




## RESEARCH ARTICLE

# Multitraits evaluation of a *Solanum pennellii* introgression tomato line challenged by combined abiotic stress

L. Vitale<sup>1</sup> , S. Francesca<sup>2</sup>, C. Arena<sup>3,4</sup> , N. D'Agostino<sup>2</sup>, L. Principio<sup>2</sup>, E. Vitale<sup>3</sup>, V. Cirillo<sup>2</sup>, M. C. de Pinto<sup>5</sup>, A. Barone<sup>2</sup> & M. M. Rigano<sup>2</sup> 

1 National Research Council (CNR), Department of Biology, Agriculture and Food Sciences (DiSBA), Institute for Agricultural and Forestry Systems in the Mediterranean (ISAFoM), Portici, Naples, Italy

2 Department of Agricultural Sciences, University of Naples "Federico II", Portici, Naples, Italy

3 Department of Biology, University of Naples "Federico II", Naples, Italy

4 NBFC – National Biodiversity Future Center, Palermo, Italy

5 Department of Biology, University of Bari "Aldo Moro", Bari, Italy

## Keywords

Combined stress; drought; heat stress; L-ascorbic acid; tomato; wild species.

## Correspondence

L. Vitale, National Research Council (CNR), Department of Biology, Agriculture and Food Sciences (DiSBA), Institute for Agricultural and Forestry Systems in the Mediterranean (ISAFoM), P.le E. Fermi 1 – Loc. Porto del Granatello, 80055 Portici, Naples, Italy.

E-mail: [luca.vitale@cnr.it](mailto:luca.vitale@cnr.it)

M. M. Rigano, Department of Agricultural Sciences, University of Naples "Federico II", Via Università 100, 80055 Portici, Naples, Italy.

E-mail: [mrigan@unina.it](mailto:mrigan@unina.it)

Vitale Luca and Francesca Silvana are co-first authors.

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## ABSTRACT

- Rising daily temperatures and water shortage are two of the major concerns in agriculture.
- In this work, we analysed the tolerance traits in a tomato line carrying a small region of the *Solanum pennellii* wild genome (IL12-4-SL) when grown under prolonged conditions of single and combined high temperature and water stress.
- When exposed to stress, IL12-4-SL showed higher heat tolerance than the cultivated line M82 at morphological, physiological, and biochemical levels. Moreover, under stress IL12-4-SL produced more flowers than M82, also characterized by higher pollen viability. In both lines, water stress negatively affected photosynthesis more than heat alone, whereas the combined stress did not further exacerbate the negative impacts of drought on this trait. Despite an observed decrease in carbon fixation, the quantum yield of PSII linear electron transport in IL12-4-SL was not affected by stress, thereby indicating that photochemical processes other than CO<sub>2</sub> fixation acted to maintain the electron chain in oxidized state and prevent photodamage. The ability of IL12-4-SL to tolerate abiotic stress was also related to the intrinsic ability of this line to accumulate ascorbic acid.
- The data collected in this study clearly indicate improved tolerance to single and combined abiotic stress for IL12-4-SL, making this line a promising one for cultivation in a climate scenario characterized by frequent and long-lasting heatwaves and low rainfall.

## INTRODUCTION

Global temperatures are expected to increase by 2–5 °C in the near future because of climate change, resulting in more intense heatwaves on a daily and seasonal scale, impacting crop productivity and food quality, especially in the Mediterranean Basin, which is already affected by a hot climate during the summer season (Collins *et al.* 2013; Hein *et al.* 2021; Francesca *et al.* 2022a). Temperatures above 35 °C can compromise plant growth and vegetative development by negatively affecting the reproductive phases, including pollen viability, and final crop yield (Pacini & Dolferus 2019; Ruggieri *et al.* 2019). Elevated temperatures are also responsible for enzyme degradation, depletion of PSII function, including a decrease in the rate of electron transport, the biosynthesis of pigments, and the

functioning of Rubisco activase (Moore *et al.* 2021). High temperatures increase evapotranspiration, thus exposing plant biomass to drought (Francesca *et al.* 2022a). Elevated temperatures and soil water depletion induce a wide range of morphological, anatomical, physiological, and biochemical changes in crops. Indeed, drought affects nutrient mobility, primary and secondary carbon metabolism, stomatal conductance, and plant developmental processes (Chaves *et al.* 2009; Shohat *et al.* 2021). Water shortage also determines a substantial alteration to oxidative metabolism, causing the accumulation of reactive oxygen species (ROS) (Francesca *et al.* 2022b).

*Solanum lycopersicum* L. has optimal growth in the temperature range of 25–30 °C during the day and 20 °C at night (Zhou *et al.* 2018), and it is also sensitive to water shortage. Several studies based on the application of different

physiological approaches have identified tomato lines tolerant to abiotic stresses (Arena *et al.* 2020); other works have led to the identification of favourable alleles underlying tolerance traits that can be transferred into elite germplasm, and to elucidation of the molecular and physiological mechanisms that control responses to abiotic stress (Ayenan *et al.* 2019; Dariva *et al.* 2020). However, only a few studies have focused on the response of plants to naturally co-occurring abiotic stresses and on the identification of genetic resources having increased flexibility to combined drought and heat stress (Zhou, Kong, Wu, *et al.* 2019; Zhou, Kong, Yu, *et al.* 2019; Francesca *et al.* 2022a). Wide differences in abiotic stress tolerance have been found between and within tomato wild species. There are accessions of wild-related species, both red-fruited (*e.g.* *S. pimpinellifolium*) and green-fruited (*e.g.* *S. pennellii*), with a high degree of tolerance to abiotic stresses (Atarés *et al.* 2011).

The wild tomato species *S. pennellii*, originating from the Andes in central Peru, exhibits adaptive mechanisms that ensure its survival in arid environments (Solankey *et al.* 2015). For this reason, it is a donor of favourable genes/alleles (Perez-Fons *et al.* 2014; Calafiore *et al.* 2016; Ruggieri *et al.* 2016) for cultivated tomato that has lost genetic variability during the processes of domestication and artificial selection. To take full advantage of the genetic variability of *S. pennellii*, scientists have developed a population of introgression lines (ILs) consisting of individuals carrying homozygous regions of the wild genome in the genetic background of the cultivated line M82 (Eshed & Zamir 1995). These ILs have so far been used for the identification of more than 2,700 quantitative trait loci (QTL), including those related to fruit traits, drought tolerance, photosynthesis, and plant growth (De Oliveira Silva *et al.* 2018; Zhou *et al.* 2018; Aliberti *et al.* 2020). Recently, one IL (IL12-4-SL) has been selected for the high content of antioxidants in the mature red fruit (Rigano *et al.* 2014; Ruggieri *et al.* 2015; Calafiore *et al.* 2016). In particular, a previous study showed that IL12-4-SL accumulates a higher amount of ascorbic acid (AsA) than the cultivated line M82, *via* increased expression of enzymes of the alternative D-galacturonate pathway leading to AsA biosynthesis (Rigano *et al.* 2018). A previous field trial also indicated the introgression line IL12-4-SL is hypothetically heat-tolerant, based on total yield and photosynthetic parameters (Arena *et al.* 2020). Indeed, the role of AsA in plant cells in controlling ROS scavenging (Akram *et al.* 2017; Hasanuzzaman *et al.* 2020), as a co-factor for some enzymes and as a signalling modulator in several cellular processes (Gallie 2013; Ruggieri *et al.* 2015; Rigano *et al.* 2018), can enhance the tolerance of crops to abiotic stress.

This study aimed to confirm the heat tolerance of IL12-4-SL and also to corroborate the hypothesis that this line, accumulating higher AsA levels, could better tolerate water shortage and combined heat and water stress. The physiological responses of the introgression line in comparison with the cultivated line M82, from which it derived, were analysed under single and combined stress. Results obtained in this study demonstrate that IL12-4-SL is a promising line for use in breeding programs, considering possible future climate scenarios characterized by frequent and long-lasting heatwaves and low rainfall.

## MATERIAL AND METHODS

### Plant material and growth conditions

The line IL12-4-SL was previously selected at the Department of Agricultural Sciences of the University of Naples "Federico II" (Italy) for its higher AsA content than the cultivated line M82 and for its putative tolerance to heat stress (Di Matteo *et al.* 2010; Arena *et al.* 2020). IL12-4-SL and M82 seeds were sown in a tray containing the commercial substrate Brill (blond tile peat 65% and German black peat 35%, pH of 6.5). After 20 days, seedlings were transferred into 20-l pots filled with the same commercial substrate and moved to two growth chambers with 29 °C/24 °C day/night temperatures, 16 h/8 h photoperiod and average daytime photosynthetic photon flux density (PPFD) 250–350  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . The experiment was arranged in a factorial design according to a completely randomized block, with three biological replicates per plant line and nine plants per treatment in each replicate.

The experiment consisted of four treatments: (1) control (C) 29 °C/24 °C day/night temperatures; (2) heat (H) 35 °C/30 °C day/night temperatures; (3) drought (D) 29 °C/24 °C day/night temperatures, without irrigation; (4) combined stress (COMB), 35 °C/30 °C day/night temperatures, without irrigation. The treatments were applied on 40-day-old seedlings and lasted 6 days. The control of environmental parameters was supported by the Arduino Mega 2560 micro-control system; the air temperature was measured every 5 min with the DHT11 sensor, which stored the data on a Secure Digital slot (Francesca *et al.* 2022b). The plants were watered daily using an automatic irrigation system based on nine soil moisture sensors for each growth chamber.

### Assessment of plant biomass and measurement of leaf functional characteristics

Plants were harvested at the end of the stress period and the fresh weight of shoots and roots, separated by cutting at the cotyledonary node, determined. All leaves from each plant were counted. Subsequently, the plant material was dried in an oven at 85 °C until constant weight was reached. The root/shoot ratio was calculated using both fresh (FW) and dry weight (DW). The number of flowers during the trial was also recorded. Leaf dry matter (LDMC), leaf area (LA), specific leaf area (SLA), and leaf thickness (LT) were determined according to Cornelissen *et al.* (2003) on five well-exposed and fully expanded leaves per line and per treatment. For the measurements, the fourth leaf from the apex in each plant was chosen. LDMC ( $\text{g}_{\text{dw}}\cdot\text{g}_{\text{fw}}^{-1}$ ) was determined as the ratio between leaf DW and the leaf turgid weight. SLA ( $\text{cm}^2\cdot\text{g}_{\text{dw}}^{-1}$ ) was calculated as LA/leaf DW, and LT as  $(\text{SLA} \times \text{LDMC})^{-1}$ . LA was measured with the Li-3100 Area Meter (Li-Cor, Lincoln, NE, USA) and turgid weight was determined by dipping the petiole in water overnight at 4 °C.

### Pollen viability

Pollen viability was analysed using at least three flowers per plant, taken from three different plants per replicate. The pollen grains were scattered onto microscope slides and a drop of DAB solution (Sigma-Aldrich, Darmstadt, Germany) added to

each sample (Dafni 1992). Scoring was made using a LEITZ Laborlux12 microscope.

### Leaf gas exchange and measurement of chlorophyll *a* fluorescence emission

Leaf gas exchange and chlorophyll *a* fluorescence were measured simultaneously on fully expanded leaves using the Li-6400 portable photosynthesis system (Li-Cor) integrated with an Li-6400-40 Leaf Chamber Fluorometer (LCF). The measurements were carried out in the morning (09:00–11:30 h) under the following environmental conditions: PPF of 1000  $\mu\text{mol}\cdot\text{photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , ambient  $\text{CO}_2$  concentration of 360  $\mu\text{mol}\cdot\text{CO}_2\cdot\text{mol}^{-1}$ , relative humidity (RH) 50–55%, and two temperature regimens, *i.e.* 26 °C (control) and 35 °C (heat treatment). Net photosynthesis ( $A_N$ ), stomatal conductance ( $g_s$ ), ambient ( $C_a$ ), substomatal ( $C_i$ )  $\text{CO}_2$  concentration and transpiration ( $E$ ) were calculated according to von Caemmerer & Farquhar (1981) using the software embedded in the Li-6400 device. Steady-state fluorescence ( $F_s$ ) and peak fluorescence ( $F_m'$ ) under illumination were measured by applying a 0.8-s saturating flash of 7,000  $\mu\text{mol}\cdot\text{photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ; the quantum yield of PSII electron transport ( $\Phi_{\text{PSII}}$ ) was calculated according to Genty *et al.* (1989). Photochemical quenching (qL) was calculated according to Kramer *et al.* (2004), while the non-photochemical quenching of excitation in PSII-associated antenna complexes ( $\text{NPQ}_T$ ) was obtained as proposed by Tietz *et al.* (2017). Instantaneous water use efficiency ( $\text{WUE}_i$ ) was calculated as the  $A_N/E$  ratio. Photosynthetic electron flow used by alternative sinks other than  $\text{CO}_2$  assimilation was estimated as the  $\text{ETR}/A_N$  ratio (Krall & Edwards 1992). The electron transport rate (ETR) was calculated as:  $\text{ETR} = 0.5 \times 0.84 \times I \times \Phi_{\text{PSII}}$ , where 0.5 is the fraction of absorbed quanta used by PSII, 0.84 is the leaf absorbance, and  $I$  is the incident PPF ( $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). All the measurements were determined on at least six well-exposed and fully expanded leaves per line and treatment.

### Analysis of the content of photosynthetic pigments

The content of carotenoids (Car) and chlorophylls (Chl) was determined according to Rigano *et al.* (2016). Samples (0.30 g) were processed with 24 ml acetone/hexane (40/60, v/v) to obtain the lipophilic extract. The mixture was centrifuged at 15,000 g for 5 min at 4 °C. The supernatants were collected and stored at –20 °C until downstream analysis. The absorbance of lipophilic extracts was read at 470, 663, and 645 nm, using a spectrophotometer (Implen, Munich, Germany) to quantify carotenoids and chlorophylls *a* and *b*. The pigment concentration was reported as  $\text{mg } 100 \text{ g}^{-1}\cdot\text{FW}$ . Three separate biological replicates for each sample and three technical assays for each biological repetition were measured.

### Estimation of hydrogen peroxide, malondialdehyde, and ascorbic acid content

The quantification of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) was carried out using a colorimetric method (Sergiev *et al.* 1997). Briefly, 500 mg frozen powder from tomato leaves was subjected to extraction with 5 ml ice-cold 0.1% trichloroacetic acid (TCA), and the mixture was then incubated for 15 min on ice and

centrifuged at 10,000 g for 15 min at 4 °C. An aliquot of 500  $\mu\text{l}$  10 mM phosphate buffer (pH 7.0) and 1 ml potassium iodide (1 M) were added to 500  $\mu\text{l}$  of supernatant. The mixtures were then incubated in the dark for 40 min and measured at 525 nm using a Nano Photometer TM (Implen, Munich, Germany). Three biological replicates per sample and three technical assays for each biological replicate were used. The concentration was expressed as  $\text{mmol}\cdot\text{g}\cdot\text{FW}^{-1}$ . The malondialdehyde (MDA) content in leaves indicates levels of membrane lipid peroxidation. Briefly, 0.2 g leaf sample was ground with 1 ml of ice-cold 0.1% TCA. The samples were incubated for 15 min on ice and centrifuged at 10,000 g for 10 min at 4 °C. Subsequently, 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 using a Nano Photometer TM (Implen). Three biological replicates per sample and three technical assays for each biological replicate were used. The concentration was expressed as the amount of MDA-TBA complex (Zhang & Kirkham 1996). Quantification of reduced AsA and total AsA (AsA + dehydroascorbate–DHA) was performed according to Francesca *et al.* (2020). Briefly, 500 mg frozen powder from tomato leaves was subjected to extraction with 600  $\mu\text{l}$  ice-cold 6% TCA and the mixture was then incubated for 15 min on ice and centrifuged at 14,000 g for 20 min. A total of 20  $\mu\text{l}$  0.4 M phosphate buffer (pH 7.4), 10  $\mu\text{l}$  double distilled (dd)  $\text{H}_2\text{O}$  and 80  $\mu\text{l}$  colour reagent solution were added to 20  $\mu\text{l}$  supernatant to estimate the reduced AsA content. This solution was prepared by mixing solution A (31% (w/v)  $\text{H}_3\text{PO}_4$ , 4.6% (w/v) TCA and 0.6% (w/v)  $\text{FeCl}_3$ ) with solution B (4% (w/v) 2,20-Dipyridyl). As for total AsA, 20  $\mu\text{l}$  5 mM dithiothreitol in 0.4 M phosphate buffer (pH 7.4) were added to 20  $\mu\text{l}$  of sample and the mixture incubated for 20 min at 37 °C. A total of 10  $\mu\text{l}$  N-ethylmaleimide (NEM; 0.5% (w/v) in water) were added and left for 1 min at room temperature. The 80  $\mu\text{l}$  dye reagent were added as previously described. Both of the final mixtures were incubated at 37 °C for 40 min and measured at 525 nm using a Nano Photometer TM (Implen). Three biological replicates per sample and three technical assays for each biological replicate were used and the concentration was expressed as  $\mu\text{mol}\cdot\text{g}\cdot\text{FW}^{-1}$ .

### Activity of antioxidant enzymes

The frozen powder (0.5 g) of 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 PMSE, 5% PVPP in a 1:4 ratio (w/v). The supernatants obtained after centrifugation at 22,000 g for 20 min were then subjected to spectrophotometric analysis. The activity of cytosolic APX (L-ascorbate: hydrogen peroxide oxidoreductase, EC 1.11.1.11) was measured following the  $\text{H}_2\text{O}_2$ -dependent oxidation of AsA at 290 nm in a reaction mixture containing 0.1 M Tris-acetate buffer, pH 6.4, 350  $\mu\text{M}$  AsA, 170  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 50  $\mu\text{g}$  protein. Catalase (CAT, EC 1.11.1.6) activity assay was performed following dismutation of  $\text{H}_2\text{O}_2$  at 240 nm in a reaction mixture consisting of 0.1 M phosphate buffer, pH 7.0, 50  $\mu\text{g}$  protein and 18 mM  $\text{H}_2\text{O}_2$  ( $\epsilon = 39.6 \text{ M}^{-1}\cdot\text{cm}^{-1}$ ). Protein content was determined according to Bradford (1976) using BSA as standard. All enzyme activities were measured using a Beckman (Fullerton, CA,

USA) DU 7000 spectrophotometer. For all analyses, three separate biological replicates for each sample and three technical assays for each biological repetition were measured.

### Statistical analysis

The data were subjected to three-way ANOVA. Duncan's test was performed on separate means if the differences were significant ( $P \leq 0.05$ ). The ANOVA was computed using SPSS (Statistical Package for Social Sciences), version 23.0. A principal components analysis (PCA) was performed and a PCA plot was generated using FactoExtra and FactoMine packages (Lê *et al.* 2008).

## RESULTS

### Phenotyping and functional leaf characteristics

Biometric and foliar traits were measured to determine the effect of drought, heat, and combined stresses on both IL12-4-SL and M82 (Table 1 and Table S1). Most of the biometric traits were affected by single and combined stresses. Under all stresses the height of IL12-4-SL was greater than that of M82. Shoot FW and DW were higher in IL12-4-SL under control, drought and combined stress, and these parameters generally decreased in both lines under stress. The FW and DW of roots was higher in IL12-4-SL than in M82, but decreased under single and combined stresses, until differences became irrelevant. Interestingly, the FW and DW shoot/root weight ratio was not affected by the different stresses in either line.

The number of inflorescences decreased in M82 under single stress, but a drastic decrease ( $-71.22\%$ ) was observed under combined stresses compared to non-stressed plants. The number of flowers extraordinarily decreased ( $-46.08\%$ ) in IL12-4-SL only under combined stress (COMB), thereby being higher than in M82 subjected to the same conditions. A decrease in pollen viability was also observed in both lines. The largest reduction in pollen viability occurred under heat stress, which led to a reduction in pollen viability of  $-89.29\%$  and  $-76.31\%$  in M82 and IL12-4-SL, respectively. In both lines, plants under drought and combined stress had withered stems, but IL12-4-SL plants appeared less stressed than M82 plants (Figure S1A, B). According to the ANOVA, only leaf area (LA) depended on the interaction between stress and the genetic background of the plants (Table S1). Under control conditions, IL12-4-SL had a higher LA than M82, and an increase in this parameter was observed in M82 under heat stress and in IL12-4-SL under COMB conditions (Table S2). The latter affected the SLA and leaf thickness (LT) of both lines. Leaves of IL12-4-SL were thinner than those of M82, whereas a decrease in relative water content (RWC) was observed in both lines under drought and COMB, and under heat in IL12-4-SL.

### Measurements of photosynthetic pigments and gas exchange

Photosynthetic pigments were analysed in leaves of plants exposed to single or combined stress. Carotenoids increased under drought and combined stress in both lines (Table S3). An increase in the content of chlorophylls *a* and *b* was observed only in IL12-4-SL under all applied stresses and in M82 under

**Table 1.** Biometric traits in two tomato lines (M82 and IL12-4-SL) grown under control (CTRL), heat (H), drought (D) and combined stress (COMB) conditions.

parameter	line	treatment			
		CTRL	H	D	COMB
Height (cm)	M82	66.5 ± 4.50 <sup>bc</sup>	75 ± 11.63 <sup>c</sup>	51.75 ± 11.14 <sup>a</sup>	58 ± 6.58 <sup>ab</sup>
	IL12-4-SL	82.5 ± 18.80 <sup>a</sup>	88.75 ± 0.95 <sup>a,*</sup>	76.25 ± 5.56 <sup>a,***</sup>	79 ± 5.88 <sup>a,***</sup>
No. leaves	M82	36.5 ± 8.85 <sup>a</sup>	37.5 ± 4.94 <sup>a</sup>	31.5 ± 13.20 <sup>a</sup>	29.25 ± 6.18 <sup>a</sup>
	IL12-4-SL	41 ± 6.73 <sup>a</sup>	28 ± 7.70 <sup>a,*</sup>	34.75 ± 7.80 <sup>a</sup>	30.25 ± 15.58 <sup>a</sup>
Shoot FW (g)	M82	168.5 ± 9.19 <sup>b</sup>	166 ± 8.48 <sup>b</sup>	109 ± 11.97 <sup>a</sup>	91.75 ± 18.13 <sup>a</sup>
	IL12-4-SL	217.25 ± 37.20 <sup>b,**</sup>	171.25 ± 30.33 <sup>a</sup>	156.75 ± 21.97 <sup>a,***</sup>	141.25 ± 11.52 <sup>a,***</sup>
Shoot DW (g)	M82	28 ± 2.82 <sup>b</sup>	24 ± 5.56 <sup>a</sup>	17.5 ± 2.08 <sup>a</sup>	15 ± 3.16 <sup>a</sup>
	IL12-4-SL	36.25 ± 4.34 <sup>c,**</sup>	25.25 ± 7.13 <sup>ab</sup>	21.25 ± 2.75 <sup>bc,*</sup>	21.5 ± 3.87 <sup>a,**</sup>
Root FW (g)	M82	35.34 ± 1.71 <sup>b</sup>	32.27 ± 12.59 <sup>ab</sup>	29.61 ± 8.83 <sup>ab</sup>	18.79 ± 7.05 <sup>a</sup>
	IL12-4-SL	51.41 ± 8.33 <sup>b,***</sup>	30.98 ± 13.69 <sup>a</sup>	35.64 ± 3.19 <sup>a</sup>	25.75 ± 8.60 <sup>a</sup>
Root DW (g)	M82	3.5 ± 1.29 <sup>a</sup>	3 ± 1.00 <sup>a</sup>	3 ± 0.81 <sup>a</sup>	2.5 ± 1.29 <sup>a</sup>
	IL12-4-SL	5.5 ± 0.57 <sup>b,**</sup>	3.25 ± 1.50 <sup>a</sup>	3.25 ± 0.50 <sup>a</sup>	2.75 ± 0.5 <sup>a</sup>
Shoot/root FW	M82	4.93 ± 0.54 <sup>b</sup>	7.44 ± 2.79 <sup>ab</sup>	3.90 ± 1.07 <sup>ab</sup>	5.25 ± 1.59 <sup>a</sup>
	IL12-4-SL	4.29 ± 0.88 <sup>a</sup>	6.41 ± 2.73 <sup>a</sup>	4.42 ± 0.73 <sup>a</sup>	5.94 ± 1.85 <sup>a</sup>
Shoot/root DW	M82	10.75 ± 6.01 <sup>a</sup>	8.22 ± 1.10 <sup>a</sup>	6.08 ± 1.29 <sup>a</sup>	5.22 ± 0.63 <sup>a</sup>
	IL12-4-SL	7.22 ± 0.49 <sup>a</sup>	7.35 ± 0.99 <sup>a</sup>	6.60 ± 1.00 <sup>a</sup>	7.83 ± 0.43 <sup>a,***</sup>
No. flowers	M82	69.5 ± 14.38 <sup>b</sup>	30 ± 14.14 <sup>a</sup>	35.33 ± 16.74 <sup>a</sup>	20 ± 5.65 <sup>a</sup>
	IL12-4-SL	57.5 ± 11.67 <sup>b</sup>	53 ± 14.13 <sup>ab,*</sup>	46.25 ± 10.5 <sup>ab</sup>	31 ± 8.48 <sup>a,*</sup>
Pollen viability	M82	0.84 ± 0.02 <sup>c</sup>	0.08 ± 0.04 <sup>a</sup>	0.57 ± 0.05 <sup>b</sup>	0.58 ± 0.01 <sup>b</sup>
	IL12-4-SL	0.76 ± 0.04 <sup>c,**</sup>	0.18 ± 0.06 <sup>a,***</sup>	0.68 ± 0.05 <sup>ab,*</sup>	0.58 ± 0.11 <sup>b</sup>

The data represent mean ± SE (n = 6). Within each tomato line, different letters correspond to significantly different values with Duncan's *post-hoc* test among treatments ( $P \leq 0.05$ ). Asterisks indicate statistically significant differences of IL12-4-SL *versus* M82 in each treatment (Student's *t* test).

\* $P < 0.05$ .

\*\* $P < 0.01$ .

\*\*\* $P < 0.001$ .

COMB. Under control conditions, IL12-4-SL had higher net photosynthesis than M82, although the degree of stomatal opening ( $g_s$ ) was comparable between lines, resulting in higher WUE (Fig. 1). In the same conditions, the quantum yield of PSII electron transport ( $\Phi_{PSII}$ ) was lower in IL12-4-SL than in M82, and this resulted in a reduction of the  $ETR/A_N$  ratio to the theoretical value of 4/5 as well as of photochemical quenching ( $q_L$ ). By contrast, an increase in thermal energy dissipation ( $NPQ_T$ ) was observed (Fig. 2). These constitutive differences between the two tested tomato lines led to a better understand of their behaviours under different applied stresses.

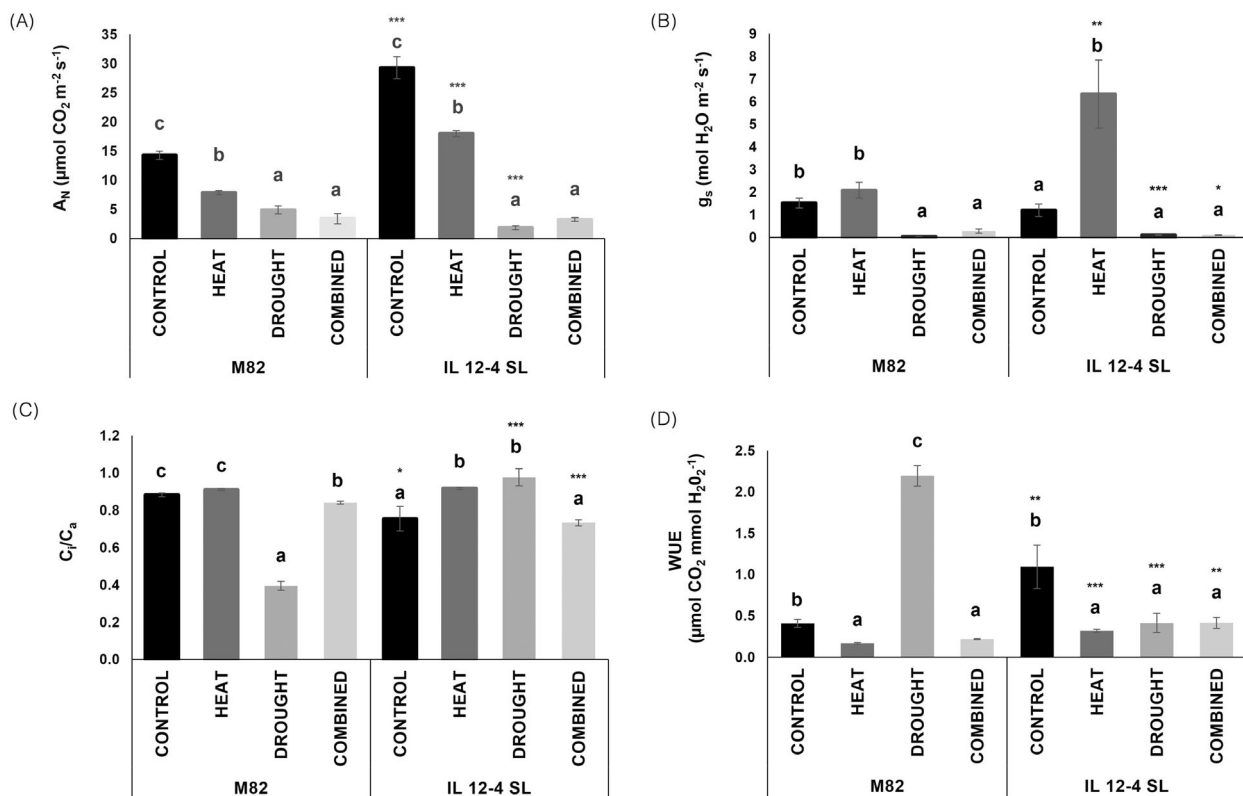
Photosynthesis was significantly affected by stresses. A single stress (heat or drought) resulted in a decreased  $A_N$  in both lines, although drought affected  $A_N$  more strongly than heat stress (Fig. 1A), especially in IL12-4-SL, whereas COMB did not appear to exacerbate a photosynthesis reduction, especially in IL12-4-SL. Heat stress did not negatively affect  $g_s$ , which was affected by drought and COMB (Fig. 1B). Although a reduction in  $A_N$  occurred, the substomatal  $CO_2$  concentration, evaluated as  $C_i/C_a$  ratio, remained high under heat treatment for both lines (Fig. 1C), whereas a decrease under drought occurred in M82. The constraint imposed by COMB resulted in decreased  $C_i/C_a$  values only in M82. WUE was affected by all stresses in both lines. WUE decreased under single stress and COMB in IL12-4-SL; the same trend was observed in M82 except for an increase observed under drought (Fig. 1D).

Single or combined stress resulted in a reduction of  $\Phi_{PSII}$  in M82, although drought caused the strongest reduction

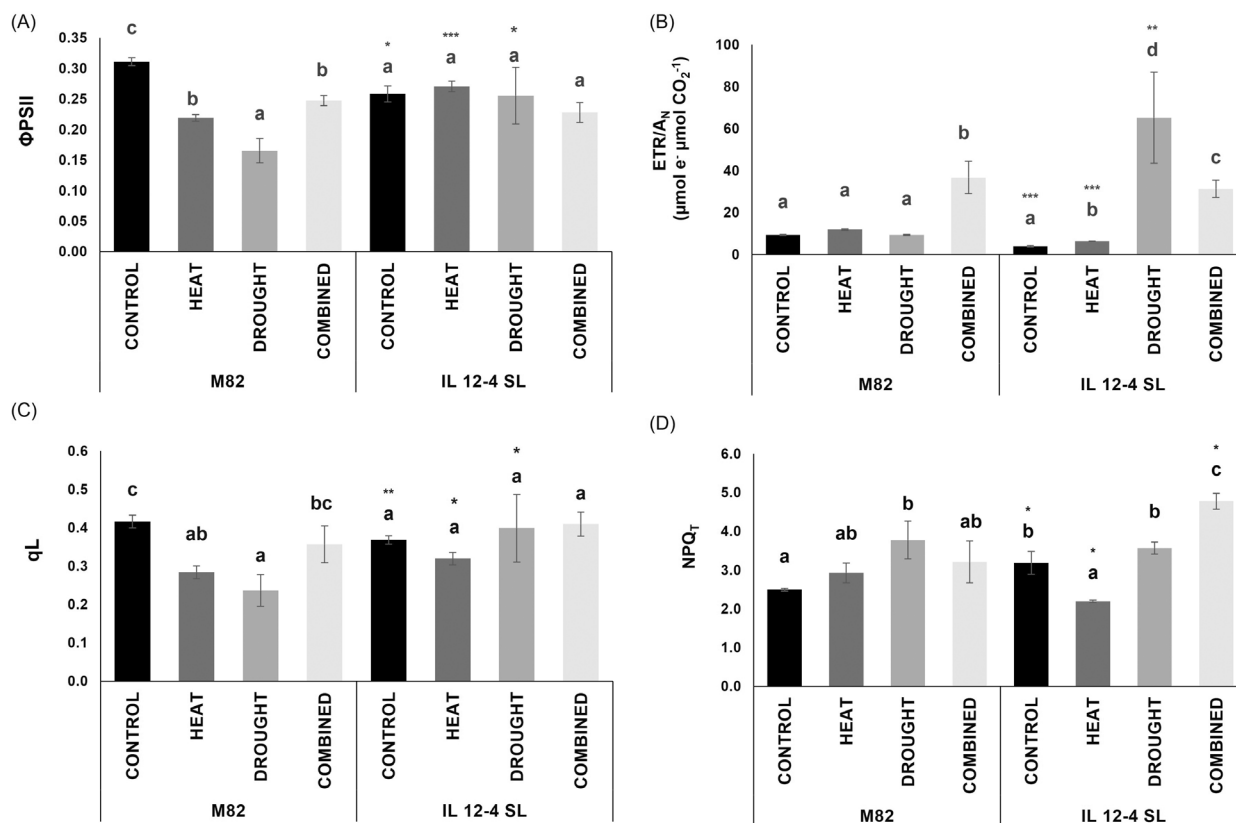
(Fig. 2A). In contrast,  $\Phi_{PSII}$  was not affected by the stress imposed on IL12-4-SL despite the  $A_N$  reduction, so indicating a higher flow of electrons towards photochemical sinks alternative to  $CO_2$  fixation as compared to the control. In M82, an increased flow of electrons to alternative sinks to  $CO_2$  assimilation was recorded under COMB, as indicated by an increase in the  $ETR/A_N$  ratio. This also occurred in IL12-4-SL under single stress and COMB, with the highest  $ETR/A_N$  ratio measured under drought. Under heat stress, the flow of electrons to alternative sinks to  $CO_2$  assimilation was lower in IL12-4-SL than in M82, whereas it was markedly higher under drought (Fig. 2B). A significant reduction of photochemical quenching ( $q_L$ ) was observed in M82 under heat and drought, whereas no influence of stress was observed in IL12-4-SL. Under single stress,  $q_L$  was higher in IL12-4-SL than in M82 (Fig. 2C). The thermal dissipation by antenna complexes ( $NPQ_T$ ) was affected by the treatments (Fig. 2D). Under heat stress  $NPQ_T$  dropped compared to the control in IL12-4-SL. In M82,  $NPQ_T$  increased under drought, whereas COMB induced a significant increase in heat dissipation in IL12-4-SL. Under combined stress, IL12-4-SL dissipated more energy than M82.

#### Measurements of parameters of oxidative stress and antioxidants

The content of total and reduced AsA was higher in IL12-4-SL than in M82 under control conditions. All the different stresses applied affected the content of this antioxidant. In M82, the



**Fig. 1.** Measurements of gas exchange and fluorescence in leaves of two tomato lines (M82 and IL12-4-SL) under control, heat, drought, and combined stress. Different panels represent (A) net photosynthesis ( $A_N$ ), (B) stomatal conductance ( $g_s$ ), (C)  $C_i/C_a$  ratio, (D) water use efficiency (WUE). Within each tomato line, different letters correspond to significantly different values with Duncan's *post-hoc* test among treatments ( $P \leq 0.05$ ). Data represent mean  $\pm$  SE ( $n = 6$ ). Asterisks indicate statistically significant differences of IL12-4-SL compared to M82 in each treatment (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; Student's *t* test).



**Fig. 2.** Measurements of gas exchange and fluorescence in leaves of two tomato lines (M82 and IL12-4-SL) under control, heat, drought and combined stress. Different panels represent (A) quantum yield of PSII ( $\Phi_{PSII}$ ), (B)  $ETR/A_N$ , (C)  $q_L$ , (D)  $NPQ_T$ . Within each tomato line, different letters correspond to significant differences with Duncan's *post-hoc* test among treatments ( $P \leq 0.05$ ). The data represent mean  $\pm$  SE ( $n = 6$ ). Asterisks indicate statistically significant differences of IL12-4-SL compared to M82 in each treatment (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; Student's *t* test).

content of total and reduced AsA increased under all applied stresses (Fig. 3A, B). In IL12-4-SL, no stress conditions applied affected total AsA content, while heat stress caused a decrease in reduced AsA.

To verify whether the stress conditions caused oxidative damage in the two lines, the concentrations of  $H_2O_2$  and MDA were determined (Fig. 3C, D). In M82, an increase in the level of  $H_2O_2$  under all applied stresses and of MDA under drought was observed. On the other hand, no significant changes in the content of  $H_2O_2$  and MDA were recorded in IL12-4-SL, except under COMB, where a significant increase in  $H_2O_2$  content was observed. The activity of APX and CAT significantly increased under drought and COMB in M82 (Fig. 3E, F) but did not show significant differences in IL12-4-SL in response to all forms of stress.

### Principal components analysis

A PCA was performed to provide a broad overview of the information content of all data gathered (Fig. 4). The first (PC1) and the second (PC2) component scores accounted for 38.65% and 21.62% of the total variability, respectively. PC1 was the main driver of differences between lines, and the key parameters leading to this separation were plant growth, photosynthetic pigments, and net photosynthesis. PC2 separated samples by treatments, driven by plant height and some gas exchange parameters, such as  $g_s$ ,  $C_i/C_a$ , WUE (Table S4).

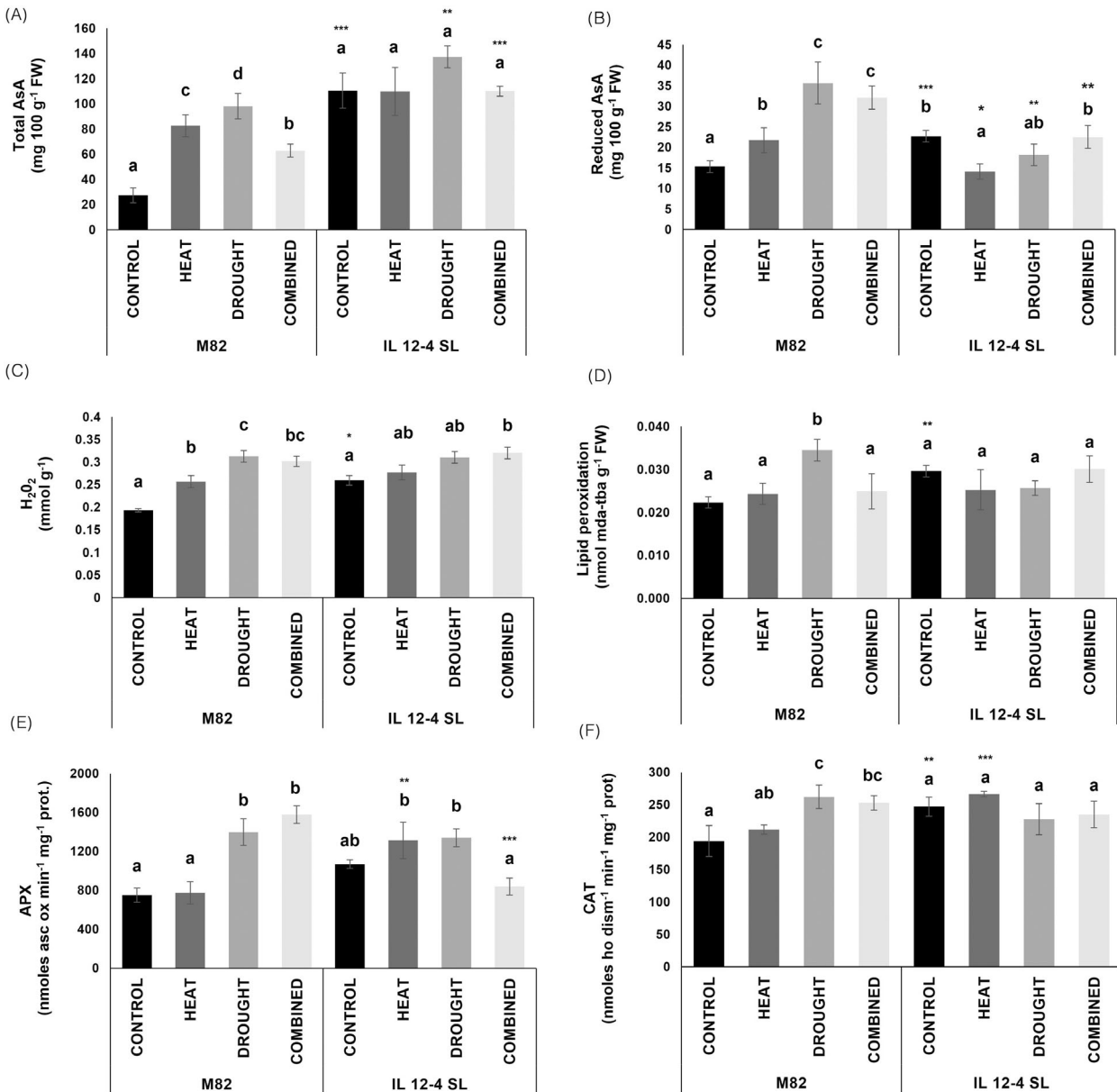
Noteworthy, the applied PCA allowed us to note the different behaviours of the two lines under different stress conditions. M82 responded differently depending on the stress applied, occupying a different square of the plot for each stress (Fig. 4). Instead, a common response seems to be activated in IL12-4-SL in the presence of stress that did not depend on the nature of the abiotic stress applied (Fig. 5).

### DISCUSSION

Global climate changes require the selection of new tomato varieties capable of responding to the challenges of global agriculture and improved knowledge of the ecophysiological strategies that the crops adopt to cope with environmental stresses. In this study, we tested the tolerance of IL12-4-SL, characterized by constitutively higher AsA production (Rigano *et al.* 2018), under heat and water stress conditions to simulate the growth environment in which these plants could be cultivated in the future. The PCA confirmed significant differences between IL12-4-SL and M82 in constitutive traits and responses to stress.

#### Tomato IL12-4-SL showed morpho-physiological differences compared to M82

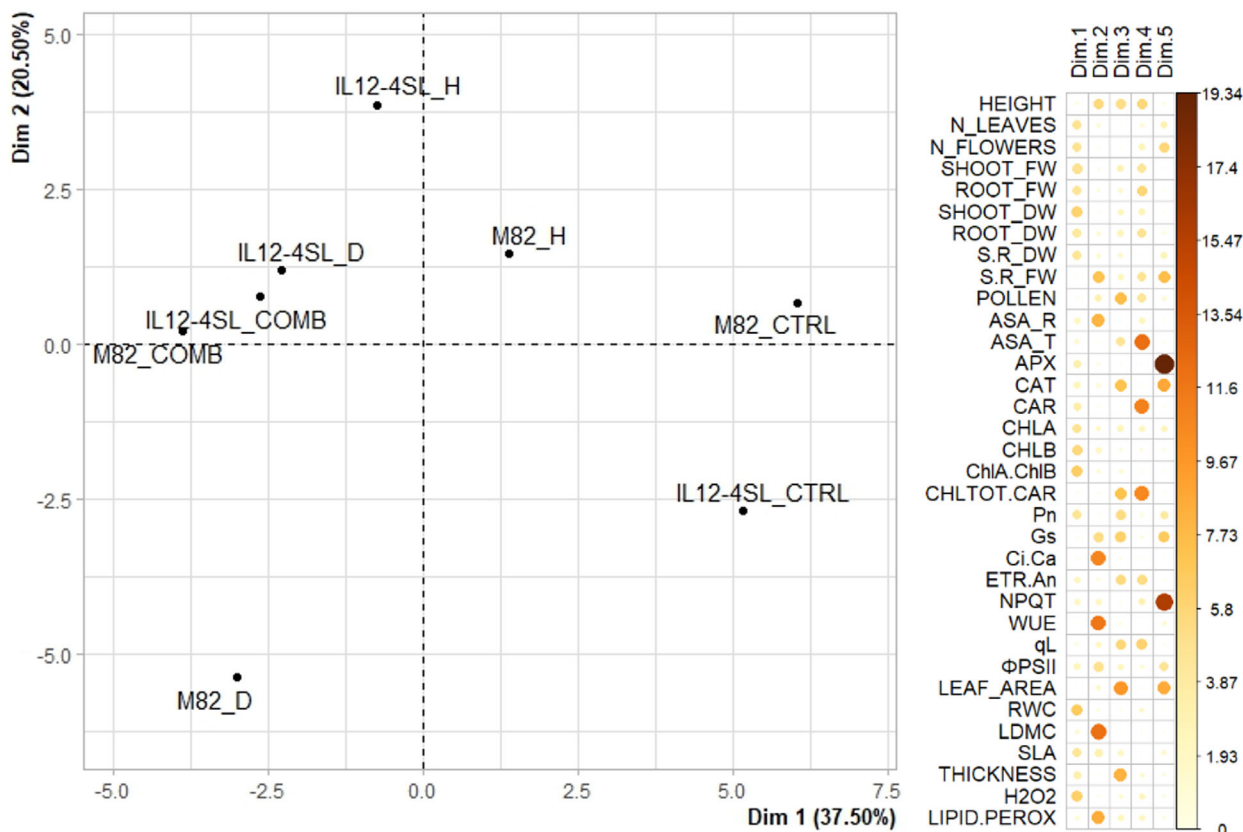
Under non-stressed conditions, IL12-4-SL produced constitutively more dry matter than M82 because of  $A_N$ . Leaf



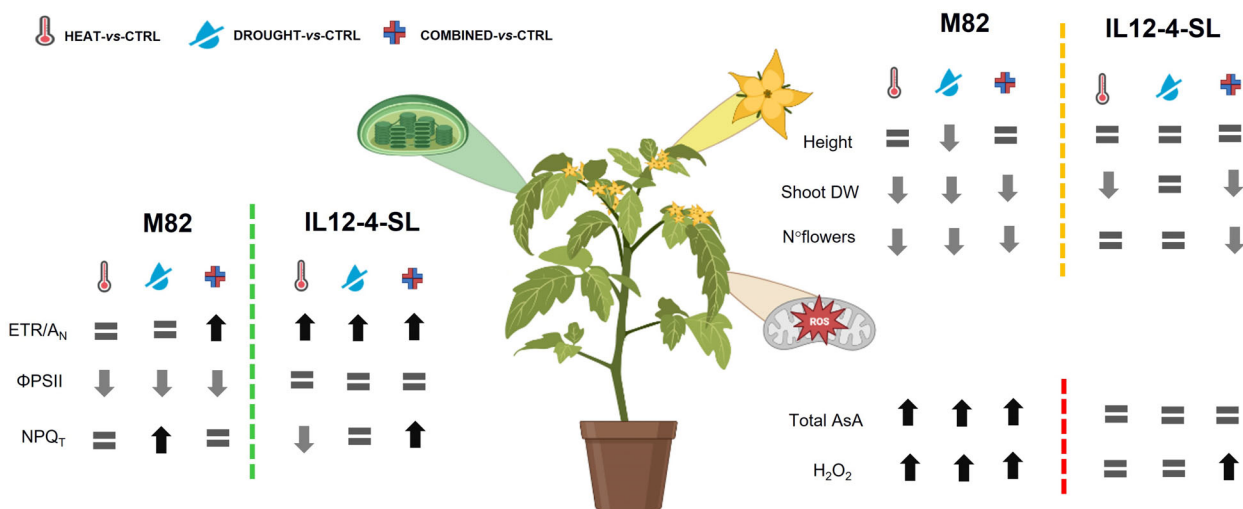
**Fig. 3.** Ascorbic acid (AsA), oxidative markers and activity of ROS-scavenging enzymes of two tomato lines (M82 and IL12-4-SL) under control, heat, drought and combined stress. Different panels represent (A) total AsA, (B) reduced AsA, (C) hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), (D) lipid peroxidation (LP), measured as malondialdehyde (MDA-TBA) content, (E) Ascorbate peroxidase (APX) and (F) Catalase (CAT). Within each tomato line, different letters correspond to significant differences with Duncan's *post-hoc* test among treatments ( $P \leq 0.05$ ). Data are mean  $\pm$  SE ( $n = 6$ ). Asterisks indicate statistically significant differences of IL12-4-SL compared to M82 in each treatment (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; Student's *t* test).

morphology and structure affect the gas diffusion through leaf tissues; thin leaves with a higher SLA have a larger mesophyll conductance, a trait that controls photosynthetic efficiency (Muir *et al.* 2014). Moreover, plants with a decreased SLA are characterized by a reduced intrinsic biomass growth rate (Poorter *et al.* 2009), indicating significant costs in terms of both photosynthesis and whole plant growth (Muir *et al.* 2014). These functional characteristics of the leaf played a key role in the introgression line in its higher carbon gain compared to M82. It is likely that a minor resistance to CO<sub>2</sub> diffusion within the leaf, due to higher SLA and lower thickness, contributed to increased chloroplast CO<sub>2</sub> concentration in

IL12-4-SL, promoting photosynthesis (Vitale *et al.* 2020) and markedly limiting the consumption of reductive equivalents towards alternative pathways to CO<sub>2</sub> fixation, as indicated by the lower values of  $\Phi_{PSII}$  and ETR/A<sub>N</sub>. Furthermore, considering the importance of AsA as a co-factor of some enzymes or protein complexes in the regulation of photosynthesis, it is highly probable that the increased accumulation of AsA in IL12-4-SL played an important role in the upregulation of enzymes related to CO<sub>2</sub> assimilation (Davey *et al.* 2000). Indeed, our results show, for the first time, that IL12-4-SL accumulates more AsA than M82, not only in fruits (Rigano *et al.* 2018) but also in leaves.



**Fig. 4.** Principal components loading plot and contribution of each parameter measured in PCA in two tomato lines (M82 and IL12-4-SL) under control (CTRL), heat (H), drought (D) and combined (COMB) stress.



**Fig. 5.** The overall effect of heat stress, drought, and combined stress in tomato lines M82 and IL12-4-SL.

**Physiological responses of IL12-4-SL and M82 to heat stress**

The increase in global warming, an expected condition through the 21st century as consequence of climate change, will negatively affect tomato growth and yield (Alsamir *et al.* 2021); therefore, the selection of heat-tolerant genotypes may represent a winning strategy. Long-term exposure to high

temperatures triggered different phenotypic responses in the two lines under investigation. While IL12-4-SL exhibited reduced carbon investment in the shoot and root when grown at high temperature, evidencing temperature-dependent changes in biomass allocation (Atkin *et al.* 2006), M82 showed phenotypic plasticity of the trait, as demonstrated by increased leaf area, which improved light interception to compensate for



the low photosynthesis rate for maintenance of plant carbon gain. In both lines, heat stress led to a large reduction in photosynthesis rates, likely attributable to non-stomatal limitations, such as impairment of enzymes involved in photosynthesis. This assumption was consistent with the unchanged or higher  $C_i/C_a$  ratio and the reduced WUE ( $A_N/E$ ) compared to the control condition. However, the stronger reduction in  $A_N$  in M82 compared to IL12-4-SL highlighted the higher thermotolerance of IL12-4-SL, which allocated more carbon towards producing flowers with a higher pollen viability than M82, with consequent prediction of better fruit production under elevated temperatures. These data are particularly important considering that pollen viability and number of flowers are among the most important traits associated with heat tolerance (Alsamir *et al.* 2021). In IL12-4-SL the increased accumulation of AsA probably compensated for the production of ROS ( $H_2O_2$ ) at high temperatures due to high activity of  $H_2O_2$ -consuming enzymes. Our results are consistent with data from crops exposed to exogenous AsA, which showed improved photosynthesis, heat tolerance, and resistance to oxidative stress (*e.g.*, Sarwar *et al.* 2018; Alayafi 2020). In IL12-4-SL, the high  $H_2O_2$  removal contributed to photochemically dissipate the excess absorbed light, also due to the increased content of photosynthetic pigments, reducing the need for thermal dissipation. It is reasonable that the enhanced AsA content in the leaves reduced the thermal dissipation activity of the photosystems.

#### Physiological responses of IL12-4-SL and M82 to water shortage

Drought is another stressor constraining crop growth and photosynthesis. Consistent with data observed under heat stress, water shortage limited  $CO_2$  uptake in both lines, but more severely than heat stress. The largest reduction in  $A_N$  was observed in IL12-4-SL. The decline in  $A_N$  was mainly due to non-stomatal limitations in IL12-4-SL and to stomatal limitations in M82, as indicated by the reduction of  $C_i/C_a$  ratio and the increase of WUE in M82. Our data indicated IL12-4-SL is a weakly drought tolerant line compared to M82, which was able to maintain higher photosynthesis rates because of leaf morphological and anatomical adjustments, such as modifications of LT and SLA, which favour leaf gas exchange. However, compared with non-stressed plants, IL12-4-SL seemed to cope better with water shortage by investing more dry matter in smaller leaves, which, due to their anatomical characteristics, could limit water losses thus preserving the water balance of the plant. Moreover, under drought, this line was able to produce many flowers with higher pollen viability compared to M82, and this represents a further advantageous trait for greater yield under water stress.

The two lines dealt with drought through different physiological strategies. Whereas IL12-4-SL dissipated absorbed light by consuming more reductive power in alternative photochemical processes to  $CO_2$  assimilation, as indicated by the increased  $ETR/A_N$  ratio (Flexas *et al.* 1999), M82 downregulated the electron transport rate to meet  $CO_2$  assimilation requirements. Under these circumstances, thermal dissipation ( $NPQ_T$ ) was increased but was inadequate to keep the reaction centers oxidized, as indicated by  $qL$  value of 0.25. It is reasonable to assume that the increased consumption of reductive power observed in IL12-4-SL was associated with

the regeneration of AsA in the ascorbate–glutathione (AsA–GDG) cycle, when reduced AsA was consumed through APX activity. Consequently, the increase in  $H_2O_2$  content was limited, and the lipid peroxidation prevented. Conversely, the diminished consumption of reduced AsA in M82 resulted in a marked increase in  $H_2O_2$  content, despite the enhanced APX and CAT activity, leading to increased membrane lipid peroxidation, highlighting higher sensitivity to drought of M82.

#### Physiological responses of IL12-4-SL and M82 to combined heat and water stress

The past few decades have witnessed severe droughts and heatwaves around the world, which are predicted to become more common and more intense in the future (Rosenzweig *et al.* 2014), exposing crops to severe limitations. Plants experience drought and heat stress in combination in the field, where the effects are more detrimental than the effect of an individual stress (Mittler 2006). Under combined stress, IL12-4-SL reduced the carbon investment towards the root and shoot but invested more carbon in leaves with a larger leaf area, when compared to stressed M82 plants. The wide leaf lamina could intercept a larger quantity of light, as also facilitated by a higher content of carotenoids compared to control conditions. By reducing carbon investment in root biomass and promoting large leaves, the IL12-4-SL line appeared to better tolerate the combined stress than the parental genotype, managing to maintain more viable flowers, a trait evidencing the heat-tolerance of this genotype (Firon *et al.* 2006; Xu *et al.* 2017). The combined stress severely reduced  $CO_2$  fixation in both genotypes, even if no more than drought alone, suggesting that both lines have the same tolerance of  $CO_2$  assimilation under combined stress. The tolerance was due to the consumption of reducing power in photochemical processes other than  $CO_2$  fixation, as indicated by the increase in  $ETR/A_N$  ratio (Flexas *et al.* 1999), thus managing to keep the electron transport chain oxidized, as indicated by high  $qL$  values, and avoiding oxidative damage to photosystems. By keeping APX and CAT activities at constitutive levels compared to non-stressed plants, IL12-4-SL avoided further excessive accumulation of  $H_2O_2$ , also thanks to higher thermal dissipation ( $NPQ_T$ ), which limited the radiative load in the reaction centers. Conversely, the M82 genotype needed to keep higher APX and CAT activities, if compared to non-stressed plants, in order to limit further increases in ROS content under combined stress.

#### CONCLUSION

The data obtained in this study clearly demonstrated that the tomato line IL12-4-SL tolerates heat stress and drought better than the cultivated line M82. In particular, the higher accumulation of AsA, likely due to activation of the alternative D-galacturonate pathway also in leaves (Rigano *et al.* 2018), proved to be advantageous for the former line to cope with single or combined water and heat stresses. IL12-4-SL was more capable than M82 of photochemically dissipating excess absorbed light under heat stress and drought, an advantageous strategy to successfully cope with the effects of climate change. We consider that IL12-4-SL is a promising tomato line for

cultivation and breeding, considering the major challenges that Mediterranean agriculture will face in response to climate change and maintenance of sustainability.

## ACKNOWLEDGEMENTS

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## AUTHOR CONTRIBUTIONS

S.F. conceived the original screening and research plans, designed the experiments and analysed the data, reviewing and editing; L.V. undertook formal analysis, investigation, writing original draft, reviewing and editing; C.A., N.D., L.P., E.V. and V.C. reviewed the manuscript and editing; M.C.d.P. and A.B. undertook formal analysis, investigation, reviewing and editing; M.M.R. was responsible for conceptualization, formal analysis, investigation, methodology, funding acquisition, supervision, writing original draft, reviewing and editing.

## DATA AVAILABILITY STATEMENT

All data generated or analysed during this study have been included in this published article [and its supplementary information files].

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** ANOVA showing the level of significance of the genotype (G), stress (S) and their interactions on different traits.

**Table S2.** Leaf traits (LA, leaf area; RWC, relative water content; LDMC, leaf dry matter content; SLA, specific leaf area; LT, leaf thickness) in two tomato lines grown under control (CTRL) heat (H), drought (D) and combined stress (COMB) conditions. The data are mean  $\pm$  SE (n = 6). Within each tomato line, different letters correspond to significantly different values with Duncan's *post-hoc* test among treatments ( $P \leq 0.05$ ). Asterisks indicate statistically significant differences of IL12-4-SL from M82 in each treatment (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; Student's *t* test).

**Table S3.** Carotenoids (Car), chlorophylls (Chl *a* and *b*), Chl *a/b* ratio and Chl/Car ratio in the two tomato lines grown under control (CTRL), heat (H), drought (D) and combined stress (COMB) conditions. The data are mean  $\pm$  SE (n = 6). Within each tomato line, different letters correspond to significantly different values with Duncan's *post-hoc* test among treatments ( $P \leq 0.05$ ). Asterisks indicate statistically significant differences of IL12-4-SL versus M82 in each treatment (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; Student's *t* test).

**Table S4.** Eigenvalues, relative and cumulative percentage of total variance and correlation coefficients for each character.

**Figure S1.** The effect of heat stress, drought and combined stress (heat stress + drought) on M82 (A) and IL12-4-SL (B).

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