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Rootstock-mediated physiological and fruit set responses in pepper under heat stress

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ABSTRACT

An increase in high temperature causes major losses in pepper yields, especially in greenhouses when extending the cropping season to late spring or summer in mild climate areas. Grafting has been identified as a possible tool to cope with this abiotic stress. The objective of this study was to analyze the heat stress impact on a sweet pepper variety grafted onto rootstocks with diverse heat stress tolerances to evaluate high-temperature effects on the leaf metabolism, pollen traits and fruit set. To do so, under two greenhouses conditions (28/22°C and 38/22°C for control and heat stress, respectively), we compared the variety grafted onto two rootstocks (VA/A57 and VA/A55, tolerant and nontolerant, respectively), and used varieties ungrafted (VA) and self-grafted (VA/VA) as controls. VA/A57 obtained the lowest electrolyte leakage, non-disturbed chlorophyll and carotenoids concentration values, increased ascorbic acid and phenols concentrations, and no hydrogen peroxide accumulation. These findings indicate better predisposition to overcome heat stress than other plant combinations. Such physiological responses in leaves conferred by the tolerant rootstock coincided with the highest proline concentration in anthers, and better pollen germination and fruit set compared to the other graft combinations. We conclude that grafting peppers onto a heat stress-tolerant rootstock, such as A57, could overcome negative high-temperature effects better than an ungrafted variety. Moreover, the better physiological performance noted in vegetative parts conferred by a heat stress-tolerant rootstock would also lead to better performance in the reproductive development phase. All this indicates that using tolerant rootstocks in pepper could be an interesting method to alleviate heat stress effects on this crop.

1. Introduction

High temperatures that affect crops are relatively frequent in protected cultivation, particularly in warm regions, when extending the cropping season to late spring or summer. This situation could be aggravated in the near future as a consequence of climate change because the average temperature is expected to rise in different regions, especially on the Mediterranean coast (IPPC, 2018). This area produces vast quantities of vegetables, particularly sweet pepper (MAPA, 2021), an economically important crop in protected cultivation that is sensitive to heat stress (HS). In such a scenario, it has been observed that temperatures above 32°C can cause serious problems for sweet pepper plants because they affect plant metabolism, growth, pollination and fertilization (Erickson and Markhart, 2002). The translocation of photosynthesis assimilates to developing flowers depends on the leaf metabolism, which is also affected by HS. High-temperature stress affects crop yield as a consequence of a series of complex morphological, physiological, biochemical and molecular changes (Wang et al., 2003), which range from diminished photosynthesis activity, a higher respiration rate, oxidative damage, effects on ion uptake-movement, membrane fluidity and ion leakage, to stunted growth and cell death (Wahid et al., 2007). All these effects end up causing significant crop productivity losses in many species (Lobell and Gourdji, 2012; Zhao et al., 2017).

These limiting temperatures are normally exceeded in the situations described above. They coincide with the flowering phase and, thus, affect fruit set (Yamazaki and Hosokawa, 2019) and fruit drop and, consequently, fruit yield and quality (López-Marín et al., 2013).

Reduced fruit set is caused partly by damage to gametophytes, which results for the male gametophytes in poor pollen viability, decreased pollen germination, limited pollen tube growth and reduced stigma receptivity, and for the female gametophytes in reduced fertilization and

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ovule viability, limited embryogenesis and increased ovule abortion (Nadeem et al., 2018).

Pollen plays a crucial function in the fruit set and is considered more HS-sensitive than both vegetative tissues and the female gametophyte in *Solanaceae* species (Reddy and Kakani, 2007). This sensitivity to HS varies during pollen development, and is more sensitive in early development stages (anther wall development, microsporogenesis and microgametogenesis) (Raja et al., 2019). Pollen viability as a key trait for detecting HS-tolerance has been studied in many crops, such as cotton (Kakani et al., 2005), wheat and rice (Mesihovic et al., 2016) and tomato (Ayenan et al., 2019), and also in *Capsicum* species (Reddy and Kakani, 2007; Yamazaki and Hosokawa, 2019). Erickson and Markhart (2002) concluded that HS affects the pollen grains development in bell peppers during the final tetrad formation period to tetrad dissolution, and results in poorer pollen viability. This reduction effectively reduces fruit set and fruit size.

Metabolic studies into anthers and pollen have shown that a higher level of specific metabolites, such as flavonols, proline and polyamines, are associated to increased pollen viability (Falasca et al., 2010; Mattioli et al., 2012; Shan et al., 2020) due to their antioxidant capacity which, thus, protects them from the ROS generation caused by high temperatures (Xie et al., 2022). In particular, proline has been directly correlated to HS-tolerance, and is also the most abundant amino acid in anthers and pollen (Santiago and Sharkey, 2019). Accumulated proline is considered a key factor for pollen viability because it is used as an energy source to enhance pollen tube growth after germination (Biancucci et al., 2015). In general, proline levels in pollen decline with exposure to high-temperature stress, which is the case of tomato (Din et al., 2015) and rice (Tang et al., 2008). In hot pepper, Fang et al. (2016) proved that proline content was higher in fertile anthers compared to sterile ones. However as far as we know, there are no references to the behavior of proline pollen levels under HS in sweet pepper.

In the same way, carbohydrates are necessary for pollen development and germination, like osmoprotection and source of energy. The main soluble sugars in mature pollen are fructose, glucose, sucrose and starch (Paupière et al., 2014), and simple sugars are used for pollen grain development (Pacini, 1996). In pepper, pollen sugar metabolism is affected by suprathermal stress, mainly because diminished invertase activity increases sucrose and starch concentrations (Aloni et al., 2001). In tomato plants under HS, Firon et al. (2006) report that the ability of mature pollen grains of heat-tolerant cultivars to accumulate sugars was greater than in non tolerant cultivars, which affects the number of fruit per plant and seeds per fruit.

Other biocompounds, such as hormonal network, polyamines, lipids or other metabolic compounds, play a role in different aspects toward successful reproductive processes and can be modified under HS with negative responses for fruit set (Madhavi Reddy et al., 2016; Paupière et al., 2014).

Consequently, plant survival under HS depends on the plant's ability to perceive the temperature stimulus and to generate the appropriate response to cope with this stress by means of appropriate morphological, biochemical and signaling changes (Madhavi Reddy et al., 2016). Grafting is a special method of adapting plants to tolerate environmental stress (Schwarz et al., 2010). The grafting technique has been successfully used so that horticultural crops can cope with different abiotic stresses (Schwarz et al., 2010). However, there are very few studies on the use of rootstocks to cope with high temperatures in pepper (Aidoo et al., 2017; López-Marín et al., 2013; Palada and Wu, 2008), and none of these have studied the effects of using rootstocks on leaf physiology and fruit set under high-temperature conditions in detail.

Some pepper accessions have been recently identified as heat-tolerant in a study carried out by our work team (Gisbert-Mullor et al., 2021). Under climate chamber conditions, we observed how some accessions were able to confer a certain degree of tolerance to HS when using them as rootstocks and maintaining membrane permeability, which ultimately maintained the chlorophyll fluorescence parameter Fv/Fm ratio and resulted in a higher relative growth rate. We also found higher fruit yields in these grafted plants compared to the ungrafted cultivar under greenhouse conditions, which was related to the number of harvested fruits. However, we did not clearly identify the causes of fruit set degree, particularly if rootstocks were able to affect some aspects related to this important yield component (Gisbert-Mullor et al., 2021).

Consequently, the objective of this work was to study the effect of high temperature on fruit set in pepper and how it can be improved by using tolerant rootstocks. For this proposal, we analyzed the HS impact on a sweet pepper variety grafted onto rootstocks with diverse HS tolerances to evaluate whether: a) the leaf metabolism in different grafted plant combinations has a differential response to heat stress; b) these responses can be involved in fruit set.

2. Materials and methods

2.1. Plant material and growth conditions

The pepper variety 'Herminio F1' (*Capsicum annuum* L., Lamuyo type (Syngenta), B2 type of Pochard (1996) classification), was grafted onto two *Capsicum annuum* L. accessions selected from previous studies (Gisbert-Mullor et al., 2021): A57 (origin Israel, considered to be HS-tolerant) and A55 (origin Algeria, considered to be HS-sensitive), respectively named VA/A57 and VA/A55. Both accessions belong to the COMAV Institute collection (Universitat Politècnica de València, UPV, Valencia, Spain). The ungrafted variety (VA) and the self-grafted variety (VA/VA) were used as controls. The four plant groups were evaluated under both HS and control (C) conditions.

Experiments were conducted in two consecutive years (2020 and 2021) at the UPV Valencia, Spain.

Sowing took place on February 1, 2020, and March 3, 2021, in 104-cell polystyrene trays in fine structure peat substrate (80% white and 20% black, pH 5.7) (Gebr. Brill, Germany). On March 16, 2020, and April 12, 2021, plants were grafted by the tube-grafting method (Penella et al., 2014).

Seedlings were transplanted on April 1, 2020, and April 29, 2021, in 6-liter pots in medium structure peat substrate (100% white, pH 5.7) (Gebr. Brill, Germany), and placed in two Venlo-type glasshouses (C and HS conditions) at a density of two plants m^{-2}. Pots were drip-irrigated using antidrain Netafim® drippers of 4 L h^{-1}, with nutrient solution containing (in mmol L^{-1}) 14.0 NO_3^-; 1.5 $H_2PO_4^-$; 2.4 SO_4^{2-}; 0.5 HCO_3^-; 1.6 Cl^-; 1.2 NH_4^+; 6.0 K^+; 5.0 Ca^{2+}; 2.5 Mg^{2+}; 0.2 Na^+, and in μmol L^{-1}: 15 Fe^{3+}; 6 Zn^{2+}; 12 Mn^{2+}; 30 B^{3+}; 0.8 Cu^{2+} and 0.5 Mo^{6+} with electrical conductivity and pH of 2.2 dS m^{-1} and 6.5, respectively. The number of irrigations, which varied according to the accumulated radiation, controlled the volume of solution applied per pot. Drainage was maintained between 15% and 20% of the total irrigation volume.

The temperature treatments were 28/22°C and 38/22°C (day/night) for the control (C) and HS conditions, respectively. The HS conditions began on June 19, 2020, and May 26, 2021, and continued until July 15, 2020, and July 21, 2021. Before this, all the plants were subjected to the C conditions. The temperature and relative humidity for both greenhouses were recorded during the experiments using an S8TH sensor (Oratge Instruments®, Valencia, Spain) with data logger MSIP801 (BSG Ingenieros, Valencia, Spain) (Fig. 1S).

Experiments were laid out to be completely randomized with four repetitions per group of plants and per treatment. Each repetition consisted of 10 plants.

2.2. Experiment 1 (2020): rootstocks' influence on pepper physiological aspects under heat stress

For the physiological leaf analysis, all the measurements were taken on July 15, 2020, after 35 DAT (days after the heat-stress treatment). Electrolyte leakage (EL), chlorophyll and carotenoids concentration,

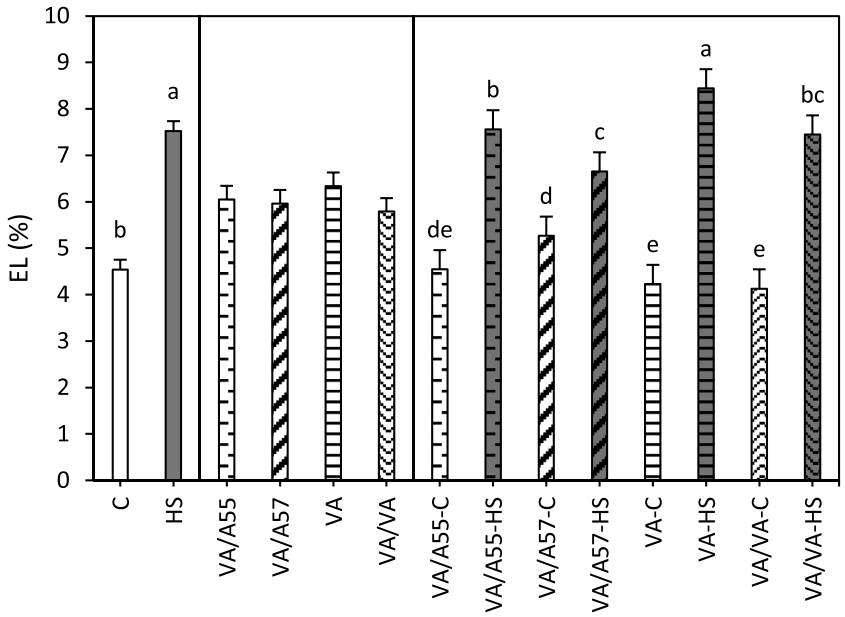

Fig. 1. Electrolyte leakage (EL) in leaves of the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively) under heat stress (HS) or control conditions (C). Bars with different letters are statistically different according with LSD test ($P \leq 0.05$). Error bars represent LSD.

ascorbate metabolism, total phenolic content and hydrogen peroxide quantification were determined. The analysis was carried out with 10 leaves per replicate (one leaf per plant; the third or fourth leaf from the apex), which were previously frozen with liquid nitrogen and stored at −80°C. Samples were ground by a mixer mill (MM400, Retsch, Hann, Germany) with liquid nitrogen to prevent melting. Fresh leaves were used for EL.

Fruit set was analyzed by tagging at least 20 flowers per plant. The fruit weighing around 25 g were considered set. The flower-tagging process began on 10 DAT to ensure that the tagged flowers developed under stress conditions. Fruit set was calculated as the number of set fruit, divided by the total number of sampled flowers, expressed as a percentage.

2.2.1. Electrolyte leakage

To analyze EL, 10 discs (1.4 cm in diameter) of freshly cut leaves were extracted from the 10 plants of each replicate with a perforator and were introduced in 50 mL matrass together with 20 mL of deionized water. Electrical conductivity measurements were taken at 0 h (EC1), at 2 h (EC2) and after leaving the matrass frozen (-40°C) for 48 h (EC3) with a conductivity meter (Model Seven Easy Mettler Toledo, Mettler-Toledo AG, Switzerland). E.L. was expressed as a percentage.

$$EL(\%) = ((EC2 - EC1) / (EC3 - EC1)) * 100$$

2.2.2. Chlorophyll and carotenoids concentration

Total chlorophyll (Chl a+b) and carotenoid (Car) contents were measured by spectrophotometry as indicated by Porra et al. (1989) with minor changes. First, 0.1 g fresh weigh (FW) of each sample was extracted in 2.5 mL of 100% (v/v) pure acetone and then centrifuged at 2000 rpm for 5 min. The supernatant was separated from the precipitate and its absorption was measured using a spectrophotometer (Lambda 25 UV/VIS, Perkin Elmer, Waltham, MA, USA) at 662nm, 645nm and 470nm, using 100% acetone for the blank. Concentration for Chl a+b and Car were expressed in μg g^{-1} FW and were calculated according to these equations:

$$\text{Chl a} + \text{b} = 7.05 * \text{Abs}_{662} + 18.09 * \text{Abs}_{645} (\mu\text{g mL}^{-1})$$
$$\text{Car} = [(1000 \times \text{Abs}_{470}) - (1.90 \times \text{Chl a}) - (63.14 \times \text{Chl b})]/214 (\mu\text{g mL}^{-1})$$

2.2.3. Ascorbate metabolism

Ascorbic acid (AsA), dehydroascorbate (DHA) and total ascorbic acid (AsAt, AsA+DHA) were quantified in parallel as described by Kampfenkel et al. (1995) with slight modifications. First, 0.4 g FW sample were extracted in 80% (w/v) trichloroacetic acid (TCA) centrifuged for 5 min at 15000 *g* and 4°C. Then two different determinations were followed (one for AsA and the other for AsAt): 50 μL of the extract supernatant were added to 150 μL of 0.2M phosphate buffer (pH = 7.4) and 50 μL of distilled water in the ascorbate tubes. In the case of total ascorbic acid tubes, the 50 μL of the extract supernatant were put together with 50 μL of 10mM dithiothreitol (DTT) and 100 μL of 0.2M phosphate buffer (pH = 7.4) and then introduced in a water bath at 42°C for 15 min. After the water bath, 50 μL of 0.5% (w/v) N-ethylmaleimide (NEM) were added only in the total ascorbic acid tubes which were then incubated for 1 min at room temperature. Afterwards, 250 μL of 10% (w/v) TCA, 200 μL of 42% (v/v) H_3PO_4, 200 μL of 4% (w/v) 2, 2'-dipyridyl and 100 μL of 3% (w/v) $FeCl_3$ were added to AsA and total ascorbic acid tubes to be incubated in a water bath for 40 min at 42°C. Absorbance at 525 nm was measured for both determinations. The DHA concentration was calculated as: [AsAt] – [AsA]. AsA and DHA concentration were expressed in μg g^{-1} FW.

2.2.4. Total phenolic content

Total phenolic content determination was performed as described by Koç et al. (2010) with some variations. First of all, 0.1 g FW of the sample was extracted in 1.5 mL of 80% (v/v) methanol and placed in an ultrasound bath for 30 minutes at 25°C. Extracts were centrifuged for 10 min at 10000 g and 4°C, and 20 μL of the resulting supernatant were diluted with 80 μL of 80% (v/v) methanol. Afterward, the diluted extract was incubated with 0.7 mL of 1:10 diluted Folin-Ciocalteu solution (Sigma-Aldrich®) for 5 minutes in the dark. Then 0.7 mL of 6% (w/v) of Na_2CO_3 were added and the solution was incubated for 1 h in the dark at room temperature. Absorbance at 765 nm was recorded, using gallic acid (GA) as a standard. Total phenolic content was expressed as mg GA g^{-1} FW.

2.2.5. Hydrogen peroxide quantification

Hydrogen peroxide (H_2O_2) was quantified as described in Velikova et al. (2000) with minor adaptations. 0.25 g FW of the sample was extracted in 2 mL of 0.1 % (w/v) TCA to be centrifuged at 10000 *g* and

4°C for 8 min. Afterward, 400 μL of the resulting extract were incubated with 600 μL of 0.1 % (w/v) TCA, 0.5 mL of 100 mM potassium phosphate buffer (pH = 7) and 2 mL of 1M KI in the dark for 1 h at room temperature. Absorbance at 390 nm was measured, and the absorbance values were interpolated on a standard curve performed with 100 mM H_2O_2. Values were expressed as nmol H_2O_2 g^{-1} FW.

2.3. Experiment 2 (2021): rootstocks' influence on pepper fruit set components under heat stress

Based on experience from previous experiments and to avoid fruit competition within the plant to fruit set, plants were pruned to four stems by cutting back the second branch at each node after evidencing fruit set of the flower at the node. In this way, it was also easier to access flowers to collect pollen or anther samples for subsequent analyses.

To analyze fruit set, at least six reproductive nodes of each stem were analyzed, which totaled 24 flowers per plant. The flower-tagging process began on 10 DAT and the fruit weighing around 25 g were considered set. The average number of seeds per fruit was analyzed in one fruit per node, which resulted in six fruit per plant.

2.3.1. In vitro germination of the pollen grain percentage (%)

To determine the *in vitro* germination of the pollen grain percentage (% GP), the pollen of each flower on the day of anthesis was evenly distributed in the germination medium proposed by Reddy and Kakani (2007).

The pollen grain was incubated for 24 h at 25°C, % GP was determined in 10 fields of each Petri dish using a Leica DM750 microscope (Leica Microsystems, Wetzlar, Germany) at 10 x magnification. Pollen grain was considered germinated when pollen tube length exceeded the diameter of grain.

To calculate % GP, a minimum of 20 flowers were collected from each repetition and treatment. % GP was calculated as the number of germinated pollen grains divided by the total number of sampled pollen grains, and expressed as a percentage.

2.3.2. Primary metabolites analysis from anthers

One day before anthesis, a minimum of 10 flowers of each repetition and treatment were harvested. Anthers were immediately separated, frozen with liquid nitrogen and stored at −80°C. Samples were lyophilized before the analysis.

A primary metabolite analysis (proline, glycine, fructose, glucose, sucrose) was analyzed at the Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC, Valencia, Spain) by the Metabolomics Platform. First 10 mg of lyophilized anther tissue were ground with liquid nitrogen and extracted in 1400 μL 100% methanol and 60 μL of the internal standard (0.2 mg ribitol in 1 mL of water). The blend was extracted for 15 min at 70°C. The extract was centrifuged for 10 minutes at 14000 rpms, and the supernatant was transferred to a glass vial. Then 750 μl of $CHCl_3$ and 1500 μL of water were added. The mixture was vortexed for 15 seconds and centrifuged for 15 minutes at 14000 rpms. Next 150 μL aliquots of the methanol/ water supernatant were speed-dried for 3 h.

For derivatization purposes, dry residues were redissolved in 40 μL of 20 mg/mL methoxyamine hydrochloride in pyridine and incubated for 90 min at 37°C. Then 70 μL of MSTFA (N-methyl-N-[trimethylsilyl]trifluoroacetamide) and 6 μL of a mixture (3.7% [w/v] mix of fatty acid methyl esters ranging from 8 to 24 C) were added and samples were incubated for 30 minutes at 37°C.

Sample volumes of 2 μL were introduced in the split and splitless modes in a 6890 N gas chromatograph (Agilent Technologies Inc. Santa Clara, CA, USA) coupled to a Pegasus 4D TOF mass spectrometer (LECO, St. Joseph, MI, USA). Gas chromatography was carried out in a BPX35 (30 m × 0.32 mm × 0.25 μm) column (SGE Analytical Science Pty Ltd., Australia) with helium as the carrier gas at a constant flow rate of 2 mL/min. The liner was set at 250°C. The oven program was 85°C for 2 min with an 8°C/min ramp until 360°C. Mass spectra were collected at 6.25 spectra s^{-1} within the 35–900 *m/z* range with 70 eV ionization energy. Chromatograms and mass spectra were analyzed using the CHROMA-TOF program (LECO, St. Joseph, MI, USA). For absolute quantification purposes, the results were compared to a standard curve performed with commercial pure compounds using ribitol as an internal standard. All the metabolites were expressed as μg g^{-1} FW.

2.3.3. Biomass production

The aerial plant biomass in fresh (FW) and dry weight (DW) was measured at the end of the experiment (58 DAT). Fresh biomass was dried at 70°C during 72 h in a laboratory oven to determine DW. To correctly analyze the influence of the thermal condition on the dry biomass of each graft combinations, this parameter was analyzed as the percentage DW loss under the HS conditions compared to the C conditions. Aerial biomass was determined in three plants per repetition and treatment.

2.4. Statistical Analysis

The data for all the parameters were evaluated using two-way ANOVA analysis via Statgraphics Centurion XVII. Graft combinations (G) and thermal conditions (TC) were employed as the factors of the analysis. For the parameter number of seeds per fruit, the statistical analysis was performed using the inverse transformation of data, and the percentage data were arcsin-transformed before analyzing. Means (n=4) were compared by Fisher's least significance difference (LSD test) at $P \leq 0.05$.

3. Results

3.1. Experiment 1: rootstocks' influence on pepper physiological aspects under heat stress

3.1.1. Electrolyte leakage

As compared to control, HS caused a significant increase (65.7%) of EL in all the graft combinations ($P \leq 0.01$; Fig. 1). No significant differences in EL were observed between graft combinations on average for temperature conditions. However, a statistically significant ($P \leq 0.01$; Fig. 1) TC x G interaction was detected: the differences in EL between the HS and C conditions were lower in the plants grafted onto the A57 rootstock (26.4%) than in the other plant combinations (66.4% for VA/A55, 80.4% for VA/VA and 99.6% for VA).

3.1.2. Chlorophyll and carotenoids concentration

The total chlorophyll (Chl a+b) and carotenoids (Car) contents presented a similar pattern, and were not affected by TC (Fig. 2 A,B). However for both thermal conditions, rootstocks A57 and A55 had a higher content of both parameters than VA and VA/VA ($P \leq 0.01$; Fig. 2 A,B). For both parameters, the TC x G interactions were also statistically significant ($P \leq 0.01$; Fig. 2 A,B). Under HS, for the variety grafted onto rootstock A57 and the ungrafted variety, chlorophyll and carotenoids contents significantly increased compared to their controls, but significantly lowered In VA/A55. No significant differences were observed in VA/VA.

3.1.3. Ascorbate and dehydroascorbate concentration

AsA and DHA contents were significantly higher under HS, on average for graft combinations, with a 5.4% and 182.3% increase, respectively, compared to the C conditions ($P \leq 0.05$; Fig. 3 A). On average for both thermal conditions, VA showed the highest AsA and DHA contents, but no significant differences with those of VA/A55 and VA/VA were found, respectively ($P \leq 0.05$; Fig. 3 A). Nevertheless, a statistically significant TC x G interaction ($P \leq 0.05$; Fig. 3 A) appeared for AsA and DHA contents. For AsA content, VA/A57 and VA had higher values under HS than under C, VA/A55 showed no differences, and VA/VA had higher AsA content under the C than the HS conditions. VA had

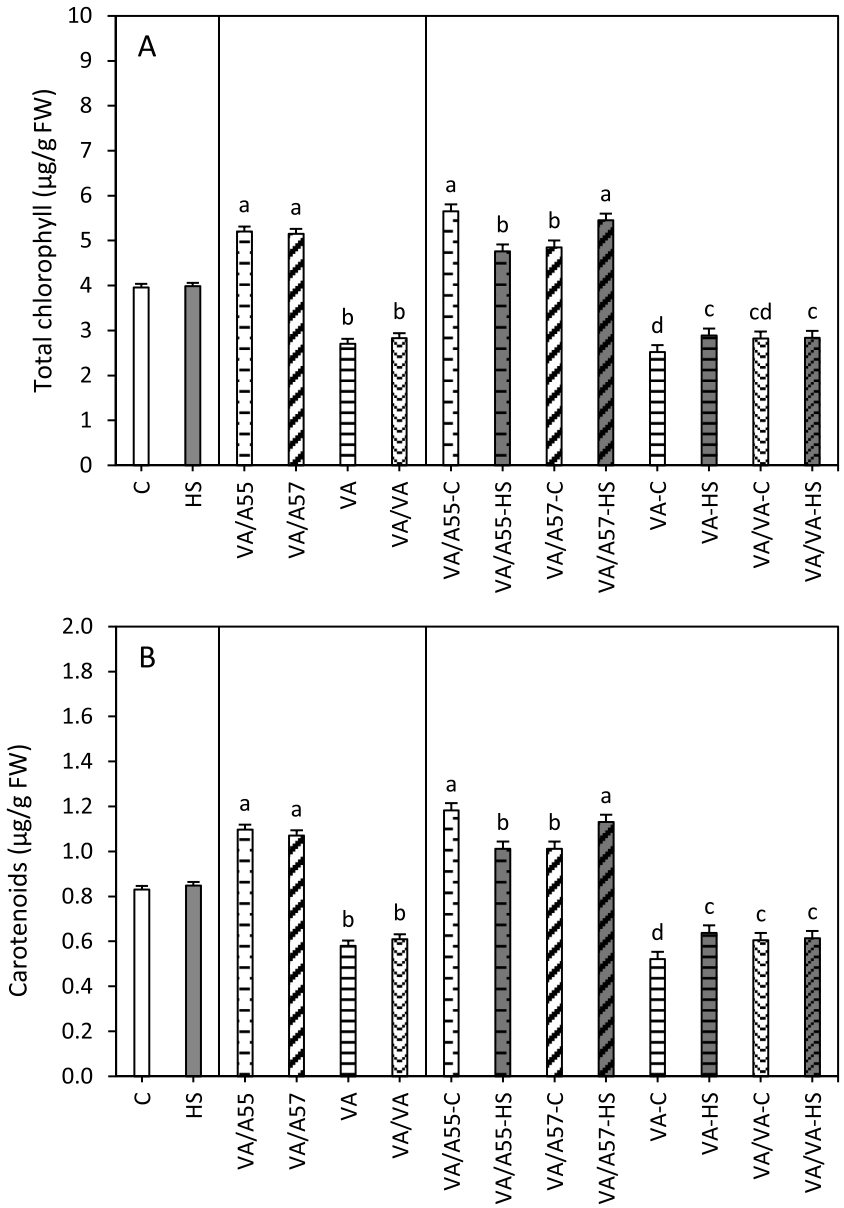

Fig. 2. Total chlorophyll (A) and carotenoids (B) in leaves of the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively) under heat stress (HS) or control conditions (C). Bars with different letters are statistically different according with LSD test ($P \leq 0.05$). Error bars represent LSD.

the highest DHA content under HS among all the graft combinations. All the graft combinations had higher DHA content under the HS than the C conditions, except VA/VA.

3.1.4. Total phenolic content

Total phenolic content was also higher under HS, on average for graft combinations, with an 18.1% increase compared to the plants under the C conditions ($P \leq 0.01$; Fig. 3 B), and VA had the highest content on average for both thermal conditions ($P \leq 0.01$; Fig. 3 B). However, the TC x G interaction was statistically significant ($P \leq 0.01$; Fig. 3 B) because, although all the graft combinations had a higher total phenols content under HS, the differences in the total phenols between the HS and C conditions were bigger in the plants grafted onto A57 (36.4%) than for the other plant combinations (9.0% for VA/A55, 17.4% for VA/VA and 12.4% for VA).

3.1.5. Hydrogen peroxide quantification

Hydrogen peroxide content (H_2O_2) globally increased to 52.9% in the heat-stressed plants compared to the control ones, and VA and VA/VA had a higher H_2O_2 content on average for the thermal conditions than VA/A57 and VA/A55 ($P \leq 0.01$; Fig. 4). The TC x G interaction was also statistically significant ($P \leq 0.01$; Fig. 4). While VA/A55, VA and VA/VA had significantly increased H_2O_2 content under HS compared to C, this parameter was not significantly modified in the VA/A57 plants under HS.

3.1.6. Fruit set percentage

Fruit set (%) was strongly affected by HS, with a reduction of 71.8% compared to the C condition on average for graft combinations, and VA/A57 was the rootstock that conferred the highest fruit set for both the HS and C conditions. Nevertheless, a statistically significant TC x G interaction ($P \leq 0.01$; Fig. 5) was detected, in which the reduction in fruit set between the HS and C conditions was less in the plants grafted onto the rootstock A57 (57.7% reduction) than for the ungrafted cultivar (VA) (67.9% reduction), and was even lower compared to VA/A55 and VA/VA (84.2% and 80.2% reduction, respectively).

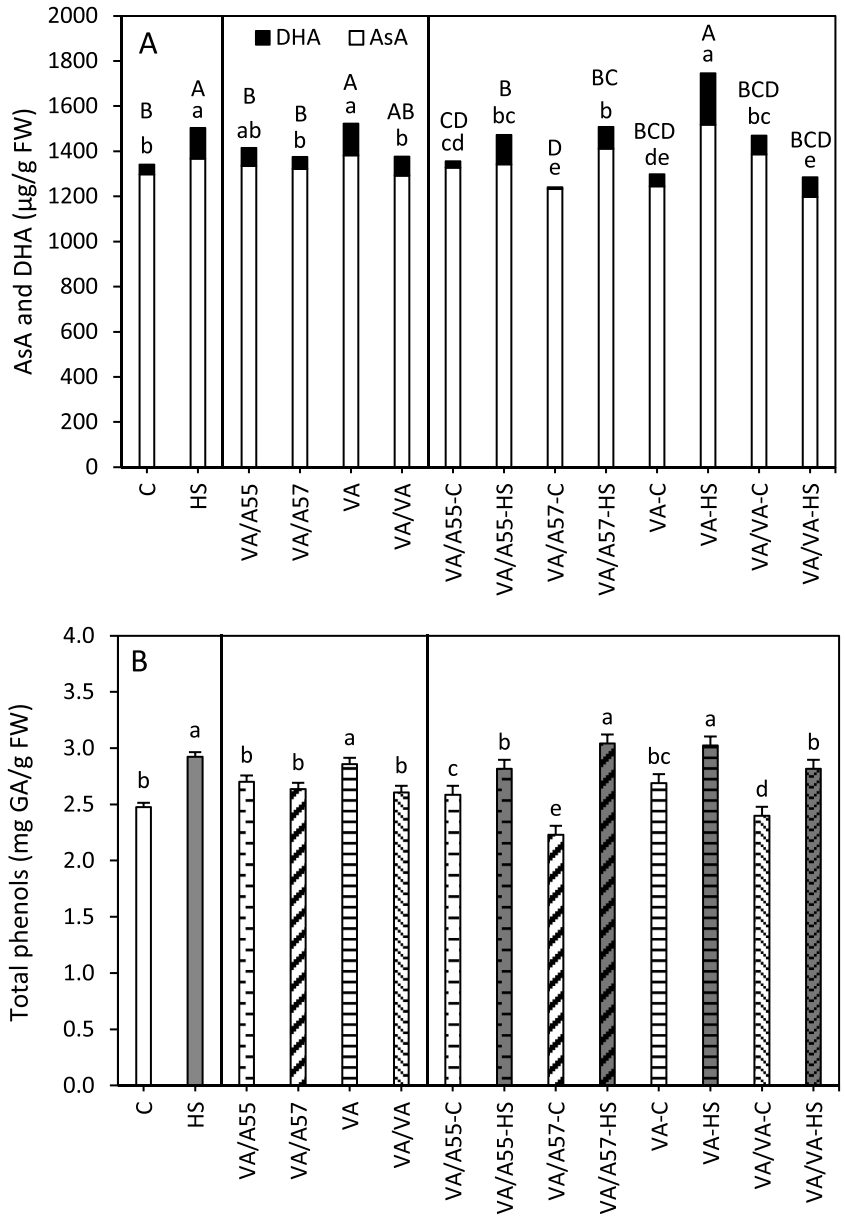

Fig. 3. Concentration of the different forms of ascorbate (AsA and DHA) (A) and total phenols (B) in leaves of the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively) under heat stress (HS) or control conditions (C). Bars with different letters are statistically different according with LSD test ($P \leq 0.05$), for the parameter AsA lowercase letters and for DHA capital letters. Error bars represent LSD.

3.2. Experiment 2: rootstocks' influence on pepper fruit set components under heat stress

3.2.1. In vitro germination of pollen grain percentage

GP (%) was significantly lower under the HS conditions, on average for graft combinations, with a 30.8% reduction compared to the C conditions ($P \leq 0.01$; Fig. 6). For both thermal conditions, VA/A57 had the highest percentage, similarly to VA/VA, and VA had the lowest germination percentage ($P \leq 0.01$; Fig. 6). However, a statistically significant TC x G interaction ($P \leq 0.01$; Fig. 6) was detected, since the reduction in % GP between the HS and C conditions was lower in the plants grafted onto rootstocks A57 and A55 (24.7% and 23.4% reduction, respectively) than in VA and VA/VA (36.9% and 37.5% reduction, respectively).

3.2.2. Primary metabolites analysis from anthers

For the primary metabolites in anthers, the plants under the HS conditions presented reductions of 30.8% in proline, 8.2% in fructose, 10.7% in glucose and 46.5% in sucrose compared to the plants under the C conditions. No significant differences between temperature conditions were detected for glycine content (Table 1). Only proline showed significant differences between graft combinations on average for thermal conditions, and rootstock A57 had the highest content ($P \leq 0.01$; Table 1). The TC x G interaction for this parameter was also statistically significant ($P \leq 0.01$; Table 1). Similarly to % GP, all the graft combinations had higher proline content under C than HS but, in this case, although VA/A57 had the highest content under the HS conditions, the reduction in proline content between HS and C was also lower in the plants grafted onto A57 (31.5% reduction) than in VA, VA/VA and VA/A55 (34.5%, 54.7% and 42.2% reduction, respectively).

3.2.3. Fruit set percentage

Similar to experiment 1, the fruit set percentage was also affected by HS ($P \leq 0.01$), with a 25.2% reduction compared to the plants under the

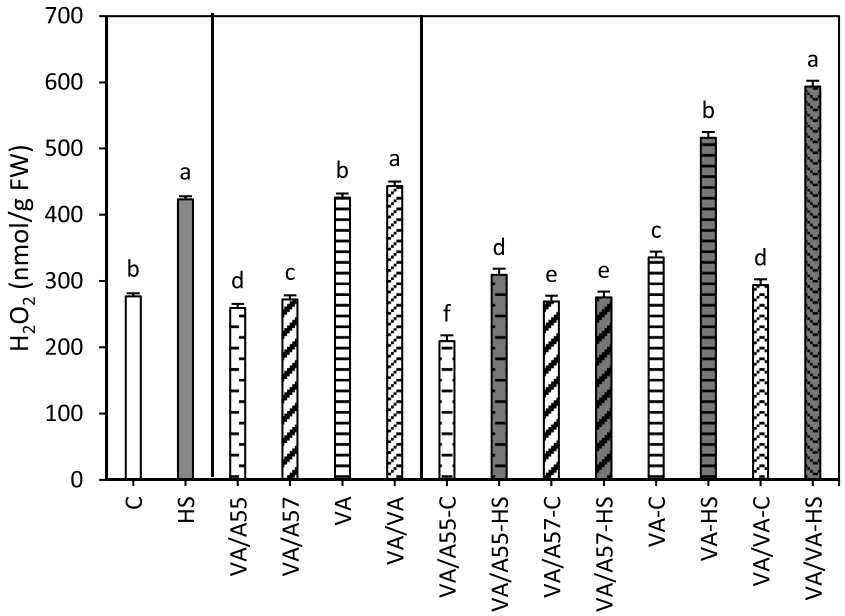

Fig. 4. Hydrogen peroxide contend (H_2O_2) in leaves of the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively) under heat stress (HS) or control conditions (C). Bars with different letters are statistically different according with LSD test ($P \leq 0.05$). Error bars represent LSD.

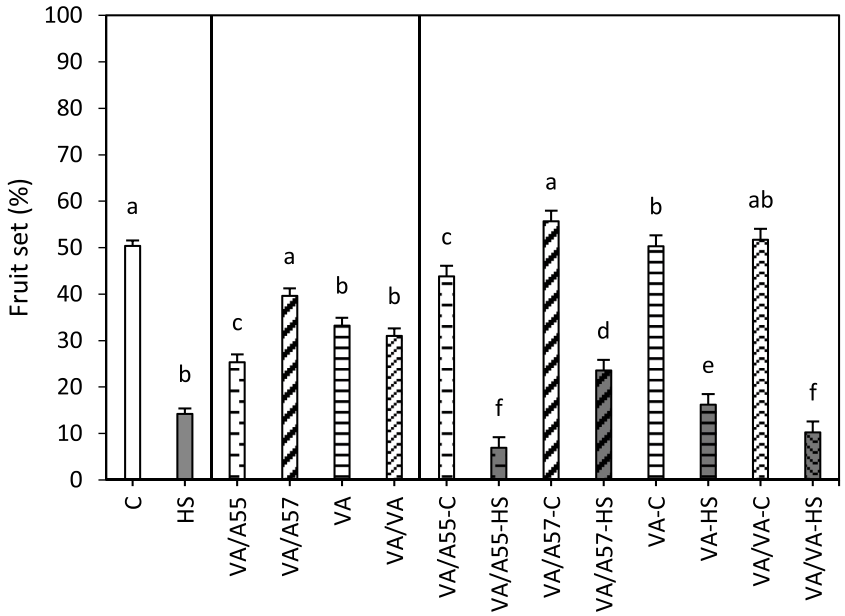

Fig. 5. Fruit set percentage for Experiment 1 in the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively) under heat stress (HS) or control conditions (C). Bars with different letters are statistically different according with LSD test ($P \leq 0.05$). Error bars represent LSD.

C conditions (Fig. 7 A), and for both thermal conditions. VA/A55 had the lowest fruit set percentage among the plant combinations ($P \leq 0.01$; Fig. 7 A). The TC x G interaction was also statistically significant ($P \leq 0.01$; Fig. 7 A) but, in this experiment, the plants grafted onto rootstock A57 were not affected by the HS conditions, while reductions of 40.5%, 29.5% and 23.5% in fruit set were obtained for the plants of VA/A55, VA and VA/VA, respectively.

3.2.4. *Number of seeds per fruit*

The fruit developed under the HS conditions, on average for graft combinations, had a smaller (59.5%) number of seeds compared to the fruit under the C conditions ($P \leq 0.01$; Fig. 7 B). For both thermal conditions, VA/A57 and VA/VA had the larger number of seeds ($P \leq 0.01$; Fig. 7 B). The interaction between both factors was also statistically significant and can be explained because, despite the fact that all the graft combinations showed similar values in this parameter under the C conditions, under HS both VA/A57 and VA/VA obtained higher values than VA and VA/A55, and the latter gave the smallest number of seeds ($P \leq 0.01$; Fig. 7 B).

3.2.5. *Biomass production*

For the DW percentage under HS *versus* C, the plants grafted onto rootstock A57 displayed less loss for the DW percentage than other graft combinations ($P \leq 0.01$; Fig. 8). No significant differences between thermal conditions were found in the other graft combinations.

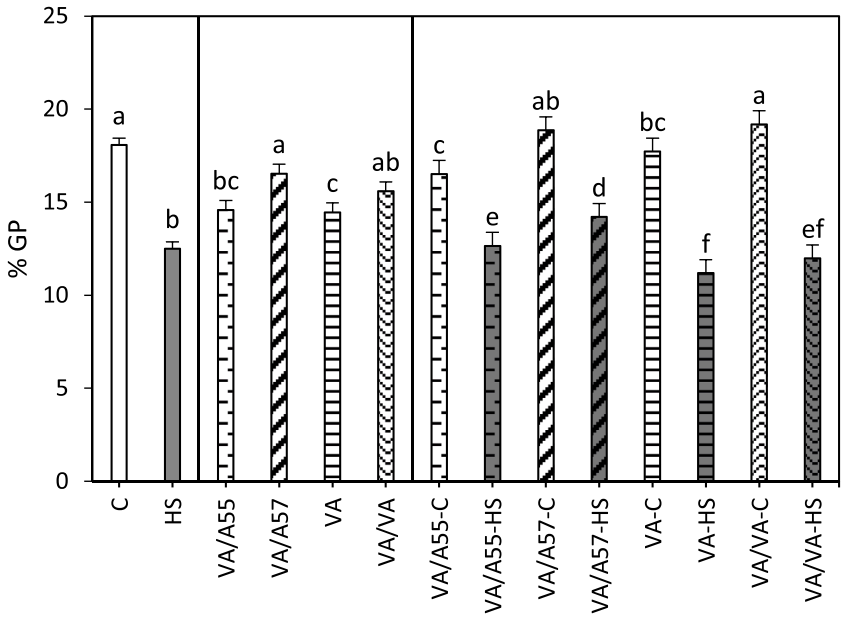

Fig. 6. In vitro germination of pollen grains (% GP) in the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively) under heat stress (HS) or control conditions (C). Bars with different letters are statistically different according with LSD test ($P \leq 0.05$). Error bars represent LSD.

Table 1
Analysis of variance (ANOVA) for Proline, Glycine, Fructose, Glucose and Sucrose (μg g^{-1}FW) expressed as mean values by Thermal Condition (TC), Graft combination (G) and interaction (TC x G). % of the sum of squares are expressed.

	Proline		Glycine		Fructose		Glucose		Sucrose	
Thermal condition (TC)										
Control	73.51	a	0.576		110.3	a	73.31	a	45.35	a
Heat stress	43.57	b	0.576		101.3	b	65.47	b	24.26	b
Graft combination (G)										
A55	58.69	b	0.567		107.5		71.51		32.50	
A57	64.67	a	0.586		106.2		69.60		39.86	
VA	57.14	bc	0.562		108.1		72.21		31.92	
VA/VA	53.66	c	0.590		101.4		64.23		34.96	
TCxG										
A55-C	74.37	ab	0.579		110.9		76.90		41.51	
A57-C	76.77	a	0.591		111.2		70.91		51.63	
VA-C	69.03	b	0.557		110.4		75.85		41.21	
VA/VA-C	73.87	ab	0.578		108.8		69.58		47.06	
A55-HS	43.02	d	0.555		104.2		66.11		23.48	
A57-HS	52.58	c	0.581		101.2		68.29		28.09	
VA-HS	45.25	d	0.568		105.9		68.58		22.63	
VA/VA-HS	33.44	e	0.601		94.1		58.89		22.86	
ANOVA (*df*)	% Sum of Squares									
TC (1)	83.27	**	0.00	n.s.	19.02	*	22.53	**	69.77	**
G (3)	5.89	**	20.66	n.s.	6.52	n.s.	14.33	n.s.	6.15	n.s.
TC*G (3)	4,25	**	12.93	n.s.	3.45	n.s.	4.06	n.s.	1.23	n.s.
Residuals (24)	6,59		66.41		71.01		59.08		22.85	
Standard Desv. (+)	4,86		0.024		10.0		7.33		6.97	

*: $P \leq 0.05$. **: $P \leq 0.01$. n.s. not significant. (+) Obtained as the square root of the residual mean square. *df*: degrees of freedom.

4. Discussion

Plants' heat tolerance is decisive for both crop growth and productivity (Wei et al., 2019). In line with this, grafting plants onto tolerant rootstocks can provide efficient mechanisms for plants to overcome high temperature stress, as far as our knowledge, there is no research about the mechanisms underlying the tolerance of pepper grafted plants to such stress (Gisbert-Mullor et al., 2021; López-Marín et al., 2013; Palada and Wu, 2008).

In this study, we encountered differential physiological responses of pepper grafted plants in VA/A55 (sensitive rootstock), VA/A57 (tolerant rootstock) and VA/VA (self-grafted), and also in VA (the ungrafted variety) induced by HS. These responses are characterized in VA/A57 by the lowest EL, non-disturbed Chl and Car concentrations, increased AsA and phenols concentrations, and non-H_2O_2 accumulation under the HS conditions. These physiological responses conferred by rootstock A57 coincided with the highest proline concentration in anthers, and better pollen germination and fruit set compared to the other plant combinations.

One of the most sensitive physiological alterations under HS is increased membrane permeability because this affects its structure and functions (Nadeem et al., 2018; Wahid et al., 2007). EL values are an indicator of membrane injury and have been used to estimate membrane thermostability (Xu et al., 2006). According to our results, EL

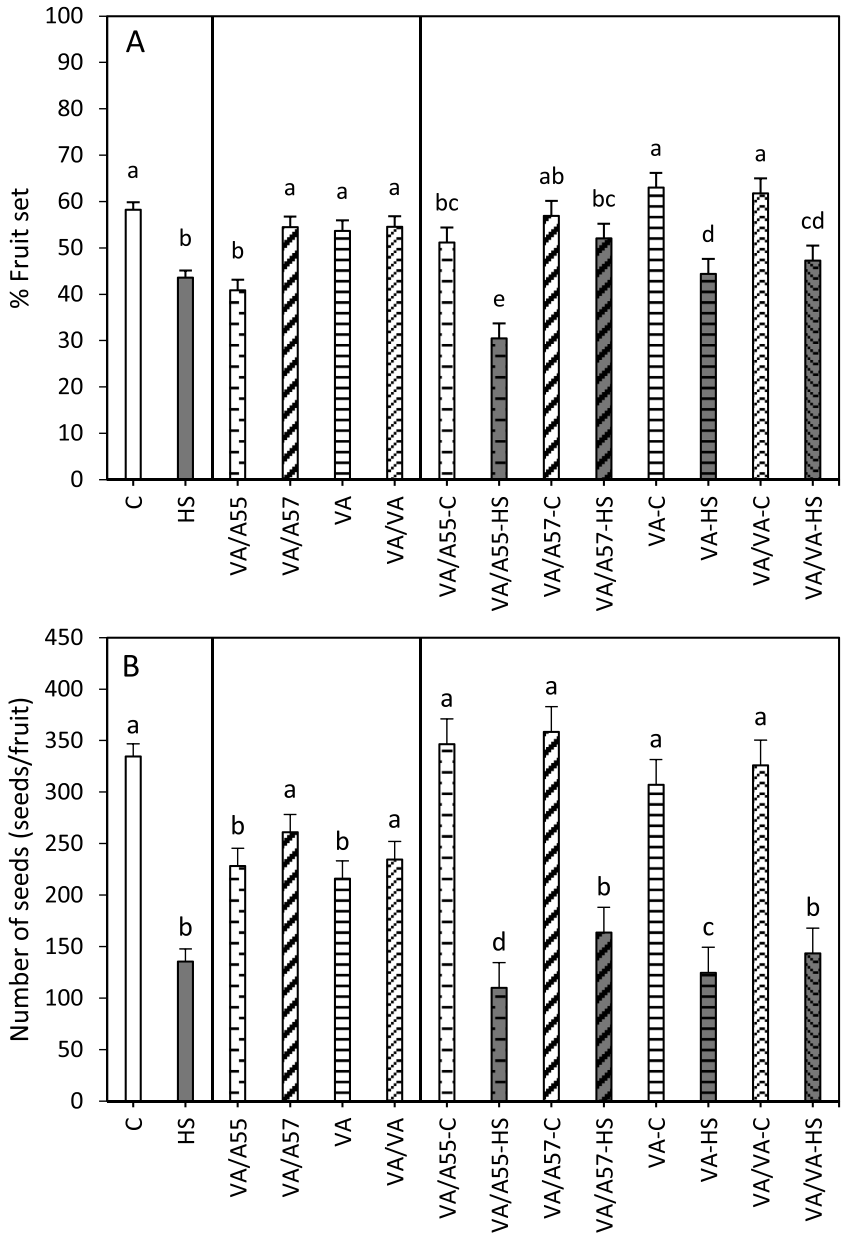

Fig. 7. Fruit set percentage for Experiment 2 (A) and number of seeds per fruit (B) in the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively) under heat stress (HS) or control conditions (C). Bars with different letters are statistically different according with LSD test ($P \leq 0.05$). Error bars represent LSD.

significantly depended on rootstock genotypes and treatments: the lowest EL values of all the plant combinations and the minor rise in relation to its controls were found in plants VA/A57. Other grafted plants, such as cucumber grafted onto *Momordica* or tomato grafted onto tomato or eggplant, have shown greater membrane stability compared to self-grafted plants or ungrafted plants under HS (Abdelhafeez et al., 1975; Abdelmageed and Gruda, 2009; Wei et al., 2019). Therefore, EL has been used as a screening test for HS tolerance (ElBasyoni et al., 2017; Nadeem et al., 2018). The EL parameter is principally influenced by the ability to balance reactive oxygen species (ROS) given that HS induces ROS generation and accumulation like H_2O_2 (Airaki et al., 2012; Xu et al., 2018). In this way, we found a positive correlation coefficient between the H_2O_2 concentration and EL ($r= 0.785$, $P< 0.05$), which indicates that the increase in EL was partially due to increased H_2O_2 production. However, the H_2O_2 concentration in VA/A57 under HS did not significantly differ from its control, which agrees with its lowest EL values. This effect could be partly due to better redox homeostasis in the VA/A57 plants supported by primary metabolites, such as carotenoids and chlorophyll accumulation, to avoid photooxidation in photosystems (Leverenz et al., 2015) and to achieve non-disturbed Fv/Fm values *versus* its control (Gisbert-Mullor et al., 2021). The same effects have been observed in cucumber grafted onto *Momordica* (Wei et al., 2019; Xu et al., 2018) at high temperature. The VA plants also had increased Chl and Car concentrations under HS compared to their control, but the photosynthetic pigments of the plants grafted onto the sensitive rootstock A55 decreased. Loss of chlorophylls and carotenoids concentration could be a consequence of the heat-induced damage to the thylakoid membrane by, thus, disrupting membrane permeability, increasing chlorophylase enzyme activity, blocking energy to reaction centers of photosystems, decreasing photosynthesis, among other causes (Cornic, 2000; Ghai et al., 2016; Komayama et al., 2007; Wahid et al., 2007).

Other plant metabolites like phenols and ascorbate are involved in HS tolerance and also play an important function in maintaining redox homeostasis (Chaudhary et al., 2020). Phenols, such as secondary

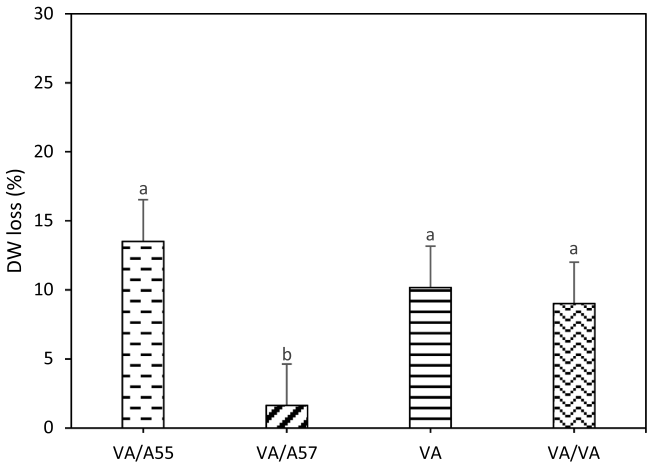

Fig. 8. Percentage dry weight loss under heat stress conditions compared to control conditions in the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively). Bars with different letters are statistically different according with LSD test ($P \leq 0.05$). Error bars represent LSD.

metabolites, are an important group of compounds that are essential for plant acclimatization and survival under different environmental conditions (Fraser and Chapple, 2011; Zandalinas et al., 2017). Their accumulation has been mentioned as a possible mechanism of tolerance to abiotic stresses (Hichem et al., 2009; Oh et al., 2009). However, contradictory results have been described in grafted plants. Rivero et al. (2003) observed greater accumulation for total phenols in tomato plants subjected to HS, and of these, lower accumulation in grafted plants compared to ungrafted ones. These authors concluded that grafted plants appeared more tolerant to HS. However, no significant differences have been observed in phenols content in fruit when pepper has been grafted onto different rootstocks (López-Marín et al., 2013). With our experimental conditions, the observed increase in phenols depended on heat treatment and rootstock genotype, with a significant increase for both VA and VA/VA57. Nevertheless, the differences in total phenols between the HS and C conditions were bigger in VA/VA57. Based on the VA/VA57 response, and by displaying the lowest EL values and the highest Chls and Car contents, it would seem that the increase in phenols could be associated with a HS tolerance factor.

AsA is considered a powerful antioxidant *per se* and an essential master compound in the AsA-glutathione cycle to scavenge ROS (Foyer and Noctor, 2005). Under our experimental conditions, the amount of AsAt increased in all the plant combinations in response to HS, and the AsA/AsAt ratio sharply dropped, which could indicate that a high AsA oxidation rate occurred in DHA (data not shown). The major increase in AsAt took place for the VA plants, followed by VA/A57. However, the higher DHA concentration observed in VA could indicate lesser AsA regeneration from DHA.

In vegetative parts, an efficient mechanism of HS tolerance plays a vital role in the formation of successful reproductive organs and, thus, positively affects final yield (Asseng et al., 2002; Wollenweber et al., 2003). We analyzed different reproductive parameters to confirm that VA/A57, which was best predisposed to overcome HS based on the physiological parameter results found in leaves, could exhibit the most suitable reproductive attributes. In most plant species, male gametophytes are more sensitive to high temperature than female gametophytes (Djanaguiraman et al., 2018). We analyzed a post-pollination event (pollen germination), primary metabolites in anthers, fruit set and number of seeds, which are all traits for thermo-tolerance studies in plants (Paupière et al., 2014). In pepper, high temperature during flowering impairs pollen germination, pollen tube growth and fertilization and, consequently, both flower abscission and fruit set lower (Aloni et al., 2001; Erickson and Markhart, 2002; Usman et al., 1999). Temperatures higher than 32°C provoke pollen abnormalities in pepper, such as shrunken and empty pollen grains with no appreciable exine (Erickson and Markhart, 2002). Under our experimental greenhouse conditions, HS reduced pollen germination despite this trait being rootstock-dependent with greater pollen germination when the variety was grafted onto A57. Reduced pollen vitality at high temperature has been described because insufficient carbohydrates are supplied from the tapetum and other anther tissues to pollen (Sato et al., 2000) in association with tapetum degeneration or malfunction (Mercado et al., 1997), as well as diminished invertase activity, which produce the hydrolysis from sucrose to hexoses and can be used by pollen (Aloni et al., 2001), among other aspects (Paupière et al., 2014). To support this idea, the amounts of fructose, glucose and sucrose in anthers according to our results revealed a significant difference between C and HS treatments, with a lower sugar concentration at high temperatures. Nevertheless, this was not rootstock-dependent, which could indicate that other factors are implicated in pollen germination because A57 displayed greater pollen viability, but not a higher sugar concentration.

Apart from sugars, proline is, among other essential metabolites (Mattioli et al., 2018, 2012; Sato et al., 2006; Xie et al., 2022), considered a key factor for pollen germination, and amino acid in the male reproductive part in the mature stage is more abundant and represents 60% of free amino acids (Paupière et al., 2014; Sangwan, 1978). Proline can act as a solute protectant during pollen development (Zhang et al., 1982) by protecting pollen grains from the desiccation that high temperature provokes, and by providing nutritive substances for pollen development (Fang et al., 2016). In tomato, Sato et al. (2006) reported decreased proline at moderately high temperature. Under HS, we observed a significant decrease in anthers' proline, but the plants grafted onto genotype A57 had the highest proline concentration under both the C and HS conditions. Observations made about the role of proline in pollen viability and development have been confirmed in a proline-deficient mutant of *Arabidopsis thaliana* (Paupière et al., 2014), and the interruption of proline synthesis leads to abortion and sterility during gametophyte development (Biancucci et al., 2015; Mattioli et al., 2018). In pepper, HS-tolerant genotypes have shown a higher proline concentration than susceptible ones (Saha et al., 2010), which confirms the master role of proline in pollen germination.

Favorable fruit set depends on several reproductive processes, including pollen germination and tube growth (Dahal et al., 2006; Madhavi Reddy et al., 2016). High temperature during pollen and/or fruit development in pepper has resulted in decreased fruit set and a smaller number of seeds per fruit (Aloni et al., 2001). In pepper, flower abortion occurs when day/night temperatures are higher than 34/21°C (Rylski and Spigelman, 1982), a situation that normally occurs in the Mediterranean Region in low-technology greenhouses. Under our experimental conditions, with temperatures of 38/22°C (day/night), fruit set significantly decreased in both experiments (2020 and 2021) at different magnitudes, and the VA/A57 plant combination was less affected by high temperature in fruit set terms, although no significant difference in fruit set was found in experiment 2 compared to its control.

In addition, significant correlations ($P< 0.05$) between fruit set and different parameters, such as number of seeds ($r=0.771$), pollen germination ($r=0.787$) and proline and sucrose concentrations in anthers ($r=0.718$ and $r=0.756$, respectively), were quantified and indicate the reliance of successful fruit set and pollen state.

As an integrated approach between both the experiments carried out in this study, and as we are aware that, because both experiments were run in different years, the parameters' correlations between them could not be established, we deduced that the physiological advantages observed in VA/A57, such as lower EL, higher Chls and Car contents, higher AsAt and also greater fruit set in Experiment 1, could be associated with the greater dry biomass maintenance and reproductive traits observed in Experiment 2 under HS. This connection might be based on the greater translocation capacity of the metabolites from photosynthesizing leaves to developing flowers (Aloni et al., 1991). In this

way, the traits associated with the vegetative and/or reproductive stage could be useful for screening tolerant plants under HS.

5. Conclusions

By way of conclusion, pepper plant growth and development under high-temperature conditions depended on plants' ability to perceive stress and to generate tolerant responses, such as tissue integrity or accumulation of protective metabolites, and grafting peppers onto a HS-tolerant rootstock, such as A57, could overcome the negative high temperature effects better than an ungrafted variety.

We also indicate that the better physiological performance noted in vegetative parts conferred by a HS-tolerant rootstock would also seem to result in better performance in the reproductive phase because fruit set, which is one of the most important yield components in fruiting vegetables, improved. This scenario is associated with better pollen germination and proline content in pollen grains.

CRediT authorship contribution statement

Ramón Gisbert-Mullor: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft. **Yaiza Gara Padilla:** Conceptualization, Methodology, Investigation. **Ángeles Calatayud:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Salvador López-Galarza:** Conceptualization, Methodology, Investigation, Writing – review & editing.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2022.111699.

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