1). Indeed, leaf water potential is an indicator of plant water status (Jongdee et al. 2002, Bartlett et al. 2012), thus underlying that plants treated with the biostimulant were less sensitive to water deprivation compared to non-

treated ones. It is possible that the presence of protective metabolites such as glycine betaine and proline in the protein hydrolysate may have enhanced the tolerance of tomato plants to water deficit. Indeed, it has been previously demonstrated that both glycine betaine and proline applied exogenously significantly increased drought tolerance of tomato plants grown under hyper-osmotic conditions, thanks to different mechanisms such as osmotic adjustment, membrane and proteins stabilization, and antioxidant activity (Makela et al. 1998, Ashraf et al. 2007). Moreover, it is known that the amino acid proline also favors the translocation of nutrients towards developing flowers (sink) (Sato et al. 2006).

6.3.3. Plant Antioxidant Activity of Leaves is Enhanced by Biostimulant Treatment

The biostimulant had a positive effect on the total antioxidant activity (FRAP) in leaves of plant grown under limited water availability (Figure 3c). The higher total antioxidant activity found in treated plants under water stress was not due to an increase in ascorbic acid, since the content of this antioxidant was significantly reduced by biostimulant application (Figure 3a,b). Possibly, treated plants had a lower need for ascorbic acid thanks to the exogenous application of molecules contained in the biostimulant formulation, such as glutamic acid, phenylalanine, glycine and proline, which can perform antioxidant activity when accumulated in plant tissues (Woodrow et al. 2017, Teixeira et al. 2017). These amino acids are found in high concentrations in the formulation of the protein hydrolysate used in this study (Table S3) (Francesca et al. 2020).In this experiment, it is probable that these metabolites worked both as compatible solutes, thus improving plant water status under drought and promoting cell enlargement, as well as antioxidant production, preventing reactive oxygen species (ROS) damage to pollen viability, with a beneficial effect on fruit set. Indeed, enzymatic and non-enzymatic antioxidant system can reduce the oxidative stress of the membranes, thus increasing the pollen integrity, viability and pollen tube development (Ren et al. 2021, Muhlemann et al. 2018). These results are consistent with a previous work, which demonstrated that treatment with this biostimulant induced the activation of the antioxidant defense system (Francesca et al. 2020). The improved water status and the protection of cellular membranes under drought could be the reason for the higher yield reported in treated plants, which was mediated by the higher drought tolerance of these plants during the sensitive stages of fruit set and enlargement. Moreover, the free amino acids present in the biostimulant may have acted as signaling molecules and may have promoted endogenous phytohormonal biosynthesis, thus stimulating plant growth and productivity (Rouphael et al. 2017).

6.3.4. Plant Antioxidant Activity of Fruits is Enhanced by Biostimulant Treatment

Regarding fruit quality, the content of carotenoids and lycopene was higher in the fruit of treated plants compared to the non-treated ones under well-watered conditions (Figure 4). These results are in agreement with the results previously obtained by Rouphael et al.2017, who showed that foliar applications of a protein hydrolysate derived from legumes had a similar effect on the lycopene content in tomatoes. Fruit vegetables, particularly tomatoes, are considered good sources of lipophilic and hydrophilic antioxidant molecules such as lycopene and ascorbic acid (Raiola et l. 2014). The beneficial effects of plant-based biostimulant on the accumulation of phytochemical compounds (i.e., lycopene) could be associated with the activation of specific molecular and physiological mechanisms related to nitrogen metabolism (Ertani et al. 2014, Van Oosten et al. 2019). In conditions of limited water availability, plants react with an increase in the content of carotenoids. According to Riggi et al. 2008, tomato plants subjected to mild water stress increase the content of lycopene and β-carotene compared to well-irrigated plants. It is conceivable that this protein hydrolysate, which contains two main osmolytes involved in osmotic stress, acts as a mild stressor to the plant. This could be the reason for the increase in lycopene content in the fruit of treated plants compared to non-treated ones under well-watered conditions, which equals the concentration of this antioxidant in the fruits grown under limited water availability (Figure 4). The higher lycopene content of treated fruits is valuable in view of the necessity to increase the nutraceutical properties of vegetables products, which are important components to supporting human health (Erba et al. 2013). On the contrary, under water limited conditions, the biostimulant treatment reduced carotenoids and lycopene concentration when compared to untreated plants (Figure 4). This could have been induced by the higher water content of the fruit from biostimulant treated plants, which diluted the concentration of these antioxidants, compared to the ones from untreated plants. These contrasting results underline the fact that it is important to evaluate the specific effects of the biostimulant products depending on the condition of its application in order to maximize the desired effect of its employment.

6.4. Materials and Methods

6.4.1. Plant Growth, Experimental Design, and Treatments

One experiment was carried out at the agronomy farm of the University of Naples “Torre Lama” located in Battipaglia, Salerno, Italy (latitude 40°31′ N; longitude 14°58′ E) on a clay-loam soil. Four weeks after seeding, at the third true leaf fully expanded, tomato plants (genotype ‘E42’, available at the University of Naples, Department of Agricultural Sciences (Olivieri et al. 2020)) were transplanted in an open field on 19 June 2019. Rainfall throughout the growing period was 10 m^3 ha^{-1} and mean daily air temperature was between 17 and 27 °C (Figure S1). The experimental design consisted of four treatments: non-treated plants, biostimulant-treated plants and two irrigation levels (100% replenishment of crop water requirements (CWR) estimated using a Class A evaporation pan

vs. 50% CWR). Plants were arranged in a completely randomized block design with three replicates per treatment and 20 plants per biological replication. The experimental field was irrigated every 10 days, using a drip irrigation system with 5 L h^{-1} (one emitter per plant). Water deficit was induced at 22 Days After Transplant (DAT) and continued until the end of the experiment. The biostimulant was applied by fertigation at a concentration of 3 g/l of water (400 mL *per* plant) at transplanting and, thereafter, every 15 days until the end of the cultivation cycle for a total of four applications. The biostimulant tested 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. Its composition was previously reported (Francesca et al. 2020). Additional details on the composition of the biostimulant and the aminogram are reported in Table S3. According to the classification of the different biostimulants provided from du Jardin et al. 2015 which define the protein hydrolysates category as "amino-acids and peptides mixtures obtained by chemical and enzymatic protein hydrolysis from agroindustrial by-products, from both plant sources (crop residues) and animal wastes (e.g., collagen, epithelial tissues)" we can define the product used in this study as a protein-hydrolysate. Harvesting started on 12 August 2019, at 54 DAT, on six plants per biological replicate per treatment. In order to further confirm the effect of the protein hydrolysate-based biostimulant on plant growth and on final yield, a second experiment was carried out in the year 2020 in another experimental field (Table 1). The experimental field was located in an agronomy farm in Apollosa, (Benevento, Campania, Italy, latitude 41°5'42"36 N; longitude 14°42'22"32 E) characterized by a clay-loam soil. Four weeks following seeding, after the third true leaf was fully expanded, tomato plants (genotype 'E42') were transplanted into an open field in May 2020. Rainfall throughout the growing period was 344 m^3 ha^{-1} and mean daily air temperature was between 14 and 29 °C (Figure S1). Tomato plants were grown following the standard agronomical practices of the area. The experimental design consisted of a completely randomized design with three replicates per treatment and 10 plants per biological replication. There were two different groups: one control, which did not receive any biostimulant, and one which was treated with the biostimulant. The same methods and quantities used in the first experiment were also maintained in the second year of the experiment.

6.4.2. Biometric, Yield and Physiology Measurements

Shoot biomass was calculated as the sum of above-ground vegetative plant parts (leaves + stems) in both experimental years. The number of fruits, the average single fruit weight and their total biomass were recorded in each experiment. During the cultivation cycle, the confirmation of plant stress was obtained measuring stomatal conductance and leaf water potential after 45 DAT (25 day after stress induction). Following procedures reported in other works [46–49], stomatal conductance was

measured with a steady state porometer (AP-4, Delta-T Devices, Cambridge, UK) on a young and healthy, fully expanded apical leaf of the third branch per plant. The value derived from the average of three measurements in different positions of the leaf abaxial side of the selected leaf. On the same leaf used for stomatal conductance measurements, the total leaf water potential (Ψt) was measured with a Scholander's pressure chamber (PMS Instrument Company, Albany, USA), following procedures reported in other works (Duc et al. 2018, Petrozza et al. 2014, Moles et al. 2018, Di Stasio et al. 2018).

6.4.3. Pollen Viability

Pollen viability was analyzed using five flowers per plant sampled from three different plants per replicate in both years. In the laboratory, pollen grains were spread on microscope slides. Then, one droplet of DAB solution (3.3′ Diaminobenzidine Sigma-Aldrich, St. Louis, MO, USA) was added to each pollen sample; slides were gently warmed with a gas lighter and mounted with a cover slip (Gracie C and Dafni A, 1994). Scoring was made using an Leitz Laborlux 12 microscope (Leica, Wetzlar, DE, Germany).

6.4.4. Total Carotenoids, Lycopene, β-Carotene and Chlorophylls

Samples of freshly harvested fully ripened tomato fruits and leaves were collected from each plot to determine pigments content by a colorimetric assay on freeze-dried and finely ground samples. The evaluation of total carotenoids, chlorophylls, lycopene and β-carotene was carried out according to the method reported by Wellburn et al. 1994 and by Zouari et al. 2014 and modified by Rigano et al. 2016. To obtain the lipophilic extract, 0.25 g of sample were extracted with 24 mL of acetone/hexane (40/60, v/v). The mixture was centrifuged at 15,000 rpm for 5 min at 4 °C. Supernatants were collected and stored at −20 °C until analyses. To determine the level of carotenoids and chlorophylls a and b, absorbance of lipophilic extracts was read at 470, 663, and 645 nm, respectively. For lycopene and β-carotene levels' absorbance was read at 505 and 453 nm, respectively. Three separated biological replicates for each sample and three technical assays for each biological repetition were measured.

6.4.5. Ascorbic Acid Content

Measurements of reduced ascorbic acid (AsA) and total ascorbic acid (AsA + dehydroascorbate—DHA) contents were carried out by using a colorimetric method (Stevens et al. 2006), with modifications reported by Rigano et al. 2014. Briefly, 500 mg of frozen powder from tomato fruits or leaves were extracted with 600 µL of 6% trichloroacetic acid (TCA). The mixture was incubated for 15 min on ice and centrifuged at 14,000 rpm for 20 min. For reduced AsA evaluation, to 20 µL

of supernatant were added 20 µL of 0.4 M phosphate buffer (pH 7.4), 10 µL of double-distilled (dd) H_2O and 80 µL of color reagent solution. This solution was prepared by mixing solution A (31% (w/v) H_3PO_4, 4.6% (w/v) TCA and 0.6% (w/v) $FeCl_3$) with solution B (4% (w/v) 2,2′ -Dipyridyl). For total AsA, to 20 µL of sample, 20 µL of 5 mM dithiotreitol in 0.4 M phosphate buffer (pH 7.4) was added and the mixture was incubated for 20 min at 37 °C. Ten microliters of N-ethyl maleimide (NEM; 0.5% (w/v) in water) were added and left for 1 min at room temperature. Eighty microliters of color reagent were added as previously described for reduced AsA. Both the final mixtures were incubated at 37 °C for 40 min and measured at 525 nm using a Nano Photometer TM (Implen, Munich, Germany). The concentration was expressed in mg/100 g of fresh weight (FW). Three separated biological replicates for each sample and three technical assays for each biological repetition were measured.

6.4.6. Antioxidant Activity Determination

The antioxidant capacity was analyzed by FRAP assay carried out by using the ferric reducing/antioxidant power method (Benzie et al. 1996) with slight modifications. The FRAP assay was carried out by adding in a vial 2.5 mL of acetate buffer at pH 3.6, 0.25 mL of TPTZ solution (10 mM) in 40 mM HCl, 0.25 mL of $FeCl_3 \cdot 6H_2O$ solution (12 mM), and 150 µL of methanolic extract obtained by adding 5 mL of 60% methanol base solution to 250 mg of frozen powder. The mixture was incubated for 30 min in the dark, and then readings of the colored products (ferrous tripyridyltriazine complex) were taken at 593 nm using a spectrophotometer. Results were expressed as micromoles of Trolox equivalents (TE) per 100 g FW. Three separated biological replicates for each sample, and three technical assays for each biological repetition, were measured.

6.4.7. Statistical Analysis

Data were subjected to analysis of variance using a two-way ANOVA. To separate means within each parameter, Duncan's test was performed. Differences of $p < 0.05$ were considered significant. ANOVA was performed by using SPSS (Statistical Package for Social Sciences) Package 6, version 23. A heat map, generated by using the http://biit.cs.ut.ee/clustvis program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage heatmap, summarized all the plant responses to both water-deficit and plant-based biostimulants.

6.5. Conclusions

In this study, a novel protein hydrolysate-based biostimulant was effective at enhancing tomato growth and productivity in different experimental fields, and also under limited water availability. This was possible thanks to the enhancement of the water status of the treated plants coupled with

higher antioxidant activity, which are part of a common tolerance strategy generally employed by plants to overcome different abiotic stresses. Even if additional research is needed to fully understand its mechanisms of action, these results can be valuable to functionalize the use of this class of biostimulants in real agricultural contexts, which is necessary to increase their efficacy. Finally, this biostimulant increased fruit quality thanks to the accumulation of antioxidant molecules, including carotenoid and lycopene, which is an added value in regard to the increasing interest in the nutraceutical properties of food.

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Supplementary material

Table S1. Pollen viability, average fruit weight, number of fruits, fruit water content, yield *per* plant and shoot fresh weight, (mean± SD) in E42 tomato plants grown in Battipaglia and treated with the biostimulant under two irrigation regimens. Asterisks indicate significant differences according to ANOVA (ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Different letters indicate significant differences according to Duncan's post-hoc test ($p < 0.05$).

	100%		50%		Significance		
	CTRL	**BIO**	**CTRL**	**BIO**	**W**	**B**	**WxB**
Pollen viability (%)	73 ± 12 b	77 ± 10 b	53 ± 8 a	80 ± 8 b	***	**	***
Average fruit weight (g)	7.69 ± 0.73 b	7.57 ± 0.27 b	4.71 ± 1.13 a	9.19 ± 1.24 b	ns	***	**
Number of fruits	162.75 ± 35.13 c	135.25 ± 15.44 c	14.00 ± 3.16 a	47.00 ± 16.10 b	***	ns	**
Fruit water content (%)	98.68 ± 0.72 bc	99.12 ± 0.33 c	91.13 ± 1.15 a	97.84 ± 0.47 b	***	***	***
Yield (kg pt^{-1})	1.25 ± 0.27 c	1.76 ± 0.60 c	0.07 ± 0.02 a	0.44 ± 0.19 b	***	ns	*
Shoot FW (kg)	2.55 ± 0.79	4.23 ±0.05	0.50 ± 0.11	2 ± 0.48	***	***	ns

Table S2. Content of total AsA, reduced AsA, carotenoids, chlorophyll a and b (Chl a, b), β-carotene, lycopene and total antioxidant activity (Frap) (mean± SD) in leaves and fruit of E42 tomato plants grown in Battipaglia and treated with the biostimulant under two irrigation regimens. Asterisks indicate significant differences according to ANOVA (ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Different letters indicate significant differences according to Duncan's post-hoc test ($p < 0.05$).

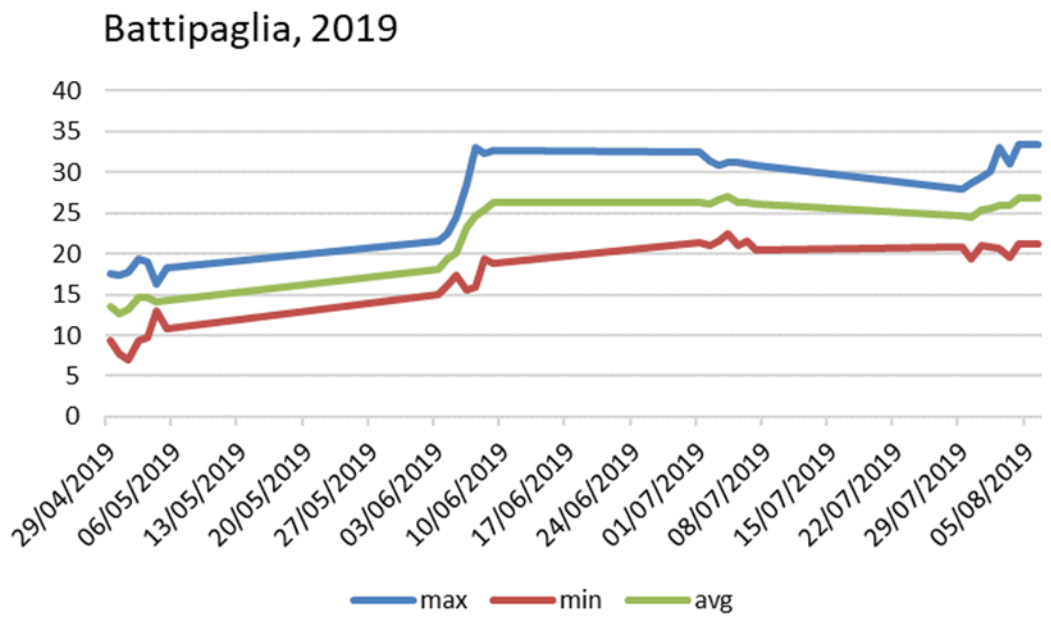
	100%		50%		Significance		
LEAF	**CTRL**	**BIO**	**CTRL**	**BIO**	**W**	**B**	**WxB**
Total Asa (mg/100 g FW)	93.51±2.53 b	65.96±9.58 a	101.82±4.80 c	94.35±2.24 b	***	***	***
Reduced AsA (mg/100 g FW)	22.26±0.47 b	15.73±2.47 a	22.81±0.42 b	22.14±2.90 b	***	***	**
Carotenoids (mg/100 g FW)	25.16±3.59	24.11±2.32	26.22±0.33	27.43±0.45	**	ns	ns
Chl a (mg/100 g FW)	132.04±0.92 b	113.09±0.60 a	130.27±3.76 b	129.54±4.45 b	***	***	***
Chl b (mg/100 g FW)	51.02±2.50 b	43.95±4.86 a	51.05±4.67 b	52.67±3.53 b	**	ns	**
Frap (mmol TE/ 100 g FW)	179.48±18.14 a	202.48±65.77 a	174.38±18.50 a	345.44±66.35 b	**	***	**
FRUIT							
Total Asa (mg/100 g FW)	115.40±11.41	100.99±6.68	111.50±7.69	102.70±8.38	ns	**	ns
Reduced AsA (mg/100 g FW)	94.20±4.90 b	84.65±7.15 a	91.11±5.03 ab	94.43±3.37 c	ns	ns	**
Carotenoids (mg/100 g FW)	11.61±0.51 a	15.47±0.95 c	16.58±0.32 d	13.31±0.41 b	***	ns	***
β-Carotene (mg/100 g FW)	0.34±0.05	0.33±0.03	0.40±0.02	0.37±0.07	**	ns	ns
Lycopene (mg/100 g FW)	0.67±0.08 a	0.88±0.06 c	0.90±0.10 c	0.76±0.06 b	ns	ns	***
Frap (mmol TE/ 100 g FW)	413.55±48.20	426.52±58.38	845.10±79.03	882.24±73.71	***	ns	ns

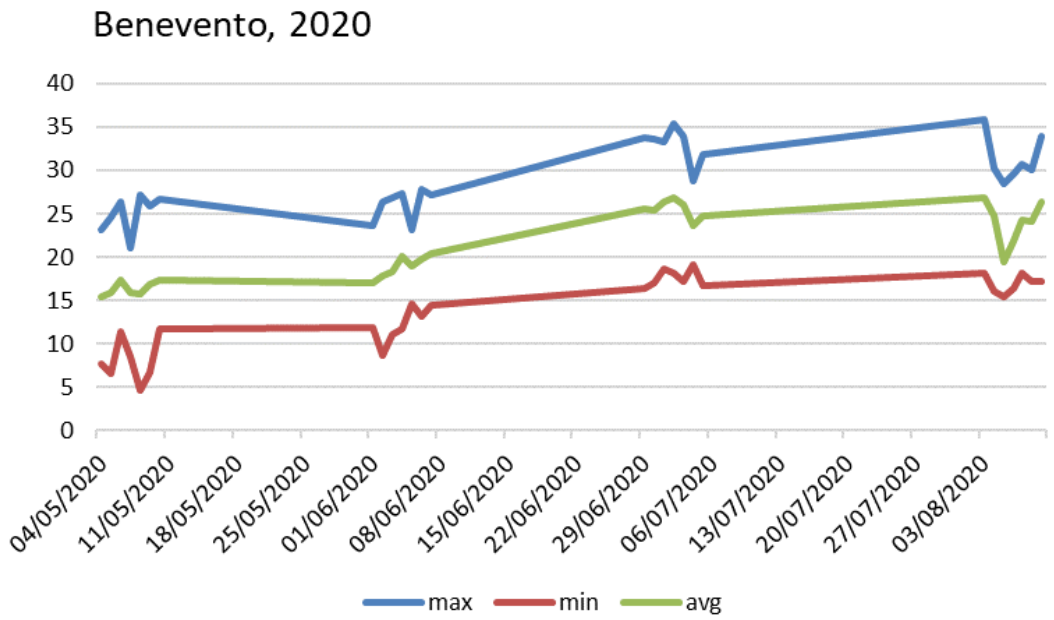
Table S3. Cycoflow composition expressed in g/100 g, modified from Francesca et al. (2020).

Glycine betaine	**3.62**
Total amino acid	
Aspartic acid (including asparagine)	2.22
Glutamic acid (including glutamine)	5.04
Alanine	1.36
Arginine	1.06
Phenylalanine	0.83
Glycine	1.02
isoleucine	1.06
Histidine	0.4
Leucine	1.48
Lysine	1.68
Proline	0.81
Serine	1.04
Tyrosine	0.76
threonine	0.98
Valine	1.23
Total cysteine and cystine	0.21
Total tryptophan	0.27
Methionine	0.32
TOTAL	**21.77**
Free Amino Acid	
Lysine	0.62
Aspartic acid	0.55
Glutamic acid	0.91
Alanine	0.79
Arginine	0.49
Phenylalanine	0.56
Glycine	0.24
isoleucine	0.58
Histidine	0.13
Leucine	0.95
Methionine	0.22

Proline	0.26
Serine	0.43
Tyrosine	0.37
threonine	0.43
Valine	0.72
TOTAL	**8.25**
Micronutrients	
Boron	0.2
Manganese	1.0
Zinc	1.2

Figure S1. Daily trends of temperatures (minimum, maximal and average temperature data) during tomato cropping cycle in 2019 and in 2020 in two different cultivation areas (Battipaglia and Benevento).





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A Novel Protein Hydrolysate-Based Biostimulant Improves Tomato Performances under Drought Stress

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Abstract: Abiotic stresses adversely affect crop production causing yield reductions in important crops, including tomato (*Solanum lycopersicum* L.). Among the different abiotic stresses, drought is considered to be the most critical one, since limited water availability negatively impacts plant growth and development, especially in arid and semi-arid areas. The aim of this study was to understand how biostimulants may interact with critical physiological response mechanisms in tomato under limited water availability and to define strategies to improve tomato performances under drought stress. We investigated the physiological responses of the tomato genotype 'E42' grown in open fields under optimal conditions (100% irrigation) and limited water availability (50% irrigation) treated or not with a novel protein hydrolysate-based biostimulant (CycoFlow, Agriges, BN, Italy). Plants treated with the protein hydrolysate showed a better water status and pollen viability, which also resulted in higher yield under drought stress compared to untreated plants. The treatment with the biostimulant had also an effect on antioxidant contents and activity in leaves and fruits depending on the level of irrigation provided. Altogether, these results indicate that the application of protein hydrolysates on tomato improved plant performances under limited water availability and in different experimental fields.

Keywords: water shortage; yield; glycine betaine; proline; pollen viability; fruit set

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1. Introduction

Tomato is one of the most important crops, with more than five millions hectares cultivated, worldwide [1]. Being a summer crop, transient or extended drought periods are common during its cultivation cycle, especially in the most sensitive periods of fruit set and enlargement [2]. These are the two phases in which even a transitory drought stress can lead to heavy yield losses [3]. Climate change will make these events more frequent in arid and semi-arid environments, with detrimental consequences for tomato productivity [4,5]. Generally, plants respond to drought with a series of physiological mechanisms including stomatal closure, repression of cell growth and photosynthesis, and activation of stress hormones and antioxidant mechanisms, which overall lead to a reduction in plant growth and productivity [6]. The forecasted lack of water and the consequent increase in competition for water resources between agriculture and other sectors require the exploration of alternative and sustainable crop management strategies that can save water for irrigation and, at the same time, still maintain satisfactory levels of crop production [7].

One of the most promising strategies that can be used to improve plant response to drought stress is the use of biostimulants. These are substances or micro-organisms whose application is beneficial for plant growth and productivity [8]. The application of biostimulants can also induce enhanced tolerance to different abiotic stresses [9,10].

Chapter 7. General conclusions

GENERAL CONCLUSIONS

Food security is affected by environmental fluctuations that can drastically decrease crop yields. For this reason, many studies aim to develop novel plant lines and practical and sustainable systems to reduce critical yield losses caused by environmental changes. However, up to date agriculture still lacks crops with stable yields under different environmental conditions and very few strategies have proven to be effective in increasing productivity. At present, new agriculture offers a lot of molecules that affect plant responses to abiotic stresses. In this regard, the use of biostimulants proved to be a promising tool to improve tolerance to heat stress and drought. Their use could also help shed light on previously under investigated aspects of plant stress tolerance.

This PhD thesis was designed to satisfy the demand for a smarter, more efficient and secure agriculture suited for future climate challenges. It aimed to unveil the mechanisms underlying responses to abiotic stress in tomato and the role of biostimulants in increasing stress tolerance. In this work we obtained novel and interestingly results that enhance the knowledge on the plant physiological mechanism in response to combined (heat and drought) stress and use of biostimulant, respectively.

The first part of this research focused on the eco-physiological screening of several tomato genotypes under elevated temperatures (**Chapter 2**). We found that some parameters associated with chlorophyll fluorescence emission (i.e., ΦPSII or NPQ) and some leaf functional traits may be used as a tool to detect high temperatures-tolerant tomato cultivars, both in the field and in the laboratory. Moreover, the chlorophyll fluorescence induction kinetics recorded on detached leaves treated for 60 min at 35 °C or at 45 °C allowed us to identify two promising tomato genotypes (LA3120, E42) that are potentially tolerant to elevated temperatures. These results have been published in the Journal "*Plants"* (Arena et al., 2020).

The selected genotypes E42 and LA3120 were further tested in a growth chamber in order to analyse the different strategies developed in response to single and combined abiotic stresses (high temperature and water shortage) (**Chapter 3**). Noteworthy, both lines seemed to be tolerant to the applied prolonged water shortage. By contrast, heat and combined abiotic stresses clearly distinguished the two genotypes that were able to employ efficient antioxidant defence mechanisms in response to single and combined stress, a trait that could be the key to the tolerance observed in both genotypes also in open fields in other papers (Olivieri et al., 2020). Moreover, a Reduced Representation Sequencing (RRS) approach was carried out that allowed to explore the genetic variability of both genotypes, in order to identify candidate genes that could regulate stress responses.

In particular, this analysis allowed to confirm the high genetic variability of the novel genotype E42 and detect mutations in candidate genes that should be further analysed, including one in a gene coding for an Arabinogalactan protein (AGP). Ultimately, this study provides a framework to explore how crops change their behavior to tackle external stresses. These results have been submitted to the *Plant biology* journal.

As previously discussed, the use of biostimulant proved to be a complementary tool to improve tolerance to abiotic stress. Recently, it has been discovered that, among biostimulants, protein hydrolysates seem to be promising, since they contain large amounts of molecules such as amino acids, small peptides and osmoactive compounds (proline, glycine betaine) which are beneficial for plant productivity under unfavorable environmental conditions (Van Oosten et al. 2017). In the fourth chapter (**Chapter 4**) we investigated if biostimulant application can promote yield and fruit quality in tomato under abiotic stress and which are the molecules present in the biostimulant that stimulate these effects. The most significant outcome from this research was that the application of one protein hydrolysate (CycoFlow/Agriges) increased plant performances in plants grown in open field under elevated temperatures. In particular, biostimulant application determined a higher plant height, a larger number of fruits, a higher pollen vitality, a higher photochemical efficiency, a higher accumulation of ascorbic acid and a higher antioxidant activity. These last results are particularly interesting considering that in plants ascorbic acid is active in the removal of reactive oxygen species (ROS), it has an important role as an enzymatic cofactor and participates in plant development, senescence, defense, division, electron transfer and also in fruit ripening (Arena et al. 2013, Xu et al. 2017, Smirnoff et al. 2000). These results have been published in the journal *Agronomy* (Francesca et al. 2020).

The adaptive physiological response to single and combined stresses and biostimulant treatment was also investigated under controlled conditions by using a phenotyping platform in the framework of an EPPN 2020 program in the selected genotypes E42 and LA3120 (**Chapter 5**). This work further demonstrated that the genotype E42 is potentially tolerant to drought stress. Moreover, we demonstrated that under water and combined stress, the treatment with the protein hydrolysate (CycoFlow) provided protection to abiotic stress in treated plants also under controlled conditions. The treatment with the biostimulant had effect dependently of the genotype it was applied on, with E42 showing a stronger response to biostimulant application compared to LA3120. Indeed, a higher biomass and height was evidenced in E42 stressed plants treated with protein hydrolysate compared to non-treated ones, also an increase in net photosynthesis level and maximal efficiency of PSII photochemistry (Fv/Fm) was noticed. Furthermore, biostimulant application increased antioxidant

content, including total and reduced ascorbic acid, and decrease hydrogen peroxide (H_2O_2) content in E42 treated plants under water and combined stress conditions.

Considering that plants grown in open field are subjected to a higher number of different variables compared with controlled environments, in the final part of this work, the performances of the genotype E42 exposed to water deficit and treated with the novel protein hydrolysate were evaluated under open field conditions (**Chapter 6**). The protein hydrolysate was effective at enhancing tomato growth and productivity in different experimental fields, and under limited water availability. This was possible thanks to the enhancement of the water status of the biostimulant-treated plants coupled with higher antioxidant activity, which are part of a common tolerance strategy generally employed by plants to overcome different abiotic stresses. Biostimulant application increased also fruit quality promoting the accumulation of carotenoid and lycopene, which is an added value in regard to the increasing interest in the nutraceutical properties of food. These results have been published in the journal *Plants* (Francesca et al. 2021).

Altogether, three were the main finding of this PhD thesis: 1) the identification of a novel genotype (E42) tolerant to heat stress and drought; 2) the elucidation of the mechanisms activated under combined abiotic stress and 3) the characterization of a novel protein hydolysate-based biostimulant able to increase plant tolerance to abiotic stress.

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