Suitable rootstocks can alleviate the effects of heat stress on pepper plants

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Abstract

In this study, different pepper rootstocks are tested for their ability to overcome heat stress situations. This work aims to evaluate: (i) the physiological mechanisms that occur during long heat stress periods (7 days) under controlled conditions in a pepper variety grafted onto accessions; (ii) the heat stress behaviour of these grafted pepper plants under greenhouse conditions in terms of marketable yields. For this purpose, plants of Lamuyo-type sweet pepper 'Herminio F1' (VA), grafted onto six accessions (VA/A25, VA/A31, VA/A34, VA/A52, VA/A57, VA/A6), and a self-grafted variety (VA/VA) were grown under controlled conditions in growth chambers (28/24 ºC, day/night temperatures and 38/24 ºC for control and heat stress, respectively) and under greenhouse conditions (38/24 ºC). For the controlled conditions, relative growth rate, leaf area, electrolyte leakage, chlorophyll *a* fluorescence and heat shock proteins were determined. For the greenhouse conditions, fresh and dry weigh, electrolyte leakage and fruit yield were determined. Our results confirmed that grafting a pepper cultivar onto appropriate rootstocks such as A6, A25 and A57 can overcome the negative effects of heat stress conditions with a higher

relative growth rate, leaf area and Fv/Fm, and lower electrolyte leakage under the controlled conditions, and with higher marketable yields under the greenhouse conditions.

Keywords

Chlorophyll *a* fluorescence; Electrolyte leakage; Grafting; Heat shock proteins; Relative growth rate; Thermal stress

1. Introduction

Sweet pepper displays a marked response to heat, the optimal temperature ranging between 20 ºC and 30 ºC. Above 32 ºC, the temperature can cause serious problems in pollination and fertilization resulting in fruit drop (Erickson and Markhart, 2002; Guo et al., 2014). However, different lines of chilli pepper from *C. chacoense*, *C. bacatum*, *C. frutescens* to *C. annuum* have been identified as heat tolerant compared with some sweet peppers based on its cumulative temperature response index (Barchenger et al., 2019; Palada and Wu, 2008). Aloni et al. (1994) associated the susceptibility to high temperature of two *C. annuum* cultivars (sweet pepper and paprika) to light intensity and ethylene production. Although, the response to heat stress is totally dependent to their genetic background (Usman et al., 2014) and needs to be evaluated for each variety and heat stress conditions.

In mild winter climates with warm springs and hot summers, the cropping season of sweet pepper crops is actually intended to extend over the 12 months of the year, normally comprising one of these three cycles: 1) planting in July/August and crop-ending in February; 2) an extended cycle, from May/June to April/May of the following year; 3) and a cycle starting in November/December to harvest during June-July-August. Depending on each cycle, flowering and fruit set normally start two months after planting

and last until the end of the cycle unless during the coldest and shortest daylength months of the season, at least December and January. During these cycles but particularly in the extended one, sweet pepper crops, normally in greenhouses, may have to withstand high temperatures, sometimes above 35 ºC, which negatively impacts growth and yields, as well as pepper fruit quality (López-Marín et al., 2013). This situation can be aggravated by the global warming scenario as temperatures grow increasingly higher worldwide, particularly in the Mediterranean Basin (IPPC, 2018), which produces huge quantities of peppers and other vegetables.

Grafting is currently an effective alternative to relatively slow breeding programmes (Schwarz et al., 2010) to obtain varieties that adapt to abiotic stresses (Penella et al., 2017; Schwarz et al., 2010). It has been shown that using appropriate pepper rootstocks is an effective, feasible and sustainable strategy mainly against water and saline stress (Gisbert-Mullor et al., 2020; López-Marín et al., 2017; López-Serrano et al., 2017). Nevertheless, very few studies have screened rootstocks for heat stress with sweet pepper (Aidoo et al., 2017; López-Marín et al., 2013; Palada and Wu, 2008), and some have pointed out that grafting is a technique capable of reducing the negative effects of high temperatures on pepper plants.

One of the most sensitive plant cell components is the membrane, and high temperature increases its fluidity and ion-permeability due to protein denaturation, accompanied by metabolism inactivation (Ayenan et al., 2019; Hansen et al., 1994). Ion leakage has been considered a bio-marker to heat tolerance. In different crops, tolerant plants to high temperatures display less membrane permeability than non-tolerant plants (Ayenan et al., 2019; Camejo et al., 2006, 2005; De Silva and Asaeda, 2017; Gulen and Eris, 2004; Hu et al., 2010; Xu et al., 2017). Hence grafting is a good tool to reduce electrolyte leakage. In fact tomato grafted onto eggplant has been found to reduce

membrane permeability under prolonged and controlled heat stress conditions (Abdelmageed and Gruda, 2009).

Heat damage affects not only membranes fluidity, but also chloroplast (and mitochondria) activities, among other metabolic processes. In fact, photosynthesis is particularly sensitive to heat stress which induces its decrease. This decline in photosynthesis is related to an increased fluidity on thylakoid membranes (Biswal et al., 2011), which disrupts the photochemical reaction in the thylakoid lamellae and also the carbon metabolism in the stroma (Hu et al., 2020a), and as a consequence an increase in the photorespiration rate occurs (Long et al., 2004). Limited photosynthesis causes a drop in photoassimilates restricting plant growth and ultimately affecting yields (Fahad et al., 2017; Taiz and Zeiger, 2015).

In grafted plants, rootstocks can influence the adaptive capacity of scion to photosynthesis under heat stress (Schwarz et al., 2010; Xu et al., 2018) as photosynthetic capacity can be dependent on the vitality of roots. Roots from rootstocks are usually larger and more vigorous, being capable of absorbing water and nutrients more efficiently than roots of the scion, which could alleviate substantially photosynthesis inhibition (Colla et al., 2008; Lee et al., 2010; López-Marín et al., 2013). In addition, the signalling compounds going through root-to-shoot like hormones, nutrients, genes, transcription factors and miRNA can alter scion perception responses to heat stress (Li et al., 2014a; Xu et al., 2018). However, how tolerant rootstocks regulate the photosynthesis processes under heat stress remains unknown (Li et al., 2016). Different plant combinations (scion/rootstock) have resulted successful in terms to improve photosynthetic apparatus protection in heat stress situations. Pepper plants grafted onto some rootstocks showed better maximum quantum yield of PSII, Fv/Fm, in contrast to ungrafted plans (López-Marín et al., 2013). Cucumber plants grafted onto *Momordica* have enhanced chlorophyll

content, Fv/Fm and net photosynthesis compared to self-grafted plants (Tao et al., 2020; Xu et al., 2018). Moreover, grafting cucumber onto heat-tolerant *Luffa* rootstock alleviates heat-induced photosynthesis inhibition and oxidative stress (Li et al., 2016).

Plants activate stress-responsive mechanisms to minimise the harmful effects of heat stress, such as antioxidant activities, osmoprotection, hormonal signals, metabolites synthesis or induction of heat shock proteins (HSPs). Different studies have also demonstrated that heat stress promotes the accumulation of HSPs, and these proteins are considered to be master players for inducing tolerance. HSPs act as molecular chaperones, and are induced under heat conditions to protect cellular proteins against irreversible heat damage (Barua et al., 2003; Boston et al., 1996; Li et al., 2014a). HSPs comprise five major families of HSPs based on their approximate molecular weights, such as HSP100, HSP90, HSP70, HSP60, and small HSP (sHSP) (Gupta et al., 2010; Kotak et al., 2007; Wang et al., 2004). Some studies have revealed an increase in HSPs in grafted plants under heat stress. The HSP70 protein has been significantly induced in cucumber grafted onto *Luffa* at an earlier stage (12-48 h) of heat treatment (Li et al., 2016, 2014a). Compared with HSP70 and HSP90, sHSPs are apparently more limited and ATP-independent, they bind to non-native proteins ranging from peptides to big proteins with high efficiency, preventing irreversible aggregation of these proteins (Haslbeck et al., 2019). However, there are no studies in pepper grafted plants about the conservation of HSPs or their effects over time (days) to confer tolerance in heat stress situations.

For all these reasons, in order to adapt the pepper crop to protected cultivation systems in the global warming scenario, it seems important to select rootstocks capable of conferring the pepper scion the ability to face the problems caused by high temperatures in the hottest part of the season by screening pepper genotypes in these situations.

Consequently, our work objectives were to evaluate: (i) in controlled conditions in growth chambers, the physiological mechanisms that occur during heat stress periods (7 days) in the *C. annuum* cultivar "Herminio" grafted onto different *C. annuum* accessions; (ii) under greenhouse conditions, the heat stress tolerance during long periods of these grafted pepper plants in terms of marketable yields.

2. Materials and methods

Six *Capsicum annuum* L. genotypes were used as rootstocks together with the scion cultivar "Herminio F1" (Syngenta) (*Capsicum annuum*, Lamuyo type, B2 type of Pochard (1966) classification) (VA). Accessions, with their country of origin in brackets, were: A25 (United States), A31 (United States), A34 (Spain), A52 (United States), A57 (Israel), A6 (Mexico). These accessions were selected according to previous testing experiments under heat stress and control conditions leading to identify both tolerant and sensitive accessions (unpublished data). All the genotypes employed in the present study belong to the COMAV Institute collection (Universitat Politècnica de València, Valencia, Spain). Two experiments were done to meet the objectives.

2.1. Experiment 1: Physiological behaviour of pepper plants under the control and heat stress conditions of growth chambers

The variety grafted onto the six accessions (VA/A25, VA/A31, VA/A34, VA/A52, VA/A57, VA/A6), the ungrafted variety (VA) and self-grafted variety (VA/VA) were evaluated under heat stress and control conditions.

The seeds of the variety and accessions were sown in 104-cell polystyrene trays in a fine structure peat substrate (80% white and 20% black, pH 5.7) (Gebr. Brill, Germany), on 27 December 2018. The graft was performed on 1 February 2019 by the

tube-grafting method (Penella et al., 2014). On 4 March 2019, plants were transferred to 0.5-litre pots filled with the same peat substrate, and eight plants for each graft combination were randomly arranged in both growth chambers (control and heat stress conditions) for 7 days, each individual plant being the experimental unit. The climatic chambers conditions were 28/24 ºC, day/night temperatures and 38/24 ºC for control and heat stress, respectively, with a 16-hour photoperiod of 450±50 µmol m^{-2} s^{-1} PPFD (photosynthetic photon flux density).

Plants were fertigated by the ebb and flow method once at the beginning of the experiment and after that using a capillary mat, with a nutrient solution containing (in mmol L^{-1}): 6.6 NO_3^-; 0.5 $H_2PO_4^-$; 0.5 SO_4^{2-}; 0.5 NH_4^+; 3.0 K^+; 1.5 Ca^{2+}, 0.8 Mg^{2+} 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+}. The electrical conductivity (EC) and pH of this nutrient solution was 0.8 dS m^{-1} and 6.5, respectively.

After 7 days under both climate conditions, relative growth rate, leaf area, electrolyte leakage, chlorophyll *a* fluorescence and heat shock proteins were determined. The relative growth rate (RGR) was calculated by the following formula:

$$RGR = \frac{\ln W_2 - \ln W_1}{\Delta t}$$

where W_1 and W_2 were the total fresh biomass on day 0 (first day in growth chambers) and day 7, respectively, and Δt was 7 days. Before carrying out each weight, the substrate was saturated by submerging the tray in the nutrient solution during 24 hours and afterwards leaving it to drain for 6 hours to avoid variances between W_1 and W_2 in relation to the weight of the substrate. The RGR was measured for the eight plants of each genotype combination and thermal conditions.

The leaf area of four plants for each combination and growth chamber was determined using an area meter (model LI-3100C; Li-Cor, Lincoln, NE, USA).

To determine electrolyte leakage (EL), 18 discs of freshly cut leaves from the eight plants of each genotype combination and thermal condition were obtained with a hole-puncher (1.4 cm in diameter) and divided into three groups. The six discs from each group were placed in 50 mL flasks together with 20 mL of distilled water. The EL in the solution was calculated from the EC measures taken at 0 h (C_1) and after 2 h (C_2) at room temperature with a conductivity meter (Model Seven Easy Mettler Toledo, Mettler-Toledo AG, Switzerland). Total conductivity (C_3) was obtained after keeping flasks frozen (-40 ºC) for 24 h. The results were expressed as a percentage of total conductivity.

$$EL\ (\%) = \frac{C_2 - C_1}{C_3 - C_1} \cdot 100$$

Chlorophyll *a* fluorescence analyses were done to evaluate the damage degree of the PSII reactions. The maximum quantum yield of PSII (Fv/Fm; where Fv = Fm – Fo) was measured on leaves after 30-minute dark adaptation with a portable pulse amplitude modulation fluorometer (PAM-2100; Walz, Effeltrich, Germany). The minimum fluorescence signal for the dark-adapted leaves (Fo) was determined with a 0.5 μmol photon m^{-2} s^{-1} measuring light at a frequency of 600 Hz. The application of a saturating flash of 10.000 μmol photon m^{-2} s^{-1} enabled maximum fluorescence (Fm) estimations to be made. Chlorophyll fluorescence parameters were measured for the eight plants of each genotype combination and thermal condition.

To measure HSP, frozen leaf samples (after 7 days in climatic chambers) were previously grounded with liquid nitrogen in a mortar until a fine powder formed. Total RNA was extracted from 100 mg of sample with the Rneasy Plant Mini Kit (Qiagen,

199 USA) and was treated with the Rnase-Free Dnase Set (Qiagen, USA) to remove the
200 remaining genomic DNA, following the manufacturer's instructions. RNA concentration
201 and purity were measured by a NanoDrop ND-1000 spectrophotometer (Thermo
202 Scientific, USA) and the samples showing a proper concentration and suitable
203 A260/A280 and A260/A230 absorption ratios were used. All the RNA samples were
204 diluted to the same concentration before reverse transcription, in which cDNA was
205 generated using the PrimeScript™ Reagent Kit (Perfect Real Time) (Takara, Japan).

206 Three primer pairs for the (HSP) genes were selected for quantitative real-time
207 PCR (qRT-PCR) (Table 1): ubiquitin binding protein gene (CaUBI-3) as well as
208 glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH), which were used as the
209 reference genes (Bin et al., 2012; Wan et al., 2011). The qRT-PCR was performed by the
210 StepOne-Plus Real-Time PCR System (Applied Biosystems, USA) and SYBR® Premix
211 Ex Taq™ II (Takara, Japan). Expression levels were calculated by the relative standard
212 curve procedure using three independent biological replicates which were, in turn,
213 technically replicated 3 times. These values were normalised with the geometric mean of
214 the two reference genes and standardized in relation to the variety values under the control
215 conditions. HSPs were measured in three plants for each genotype combination and
216 thermal condition.

217 **Table 1.** The primer sequences of the target HSP genes.

Gene name	Gene ID	Primer sequence	References
CaHsp70-13	CA00g89640	5' ACTTTCTACCTCAGGCGACA 3' (F) 5' CATAACTCTTCAAACTTGGCTC 3' (R)	Guo et al. (2016)
CaHsp3-Q	CA03g21390	5' CTCGATGTCTCCCCTTTCGG 3' (F) 5' TGATGCCCTGTTCCTTCCTG 3' (R)	Li et al. (2015)
CaHsp22.7	CA06g20260	5' AATGTTTCCACAAGAGGCTGATCC 3' (F) 5' CCTCCGTCTTCATCCCTGGTAT 3' (R)	Self-designed

F: Forward Primer; R: Reverse Primer.

218

219 *2.2. Experiment 2: Agronomic evaluation of the pepper grafted plants under heat stress*
220 *conditions in a greenhouse*

221 The scion (cv. "Herminio") grafted onto the six accessions (VA/A25, VA/A31,
222 VA/A34, VA/A52, VA/A57, VA/A6), the ungrafted variety (VA) and the self-grafted
223 variety (VA/VA) were evaluated under greenhouse conditions in soilless cultivation.

224 On 16 February 2019, the seeds of the cultivar and accessions were sown in 104-
225 cell polystyrene trays in a fine structure peat substrate (80% white and 20% black, pH
226 5.7) (Gebr. Brill, Germany). The graft was performed on 27 March 2019. On 15 April
227 2019, plants were transplanted in 6-litre pots, in a medium structure peat substrate (100%
228 white, pH 5.7) (Gebr. Brill, Germany), and placed in a Venlo-type glasshouse at a density
229 of 2.5 plants m^{-2}. Pots were drip-irrigated, using anti-drain Netafim® drippers of 4 L h^{-1}
230 , with a nutrient solution containing (in mmol L^{-1}): 14.0 NO_3^-; 1.5 $H_2PO_4^-$; 2.4 SO_4^{2-};
231 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
232 Fe^{3+}, 6 Zn^{2+}, 12 Mn^{2+}, 30 B^{3+}, 0.8 Cu^{2+} and 0.5 Mo^{6+}. The EC and pH of this nutrient
233 solution were 2.2 dS m^{-1} and 6.5, respectively. The volume of the solution was controlled
234 by the number of irrigations, which varied according to accumulated radiation. In
235 addition, an attempt was made to maintain drainage between 15% and 20% of the total
236 irrigation volume.

237 The heat stress conditions (day/night set point temperatures of 38/24 ºC) began on
238 25 May 2019, 40 days after planting, when flower buds were observed in the second node.
239 The set fruits in the first node, if any, were removed. The temperature, relative humidity
240 and accumulate radiation values were recorded during the experiment using S8TH sensor
241 (Oratge Instruments®, Valencia, Spain) with data logger MSIP801 (BSG Ingenieros,
242 Valencia, Spain) and are presented in Fig. 1.

243

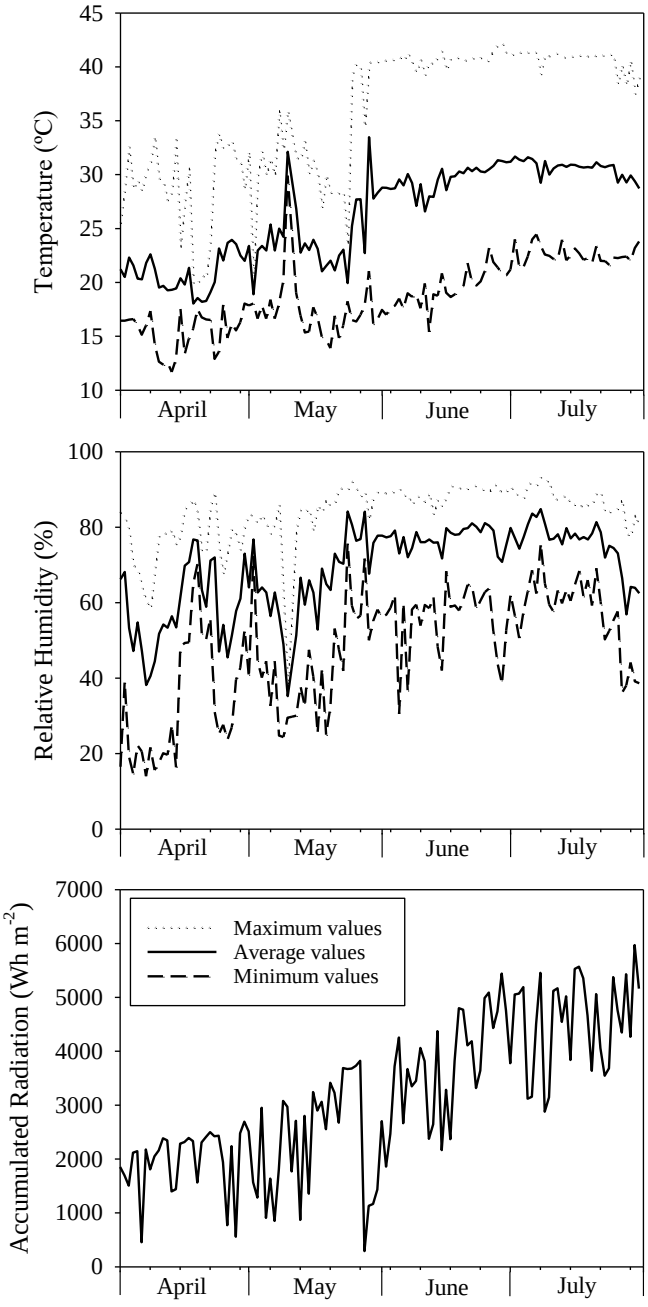


Fig. 1. Temperature (ºC), relative humidity (%) and accumulated radiation (Wh m^{-2}) values inside the greenhouse.

The layout took a completely randomised design, based on our previous experience in this greenhouse, with three replications for each genotype combination and 10 plants per replication.

Fresh weight per plant was measured weighing leaves and stems which were later exposed to dry heat at 70 ºC for 72h in a laboratory oven to measure dry weight.

EL was measured as it is described in the Experiment 1 (2.1. section).

Harvests were staggered between the end of June and the end of July and consisted in one harvest per week, matching five harvest rounds. Marketable and non-marketable production were evaluated following the criteria described by European Regulations (Official Journal of the European Union, 2011). First, fruits were partitioned into two categories: «Extra» Class and Class I (together hereafter they are referred to as marketable yield; MY). The fruit which, due to their defects (Blossom End Rot (BER), cracking, sunscald) did not reach these categories, are referred to as non-marketable yield (NMY). Then, fruit yield was measured as the weight of the fruits per plant (g/plant), number of fruits per plant and average marketable fruit weight (g/fruit) for all the plants of each genotype combination and replication.

2.3. Statistical analysis of data

For both experiments, the results for the different parameters were evaluated by an analysis of variance (ANOVA) using the Statgraphics Centurion XVII software (Statistical Graphics Corporation 2014). The RGR and EL data were *arcsin*-transformed before the analysis. Means were compared by the Fisher's least significance difference (LSD test) at $P \leq 0.05$. Stepwise multiple regression analysis for RGR as dependent variable and Fo, Fm, Fv/Fm, leaf area and EL as independent variables was performed as well as correlation analyses in Experiment 1 between the abovementioned parameters using the previously cited Statgraphics software.

3. Results

3.1. Experiment 1: Physiological behaviour of pepper plants under the control and heat stress conditions in growth chambers

3.1.1. Relative growth rate

The RGR was, on average for the genotypes, lower under heat stress, with a 28.1% reduction compared to the plants under the control conditions ($P \leq 0.01$; Table 2).

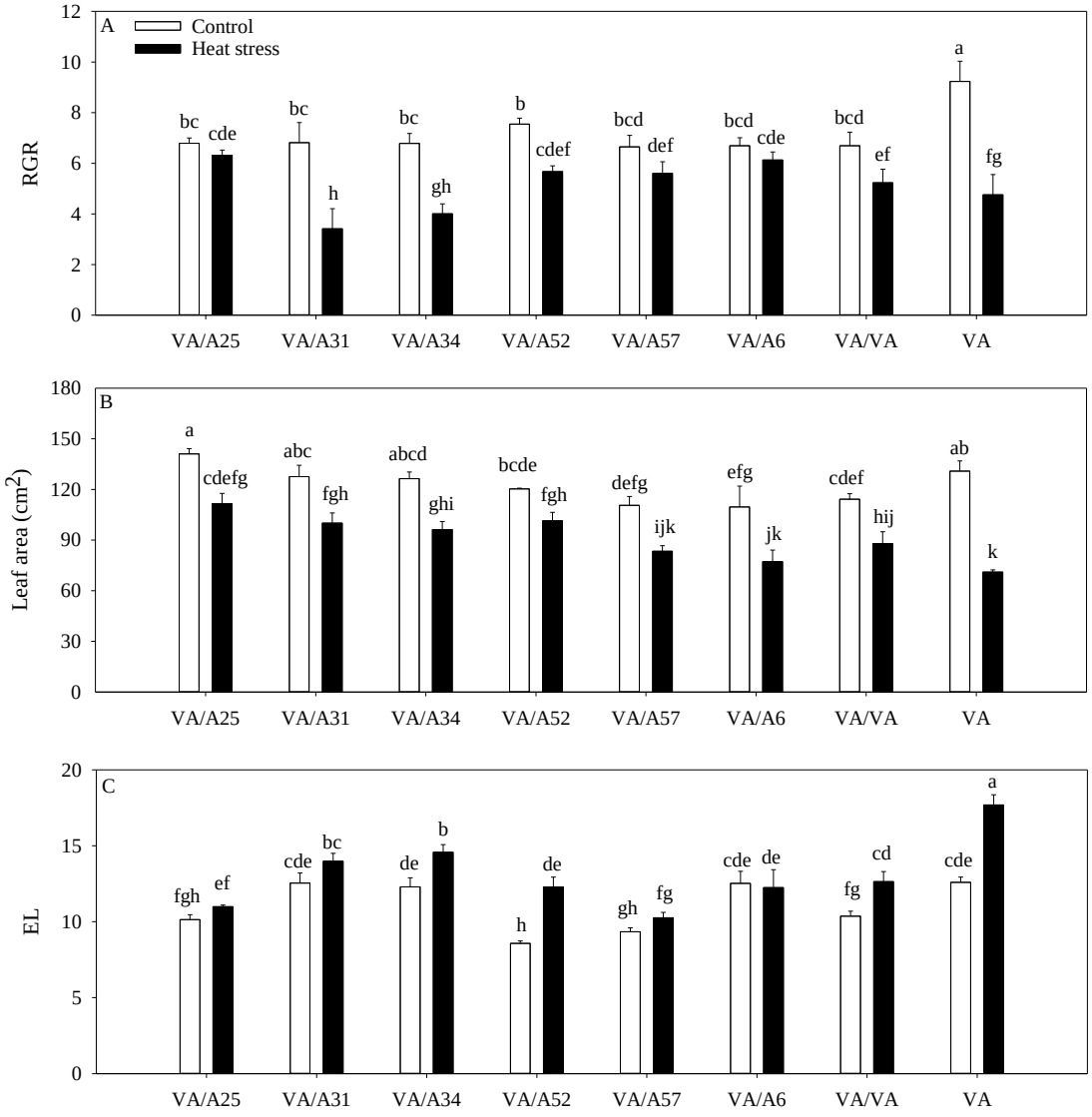
The interaction between the thermal conditions (TC) and genotypes (G) was also statistically significant ($P \leq 0.01$; Table 2). The RGR of VA, VA/VA, VA/A31, VA/A34 and VA/A52 was negatively affected by heat stress, whereas the VA/A25, VA/A57 and VA/A6 combinations were not affected by stressing temperatures (Fig. 2A). The RGR of the ungrafted cultivar (VA) under the control conditions was much higher than those of the grafted plants, but sharply dropped under stress conditions (Fig. 2A). The RGR of the self-grafted cultivar (VA/VA) under stress was less affected than that of VA, but was also negatively affected by stress. Furthermore, the RGR of VA/A25 and VA/A6 was higher than that of VA under stress, but similar to the VA/VA plants under heat stress.

3.1.2. Leaf area

Similarly to the RGR, the leaf area was higher on average in all the plants under the control conditions, with a 34.5% increase in relation to the plants under stress ($P \leq 0.01$; Table 2).

The interaction between the TC and G was also statistically significant, but explained very little about variation ($P \leq 0.05$; Table 2). The reduction in leaf area in the ungrafted variety (VA) under the stress conditions (46% in relation to its control) was much higher than in the grafted plants, which were 23% on average, including the self-grafted (VA/VA) one. Under the heat conditions, all the grafted combinations underwent significant reduction in the leaf area compared to their controls (Fig. 2B). The leaf area

300 of the VA/A25 combination under heat stress was statistically higher than those of VA
301 and VA/VA (Fig. 2B).
302 *3.1.3. Electrolyte leakage*
303 The EL value was higher under heat stress, with an 18.4% increase compared to
304 the plants under the control conditions ($P \leq 0.01$; Table 2).
305 The interaction between the TC and genotypes G was statistically significant ($P \leq$
306 0.01; Table 2). The EL of the plants of the ungrafted cultivar (VA) under the stress
307 conditions was much higher than for the other combinations (Fig. 2C). Compared to VA,
308 the EL in the self-grafted cultivar (VA/VA) was 28.5% lower under the stress conditions
309 and 17.7% lower under the control conditions.
310 Of all the studied combinations, VA/A25, VA/A31, VA/A57 and VA/A6 showed
311 no significant differences in EL between the stress and control conditions. Moreover, the
312 EL in VA/A57 and VA/A25 lowered compared to VA/VA under stress, and was similar
313 under the control conditions. The EL in VA/A57 under the heat stress conditions was less
314 than in the VA grafted onto the other rootstocks, except for VA/A25 (Fig. 2C). Finally,
315 VA/A31 and VA/A34 had a higher EL than VA/VA under both conditions (Fig. 2C).



316

317 **Fig. 2.** Relative growth rate (RGR) (A), leaf area (B) and electrolyte leakage (EL) (C) in
318 pepper genotypes under heat stress and the control conditions. Different letters indicate
319 significant differences at $P \leq 0.05$ (Fisher's LSD test). Data are the mean of eight
320 replicates for the RGR, four replicates for leaf area and three replicates for EL. Error bars
321 represent standard error.

322 *3.1.4. Chlorophyll a fluorescence*

323 The maximum quantum yield of chlorophyll *a* fluorescence (Fv/Fm) was
324 significantly lower in all the combinations subjected to heat stress, with an 8% reduction
325 on average compared to those of the control conditions ($P \leq 0.01$; Table 2).

326 The interaction between the TC and G was also statistically significant ($P \leq 0.05$;
327 Table 2). It is highlighted that the Fv/Fm under the control conditions was similar for all
328 the G, but significant differences appeared under heat stress, where VA was much lower
329 under stress conditions than in the other genotypes, except for VA/A34 and VA/A6 (Fig.
330 3A). Significant differences between the control and stress conditions also appeared. The
331 Fv/Fm in the VA and VA/VA, VA/A6, VA/A34 and VA/A31 combinations were
332 significantly higher in the control than under stress, whereas no significant differences
333 were observed in VA/A25, VA/A52 and VA/A57 among thermal conditions (Fig. 3A).
334 In the self-grafted cultivar (VA/VA), Fv/Fm was 10.4% higher in stress compared to the
335 ungrafted VA.

336 The minimum chlorophyll fluorescence (Fo) was, on average, lower in all the G
337 under the control conditions, with a 12% reduction compared to the plants under the heat
338 stress conditions ($P \leq 0.01$; Table 2). The interaction between the TC and G was not
339 statistically significant (Fig. 3B)

340 The maximum chlorophyll fluorescence (Fm) was lower in all the plants subjected
341 to heat stress, with a reduction of 6.1% on average compared to the plants under the
342 control conditions ($P \leq 0.05$; Table 2). The interaction between the TC and G was also
343 statistically significant ($P \leq 0.01$; Table 2). In VA/A6, Fm was significantly lower under
344 stress than under the control conditions, whereas the contrary took place in VA/A57,
345 which was higher under stress than under its control. With the other combinations, Fm
346 was not significantly different between both the stress and control conditions (Fig. 3C).

Table 2. Analysis of variance (ANOVA) of parameters relative growth rate (RGR), leaf area, electrolyte leakage (EL), Fv/Fm, Fo and Fm. % of the Sum of Squares for the factor thermal condition (TC) and genotype (G) as well as their interaction (TC*G).

ANOVA (*df*)	% Sum of Squares					
	RGR	Leaf area	EL	Fv/Fm	Fo	Fm
TC (1)	33.1 **	51.6 **	19.8 **	23.2 **	7.5 **	3.6 *
G (7)	11.6 **	21.1 **	55.5 **	12.1 **	6.3 n.s.	21.1 **
TC*G (7)	14.7 **	6.7 *	12.3 **	8.2 *	4.6 n.s.	12.4 **
Residuals	40.6	20.6	12.4	56.5	81.7	62.9
Residuals df	112	45	32	112	112	112
Standard Dev. (+)	1.19	11.5	0.99	0.05	58.2	133

*,** indicates significant differences at $P \leq 0.05$ and $P \leq 0.01$, respectively. n.s. denotes no significant differences. (+) Calculated as the square root of the residual mean square. *df*: degrees of freedom.

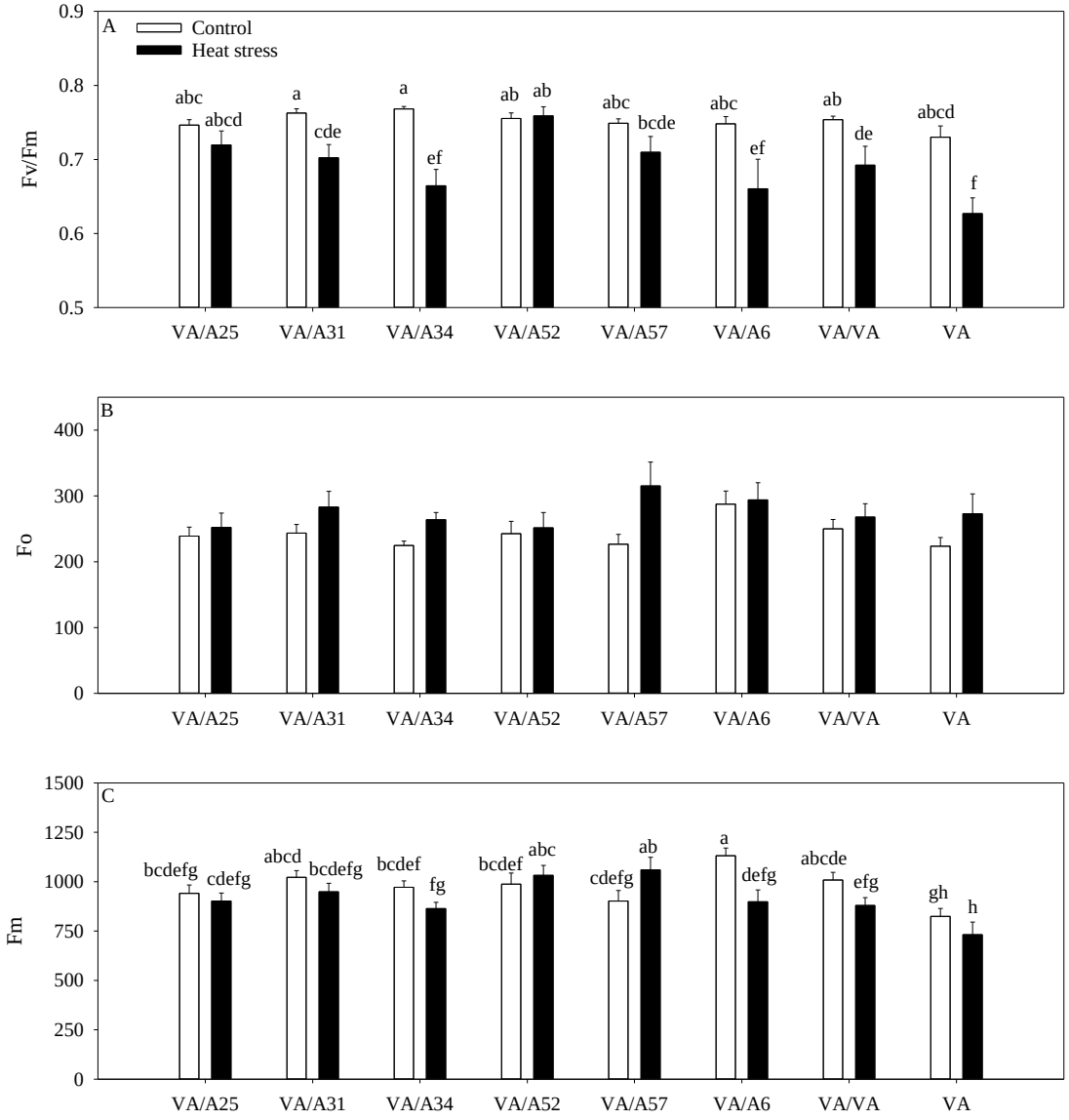


Fig. 3. Fv/Fm (A), Fo (B) and Fm (C) chlorophyll fluorescence parameters in pepper genotypes under heat stress and the control conditions. Different letters indicate significant differences at $P \leq 0.05$ (Fisher's LSD test). Data are the mean of eight replicates. Error bars represent standard error.

3.1.5. Heat Shock Proteins

To analyze whether HSPs contributed to the rootstock-induced thermo-tolerance of pepper plants, we compared the responses of two sHSP (HSP25.8 and HSP22.7) and one HSP70 (70-13) to heat stress between plant combinations. The qRT-PCR analysis

indicated that the increased abundance of the transcripts of three HSP caused by heat stress was greater than under the control conditions after 7 days in climatic chambers for both grafted and ungrafted plants (Table 3).

The interaction between the TC and G was statistically significant for the three analysed HSPs, with $P \leq 0.05$ for HSP70-13 and $P \leq 0.01$ for sHSPs (Table 3).

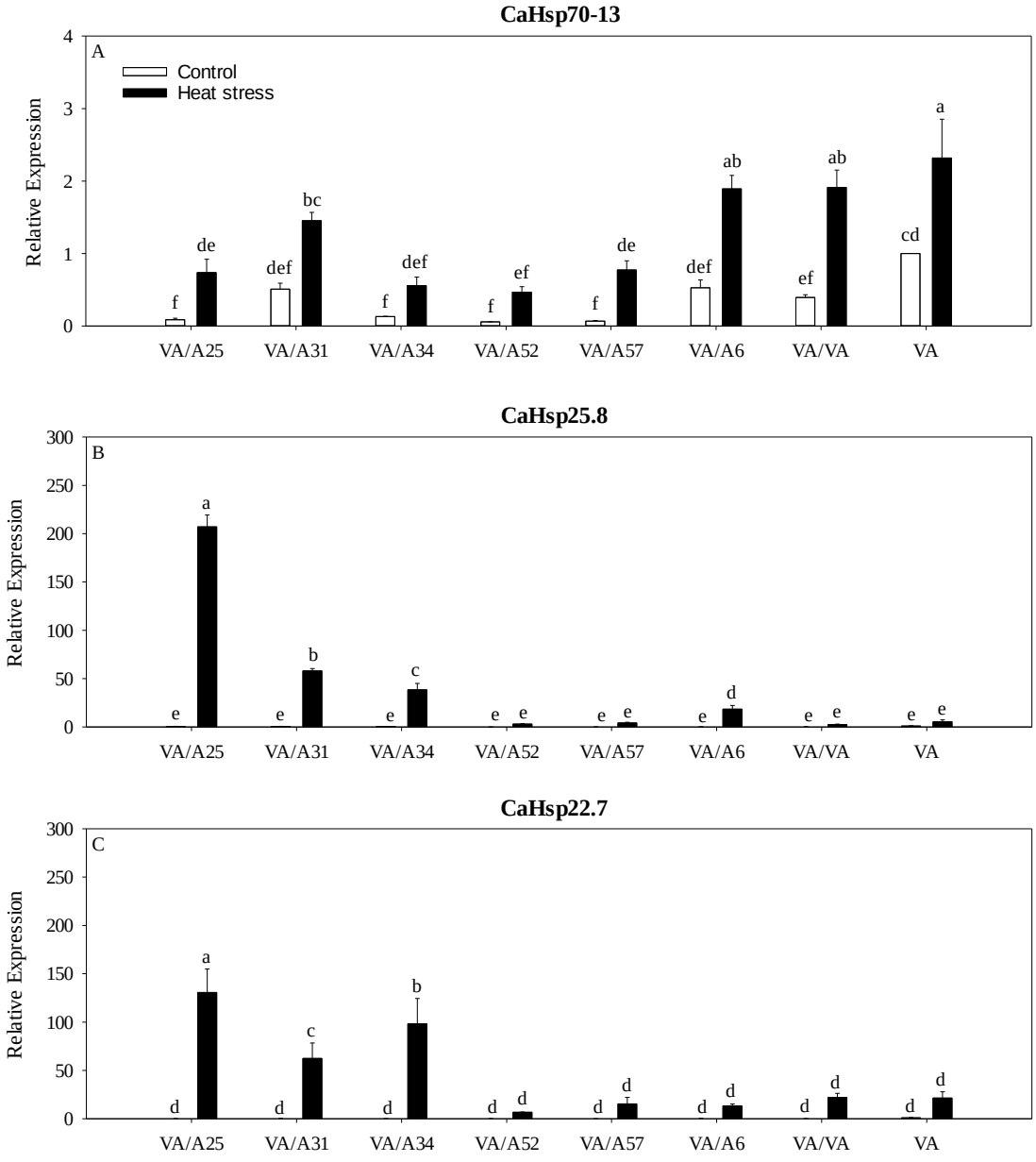
The expression levels in HSP70 (70-13) were lower than the values obtained in sHSP (Fig. 4A). Under heat stress, the highest values went to VA, VA/VA and VA/A6. Under the control conditions, the values for the grafted plants were lower than in VA and with significant differences.

The levels in HSP25.8 and HSP22.7 were higher in VA/A25, followed by VA/A31 and VA/A34 (Fig. 4B, C) for both sHSPs.

Table 3. Analysis of variance (ANOVA) of the expression levels for HSP70-13, HSP25.8 and HSP22.7. % of the Sum of Squares for the factor thermal condition (TC) and genotype (G) as well as their interaction (TC*G).

ANOVA (*df*)	% Sum of Squares		
	HSP70-13	HSP25.8	HSP22.7
TC (1)	38.6 **	16.7 **	31.6 **
G (7)	42.6 **	41.2 **	27.9 **
TC*G (7)	7.7 *	41.0 **	28.1 **
Residuals (32)	11.1	1.1	12.3
Standard Dev. (+)	0.67	0.36	0.44

*,** indicates significant differences at $P \leq 0.05$ and $P \leq 0.01$, respectively. n.s. denotes no significant differences. (+) Calculated as the square root of the residual mean square. *df*: degrees of freedom.



374

375 **Fig. 4.** Relative expression for CaHsp70-13 (A), CaHsp25.8 (B) and CaHsp22.7 (C) in
376 pepper genotypes under heat stress and the control conditions. Different letters indicate
377 significant differences at $P \leq 0.05$ (Fisher's LSD test). Data are the mean of three
378 replicates. Error bars represent standard error.

379 *3.1.6. Multiple regression analysis*

A stepwise multiple regression analysis was performed to predict RGR from the variables Fo, Fm, Fv/Fm, Leaf area and EL. The variables Fm, Leaf area and EL, statistically significantly predicted RGR, with an F (3, 95) = 9.24, $p < 0.0001$, and $R^2 = 0.43$, according to the model: RGR = 0,000646957 + 0.0000129391*Fm + 0.00003678*Leaf area – 0.116089*EL.

3.2. Experiment 2: Agronomic evaluation of pepper grafted plants under the heat stress conditions in greenhouses

In the greenhouse experiment, we compared the ungrafted variety and the self-grafted to the variety grafted onto all the studied accessions under the heat stress conditions. Fresh weight of aerial part didn't show significant differences between genotypes, however dry weight and EL showed differences between genotypes with $P \leq 0.05$ and $P \leq 0.01$, respectively (Table 4). DW was lower in VA/A6 compared to the other genotypes. For EL, highest values belong to VA and VA/VA, followed by VA/A25 and VA/A34, being the lowest values from VA/A52, VA/A6, VA/A57 and VA/A31.

Table 4. Analysis of variance (ANOVA) for fresh weight (FW), dry weight (DW) and electrolyte leakage (EL) from greenhouse experiment expressed as mean values by Genotype (G) and % of the sum of squares. Different letters in each column indicate significant differences at $P \leq 0.05$ using the LSD test.

Genotype (G)	**FW** (g plant^{-1})	**DW** (g plant^{-1})	**EL** (%)
VA/A25	919	193.5 a	8.89 bc
VA/A31	1184	166.2 a	7.00 c
VA/A34	1235	185.8 a	8.16 bc
VA/A52	944	177.1 a	7.62 c
VA/A57	988	167.4 a	7.10 c

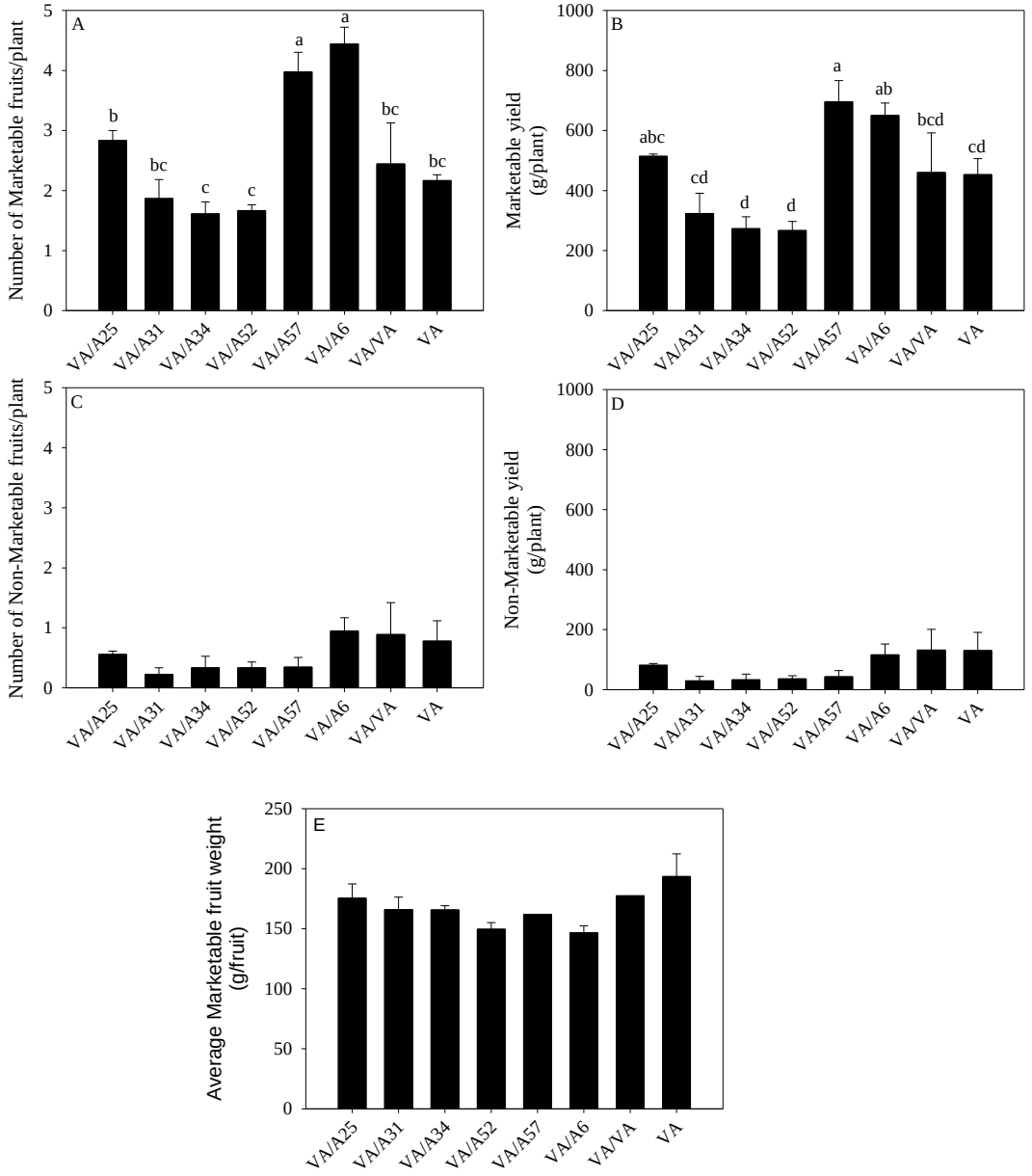
VA/A6	985	121.4	b	7.11	c
VA/VA	1390	178.0	a	10.66	ab
VA	1261	202.5	a	11.72	a

ANOVA (df)	% Sum of squares				
G (7)	64.07	80.88	*	50.89	**
Residuals	35.93	19.12		49.13	
Standard Dev. (+)	174	15.8		1.89	

* and ** indicate significant differences at $P \leq 0.05$ or $P \leq 0.01$ respectively, using the LSD test.
(+) Calculated as the square root of the residual sum of squares.
df degrees of freedom.

The marketable fruit number per plant of combinations VA/A57 and VA/A6 were higher than those of the cultivar (VA), the self-grafted cultivar (VA/VA) and the other combinations ($P \leq 0.01$; Fig. 5A). The MY of VA/A57 was significantly higher than those of VA and VA/VA (Fig. 5B). The MYs of VA/A25 and VA/A6 were also high, but not significantly different from that of VA/A57, but were also significantly different to those of VA and VA/VA.

No significant differences between the different grafted genotypes and the ungrafted cultivar were observed for the number of non-marketable fruits per plant, non-marketable yield and average marketable fruit weight ($P \leq 0.05$; Fig. 5C, D, E).



412

413 **Fig. 5.** Number of marketable fruits per plant (A), marketable yield (g/plant) (B), number
414 of non-marketable fruits per plant (C) and non-marketable yield (g/plant) (D) and average
415 marketable fruit weight (g/fruit) (E) in pepper genotypes under heat stress conditions.
416 Different letters indicate significant differences at $P \leq 0.05$ (LSD test). Data are the mean
417 of three replicates. Error bars represent standard error.

418 **4. Discussion**

It is well-known that heat stress causes deleterious effects on growth. Temperature levels above 35 ºC have been found to limit *Solanaceae* cultivation (Schwarz et al., 2010). These high temperatures as either heat shock or prolonged high temperatures dramatically affect growth, although studies of physiological responses to prolonged warming are rare (Wang et al., 2020).

In our experiments, we analysed the effect of high temperatures (38 ºC, 10 ºC above the control) for 7 days, which are usual conditions in the Mediterranean Region or hot climates in greenhouse crops (López-Marín et al., 2013) to simulate a heat wave. Our results showed both the negative effect of high temperatures on pepper plants and the possibility of tolerating heat stress using appropriate rootstocks.

One of the most important effects of high temperature on pepper crops from the economic-agronomy point of view is reduced growth and yields. The present study found that the highest RGR in pepper plant seedlings was observed in the genotypes grown under the control conditions, and the RGR was dependent on the accessions used as a rootstock. The pepper cultivar grafted onto accessions A25, A57 and A6 maintained plant growth, with no significant differences between heat stress and the control treatment. However, the cultivar grafted onto accessions A31, A34, A52, as well as the self-grafted cultivar and the cultivar itself, displayed reduced growth. These results suggest that RGR is dependent on the adaptability of roots to aerial temperature. Similar effects have been made by Rivero et al. (2003), who also observed that the dry weight of tomato plants grown at 35 ºC lowered more in non-grafted plants than grafted plants. Li et al. (2014b) observed better shoot growth in cucumber grafted onto tolerant luffa at 36 ºC than in ungrafted cucumber. However, in our experiments, the conservation into RGRs in VA/A25, VA/A57 and VA/A6 at high temperature was unable to preserve the leaf area. We observed reduced leaf areas in all the plant combinations under high temperature.

444 This result could indicate that these grafted plants increased leaf thickness that acted as
445 an adjustment strategy to acclimate at high temperature (Shu et al., 2016; Wahid et al.,
446 2007) and to sustain the RGR.

447 The reduction in the RGR by high temperature is related to physiological and
448 metabolic changes in plant cells. One of the most sensitive physiological alterations to
449 heat stress is increased membrane fluidity due to protein denaturation and increased levels
450 of unsaturated fatty acids with effects on plasma membrane stability (Hu et al., 2020b;
451 Wang et al., 2019). The amount of EL associated with membrane thermostability has been
452 successfully employed to evaluate heat stress in several crops (Nadeem et al., 2018),
453 including pepper (Wang et al., 2019), where heat-sensitive genotypes underwent greater
454 membrane injury than heat-tolerant ones (Li et al., 2015). Some studies found low EL in
455 tomato-grafted plants under heat stress conditions compared to ungrafted plants
456 (Abdelhafeez et al., 1975; Abdelmageed and Gruda, 2009). Our study at growth chambers
457 observed no significant differences in EL between the plants under heat stress and the
458 control in the VA/A25, VA/A57, VA/A6 and VA/A31 combinations. What is more, under
459 the stress conditions the EL values in VA/A25 and VA/A57 were lower than in the VA
460 and VA/VA ones. This agrees with the RGR values of VA/A25 and VA/A57. Similar
461 results were obtained in greenhouse conditions, where EL values were higher for VA and
462 VA/VA.

463 The diminished membrane stability under heat stress could lead to increased
464 thylakoid membrane fluidity (Prasad et al., 2008). Photosynthesis is one of the most
465 sensitive processes to abiotic stresses via alterations to the photosynthetic apparatus
466 (Zhou et al., 2015), and photosystem II (PSII) is regarded a sensitive and heat-labile
467 component (Čajánek et al., 1998; Mathur et al., 2011). In our experiments, Fv/Fm under
468 heat stress was lower than for the control conditions, which demonstrates that heat stress

affects Fv/Fm. This ratio lowered in combinations VA/A31, VA/A34, VA/A6, VA/VA and VA under heat stress compared to their controls. A downfall of Fv/Fm may be the result of a drop in the rate constant of PSII that leads to a rise in Fo, whereas an increase in non-radiative energy dissipation leads to Fo and Fm to lower (Guidi et al., 2019; Kitajima and Butler, 1975). Fo remained unchanged in all the treatments and plant combinations, which indicates that heat stress did not induce modifications either at the antenna pigment level or in the excitation trapping efficiency at the active centres of PSII (Calatayud and Barreno, 2001). Fm behaviour was more erratic and dependent on plant combinations. Fm showed significant differences between heat stress and the control in VA/A6 and VA/A57, of which the last one showed an increased Fm vs. its control which could be due to an enhanced electron transport rate as Fv/Fm was unmodified.

The Fv/Fm ratio has been used as an early indicator of heat stress (Poudyal et al., 2018; Tsai et al., 2019; Zhou et al., 2015) and it allowed screening plants to heat stress by validating a negative correlation between high Fv/Fm values and the heat injury index in fruit set or the RGR (Poudyal et al., 2018). These results could indicate that Fv/Fm under our heat stress conditions could not be used as an earlier indicator of heat stress because the drop in Fv/Fm observed in some plant combinations could be due to a non-photochemical increase (Calatayud and Barreno, 2001; Wang et al., 2020) and it was not possible to separate heat damage from dynamic photoinhibition to preserve PSII.

In order to determine which physiological index contributed most to RGR under heat stress in growth chambers the regression analysis was analysed. EL is the physiological index that most contributed to explain the RGR variation. In fact, the most sensitive component under heat stress is the plasma membrane and it is the primary sites of injury driving to increase in EL (Wise et al., 2004).

493 Protein biosynthesis motivates plant growth and development as an essential
494 biological process (Hu et al., 2020b; Li et al., 2018; Shalgi et al., 2013). HSPs have
495 evolved in plants as chaperon proteins to prevent protein denaturation and aggregation,
496 and represent an essential role played under heat stress (Barua et al., 2003; Hu et al.,
497 2020b). It is well established that plant exposure to heat shock increases HSP
498 accumulation, but very few studies have examined HSP accumulation over many
499 consecutive days (Wang et al., 2020), which often occurs during heat waves under natural
500 conditions. In this study, we analysed the relative expression of HSP70 and two small
501 HSPs, sHSP25.8 and sHSP22.7, in grafted and ungrafted pepper plants under the control
502 and high temperature for 7 days. The expression of the three HSPs was higher under heat
503 stress compared to the control conditions. These results indicate that high temperature up-
504 regulates the expression of HSPs, which agrees with Li et al. (2014b) for cucumber
505 grafted plants and Wang et al. (2020) for *Arabidopsis*. However, relative HSP expression
506 levels differ depending on HSPs and plants combinations. Our results showed that HSP70
507 expression increased significantly in relation to the control in all the plants, except for
508 VA/A34 and VA/A52. Higher HSP70 expression has been detected in leaves of cucumber
509 grafted onto luffa after 12 h, 36 h or 25 days (Li et al., 2016, 2014a, 2014b) at high
510 temperature, but not in roots (rootstock) as HSP70 was not present in the xylem (Li et al.
511 2014a). The leaves of our VA plant displayed the highest HSP70 expression. This
512 indicates that aerial parts induced HSP70 synthesis but it can't be discarded that stress
513 signal comes from root to shoot (Li et al., 2014a) that can modulate the response of HSP70
514 synthesis in leaves similarly to the grafted plants with a low HSP70 expression. However,
515 different heat temperature acclimation may induce distinct heat response pathways or
516 other HSP70 or HPSs can act in heat response after 7 days.

We observed differential expression behaviour between sHSPs and HSP70. The sHSPs expression levels were higher than in HSP70 and were up-regulated by heat stress in all the plants. The accumulation of sHSPs was greater in VA/A25, and also in VA/A31 and VA/A34, but VA/A6 only showed significant differences in sHSP25.8 comparing heat stress and control conditions. Unlike HSP70, no expression was observed in sHSPs in VA or VA/VA at high temperature, which indicates different plant combinations affect the expression of sHSPs that can be modulated by rootstocks with varying degrees of sHSPs synthesis. To date, as we are unaware of any research studies on sHSPs in pepper grafted plants, we herein present the first evidence that sHSPs were up-regulated by 7 day heat stress period.

In fact the accumulation of HSPs and sHSPs plays a key role in both the heat stress response and acquired thermo-tolerance in plants (Wang et al., 2020; Zhou et al., 2011). Nevertheless, the mechanism by which the protective effects of HSPs on plant cells can be achieved is attributed to the chaperone machinery network, in which different HSPs/chaperones act cooperatively in connection with other signal and metabolic processes (Wang et al., 2004). Furthermore, distinct heat response pathways for thermo-tolerance or thermo-acclimation can be induced depending on the applied heat regime. Nevertheless after the analysis of HSPs, as we were unable to distinguish if rootstocks' different degrees of tolerance correlated positively with fruit yield or the RGR, additional studies will be necessary to understand the complex network of HSPs.

Regarding the results obtained in experiment 2, the low yields were the result of both the severe heat stress suffered by plants and stressing temperatures, which reached 40 ºC and strongly affected the flowering and fruit set processes (Erickson and Markhart, 2002; Yamazaki and Hosokawa, 2019), as well as the employed short crop cycle. However, the obtained low yields confirmed the observations made in the controlled

experiment, where we observed that some rootstocks, A6, A25, and particularly A57, could better tolerate heat stress than the cultivar itself or the self-grafted cultivar according to the measured physiological parameters.

Apart from all these observations, it is interesting to point out that Penella et al. (2016) and López-Serrano et al. (2019) observed how the A25 accession used as a rootstock displayed higher photosynthesis and biomass than the ungrafted cultivar under saline or water stress. In the present study, we confirmed that the VA/A25 combination had higher RGR, leaf area and Fv/Fm values and lower EL than the ungrafted cultivar under the heat stress conditions. These results may indicate that, despite heat stress affecting aerial plant parts more, heat stress tolerance may be mediated by radical plant parts. Nevertheless, more studies are needed to confirm this hypothesis.

5. Conclusions

By way of conclusion, we observed how some genotypes can confer a certain degree of heat stress tolerance when used as rootstocks for a pepper cultivar by maintaining EL, which finally maintained the Fv/Fm ratio and resulted in a higher RGR and bigger fruit yields compared to the ungrafted cultivar. Accession A57 performed the best of all those we tested, and clearly its advantages are due to its genetic performance and not to a significant effect of the graft itself.

Author contribution statement

RG-M, SL-G and AC conceived and designed the experiments. RG-M. YGP, M-RM-C and AC performed the experiments. All authors have analyzed the data and discussed the study results. RG-M, SL-G and AC wrote the paper.

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Declaration of Competing Interest

The authors report no declarations of interest.

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