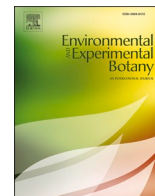


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Grafting improves tolerance to combined drought and heat stresses by modifying metabolism in citrus scion

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ABSTRACT

Mediterranean basin and other citrus-growing areas, such as Florida or California, are among the most vulnerable regions to the impacts of global warming. Therefore, citrus will be likely subjected to increasing periods of water scarcity combined with high temperatures that will impair plant growth, development and yield. In citrus industry, grafting is used to improve varietal characteristics, such as abiotic stress tolerance. Previous research showed that under drought and heat stress conditions, Carrizo citrange (*Poncirus trifoliata* × *Citrus sinensis*) is a better rootstock than Cleopatra mandarin (*Citrus reshni*) because it induces a higher antioxidant activity on the scion, reducing oxidative damage and increasing plant tolerance. Here, it is shown that metabolic reconfiguration, including changes in carbohydrate and amino acid fluxes, are key responses for plant acclimation to stress conditions. Moreover, the importance of the rootstock on scion metabolic and hormonal responses to drought and heat stress combination has been addressed by using reciprocal grafting between Carrizo and Cleopatra genotypes. Thus, Carrizo as a rootstock improves the metabolic and hormonal response of Cleopatra scions to the stress combination by inducing the accumulation of protective compounds such as raffinose, galactinol and salicylic acid. In turn, Cleopatra as a rootstock reduces levels of raffinose, galactinol, proline, phenylalanine and tryptophan in Carrizo scions, which impairs plant tolerance to the stress combination. Our findings show the effect of the rootstock on scion metabolic response to stress combination and remark the importance of the rootstock in citrus plants exposed to harsh environmental conditions.

1. Introduction

Agricultural production is highly conditioned by environmental fluctuations that affect plant growth and development. Water scarcity and high temperatures are among the most common adverse situations worldwide. Drought induces stomatal closure, reduces CO₂ intake and photosynthetic activity, increases oxidative stress and causes membrane damage (Ahuja et al., 2010; Vincent et al., 2020). Heat stress enhances transpiration and water consumption, alters cell membrane structure, causes protein denaturation and enzyme inactivation, damages photosystem reaction centers, reduces rubisco activity and increases oxidative damage (Wahid et al., 2007; Szymańska et al., 2017). In addition, these adverse situations often appear simultaneously in the crop fields and the occurrence of one can exacerbate the impact of the other on plants (Zhao et al., 2017; Zandalinas et al., 2018; Teuling, 2018). Therefore, the co-occurrence of two or more abiotic stress situations triggers a new

stress condition that usually is more damaging for the plant than the addition of the effects of the individual stress situations (Rizhsky et al., 2004; Pandey et al., 2015; Suzuki et al., 2016; Balfagón et al., 2019, 2020; Zandalinas et al., 2021). Specifically, drought and heat stress combination is detrimental for different crop species, causing important yield reductions (Fahad et al., 2017; Sehgal et al., 2017; Elferjani and Soolanayakanahally, 2018; Fábian et al., 2019).

Grafting is an ancient practice that provides many agronomical advantages to crops. The use of a proper rootstock can have important improvements for the scion such as reduction of the juvenility period, high yield, improved fruit quality, homogeneous plant architecture, protection against pests and diseases, and suitable tolerance to abiotic stress factors (Albacete et al., 2015; Han et al., 2019; Rasool et al., 2020). Therefore, the selection of an appropriate scion-rootstock combination is pivotal for successful agricultural production and, in particular, grafting is a widely used technique in citrus industry (Benjamin

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et al., 2013; Bowman and Joubert, 2020). Despite the importance of the rootstock-scion combination for citrus industry, there is an important lack of information on the communication between roots and shoots and on the modifications that the rootstock causes on scion physiology and metabolism. Santana-Vieira et al. (2016) demonstrated that the citrus rootstock Sunki Maravilha mandarin under drought stress was able to transfer its survival strategy to the scion by modulating the accumulation of hormones and antioxidant compounds. Another study showed that the citrus rootstock sour orange was able to transmit to the scion a metabolic response against spider mite attack. The increase in glutamate levels triggered higher accumulation of jasmonic acid in the scion and resulted in an enhanced resistance to the pest (Agut et al., 2016).

Carrizo citrange genotype presents a higher tolerance to drought and heat stress combination than Cleopatra mandarin and this is, in part, due to a higher antioxidant capacity, a better stomatal regulation that allows leaf cooling, and the accumulation of protective proteins (Zandalinas et al., 2016, 2017a; Balfagón et al., 2018). A previous study in Carrizo and Cleopatra plants under drought and heat stress combination demonstrated that the induction of a metabolic reprogramming in the plant (specially of the secondary metabolism), relied on plant ability to cope with oxidative stress (Zandalinas et al., 2017b). Furthermore, when used as a rootstock, Carrizo increases scion tolerance to drought and heat stress combination, whereas other rootstocks such as Cleopatra have the opposite effect. This is due, at least in part, to the ability of Carrizo to activate the antioxidant system of the scion, enhancing protection against oxidative stress and reducing cell damage (Balfagón et al., 2021). In the present work, we hypothesized that the influence of the rootstock on scion metabolic response to stress combination could determine plant tolerance to the adverse condition. Reciprocal grafting between Carrizo and Cleopatra revealed that the accumulation of key stress-responsive metabolites in the scion is strongly influenced by the rootstock, which can be crucial for the plant to cope with stress combination.

2. Materials and methods

2.1. Plant material

Reciprocal and self-grafted plants of Carrizo citrange (*Poncirus trifoliata* L. Raf. × *Citrus sinensis* L. Osb., CC) and Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan., CM) were acquired from an authorized commercial nursery (Beniplant S.L., Peníscola, Spain). Two-year-old seedlings of each citrus grafting combination ($\frac{CC}{CC}$, $\frac{CC}{CM}$, $\frac{CM}{CC}$ and $\frac{CM}{CM}$) were cultivated in plastic pots filled with perlite under greenhouse conditions, with natural photoperiod and day and night temperatures averaging 25.0 ± 3.0 °C and 18.0 ± 3.0 °C, respectively. Plants were watered three times a week with a half-strength Hoagland solution. After three months, plants were transferred to growth chambers and maintained for 2 weeks for acclimation to a 16-h photoperiod at 25 °C.

2.2. Stress treatments

Similar to Zandalinas et al. (2017a), four different groups of plants were established (Figure S1): well-watered plants at 25 °C (CT) or 40 °C (HS) and plants subjected to water stress at 25 °C (WS) or 40 °C (WS+HS). Water stress was imposed by transferring plants to dry perlite for 24 h and heat stress was applied for 6 days. Fully expanded leaves with an intermediate position in the canopy were harvested after 24 h. Harvesting was performed during daytime and illuminated leaves were immediately submerged in liquid N₂ to deter all metabolic activity. Frozen leaves were crushed and ground to fine powder and stored at – 80 °C for subsequent hormonal and metabolomic analyses.

2.3. Leaf damage and photosynthetic rate

Leaf damage was evaluated after stress treatments. Chlorotic or wilted leaves were considered as damaged leaves. Photosynthesis rate (A) was measured with a LCpro+ portable infrared gas analyzer (ADC BioScientific Ltd., Hoddesdon, UK) under ambient CO₂ and moisture. Supplemental light was provided by a photosynthetic active radiation (PAR) lamp at $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ and air flow was set at $150 \mu\text{mol mol}^{-1}$. After instrument stabilization, at least 10 measurements were taken on three leaves in three replicate plants from each stress treatment.

2.4. Determination of primary metabolites

The relative levels of polar metabolites were determined similarly as described in Zanol et al. (2009). Fifteen mg of crushed lyophilized leaf tissue were weighed. Polar metabolites were extracted in 1.4 mL of methanol and 60 μL of an aqueous solution with 0.2 mg mL^{-1} of ribitol, which was used as internal standard. Extraction was performed at 70 °C for 15 min in a water bath. The extract was centrifuged at 14,000 rpm for 10 min, and the supernatant was recovered and fractionated adding 750 μL of chloroform and 1.5 mL of Milli-Q water. After vigorous vortexing for 15 s and 15 min centrifugation at 4000 rpm, 50 μL of the aqueous phase were recovered and vacuum-drying. The dry residue was derivatized by the addition of 40 μL of a 20 mg mL^{-1} methoxyamine hydrochloride solution in pyridine, (Sigma) and incubation at 37 °C during 2 h with 950 rpm agitation in a Thermomixer (Eppendorf). Then, 70 μL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) (Macherey-Nagel, Düren, Germany) were added, and vials further incubated for 30 min in the same conditions of temperature and agitation. Fatty acid methyl esters (C₈–C₂₄) were added and used as retention index (RI) markers. Analyses were performed on a 6890 N gas chromatograph (Agilent Technologies, USA) coupled to a Pegasus 4D TOF mass spectrometer (LECO, St. Joseph, MI). One μL of the derivatized extract was injected with a split ratio 1:10. Chromatography was performed with a BPX35 (30 m, 0.32 mm, 0.25 μm) capillary column (SGE Analytical Science Pty Ltd., Australia) with a 2 mL min^{-1} helium flow. Oven programming conditions were as follows: 2 min of isothermal heating at 85 °C, followed by an $8 \text{ }^\circ\text{C min}^{-1}$ temperature ramp up to 360 °C. Injection port and ion source temperatures were set at 250 °C. Data were acquired after EI ionization at 70 eV, and recorded in the 70–800 *m/z* range at 20 scans s^{–1}. Chromatograms were analyzed by means of the ChromaTOF software. Metabolites were identified by comparison of both mass spectra and retention time with those of pure standards injected under the same conditions. Peak area of each identified compound was normalized to internal standard area (ribitol) and sample dry weight. Four independent samples per treatment were analyzed.

2.5. Determination of hormonal content

Hormones analyzed were abscisic acid (ABA), phaseic acid (PA), salicylic acid (SA), indoleacetic acid (IAA), jasmonic acid (JA), JA-Issoleucine (JA-Ile) and 12-oxo-phytodienoic acid (OPDA). Extraction and analysis were carried out as described in Durgbanshi et al. (2005) with few modifications. Briefly, 0.1 g of dry tissue was extracted in 2 mL of ultrapure water after spiking with 50 ng of [²H₆]-ABA, [²H₃]-PA, [¹³C]-SA, [²H₂]-IAA and dihydrojasmonic acid in a ball mill (MillMix20, Domel, Železniki, Slovenija). After centrifugation at 4000 g at 4 °C for 10 min, supernatants were recovered and pH was adjusted to 3 with 30% acetic acid. The aqueous extract was partitioned twice against 2 mL of diethyl ether and the organic layer was recovered and vacuum-dried. The residue was resuspended in a 10:90 MeOH:H₂O solution by gentle sonication. The resulting solution was filtered through 0.22 μm polytetrafluoroethylene membrane syringe filters (Albet S.A., Barcelona, Spain) and directly injected into an ultra performance LC system

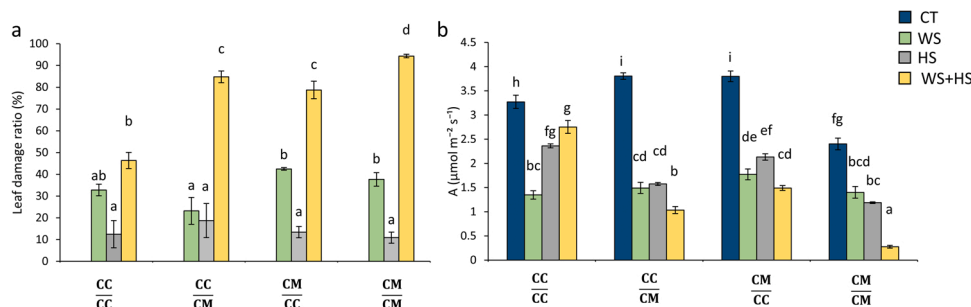


Fig. 1. Leaf damage index (a) and photosynthetic rate (b) in Carrizo and Cleopatra plants self-grafted and grafted into each other subjected to control conditions (CT), drought (WS), heat stress (HS) and drought and heat stress combination (WS+HS). Data are mean values \pm standard errors. Different letters show statistical significance at $p \leq 0.05$.

(Acquity SDS, Waters Corp., Milford, MA, USA). Chromatographic separations were carried out on a reversed-phase C18 column (Gravity, 50×2.1 mm, $1.8\text{-}\mu\text{m}$ particle size, Macherey-Nagel GmbH, Germany) using a MeOH:H₂O (both supplemented with 0.1% acetic acid) gradient at a flow rate of $300 \mu\text{L min}^{-1}$. Hormones were quantified with a TQS triple quadrupole mass spectrometer (Micromass, Manchester, UK) connected online to the output of the column through an orthogonal Z-spray electrospray ion source. Peak area of ABA was normalized to internal standard [²H₆]-ABA, PA to [²H₃]-PA, SA to [¹³C]-SA, IAA to [²H₂]-IAA and JA, JA-Ile and OPDA to dihydrojasmonic acid and sample dry weight. Results were processed using Masslynx v4.1 software. Analyses were repeated three times.

2.6. Statistical analysis

Results are presented as the mean \pm SD. Statistical analysis were performed by two-way ANOVA followed by a Tukey post hoc test when a significant difference was detected (different letters denote statistical significance at $p \leq 0.05$). Principal component analysis (PCA) was performed by means of the SIMCA 13.0.3 software (Umetrics), using the log₂ transformed data and unit variance normalization.

3. Results

3.1. Citrus rootstock alters the tolerance of the scion to individual and combined drought and heat stress

Leaf damage on Carrizo and Cleopatra plants (self-grafted) and reciprocal grafts of these genotypes was analyzed under water deficit, high temperatures and combination of both stress factors (Fig. 1A). WS caused similar types of injuries in Carrizo and Cleopatra leaves, although $\frac{CC}{CM}$ plants (Carrizo scion grafted onto Cleopatra rootstock) were less affected than $\frac{CM}{CC}$ and $\frac{CM}{CM}$. HS caused slight damage in plants in a uniform way (10–18% without significant differences among plant groups). Finally, WS+HS was very harmful for both citrus genotypes, but important differences were observed among grafting groups: whereas the percentage of damaged leaves in $\frac{CC}{CC}$ was 46.3%, in $\frac{CM}{CM}$ plants it was 94.3%, which accounts for severely damaged plants. The percentage of damaged leaves in reciprocal grafts, $\frac{CC}{CM}$ and $\frac{CM}{CC}$, showed intermediate values (84.7% and 78.7%, respectively).

Photosynthetic rate (A) was negatively affected by individual and combined stress conditions (Fig. 1b). Interestingly, plants grafted onto Cleopatra rootstock were more affected by heat stress than those grafted onto Carrizo, while drought reduced A similarly in all plant groups. The combination of drought and heat stress negatively affected A of both genotypes in a rootstock-dependent manner: in Carrizo scions under stress combination, A decreased to a greater extent in plants with Cleopatra rootstock ($\frac{CC}{CM}$) than in self-grafted ones ($\frac{CC}{CC}$). On the opposite way, A was severely reduced in $\frac{CM}{CM}$ plants, whereas the reduction was

lower in $\frac{CM}{CC}$.

3.2. Metabolic and hormonal profiles are modified by the rootstock and the stress conditions

To study the rootstock influence on citrus primary metabolism and how WS, HS and WS+HS modify stress-associated metabolite levels, analysis of polar compounds together with hormonal quantification were performed. Principal Component Analyses (PCA) revealed that the metabolic profile of the samples was differentiated by the stress conditions and the grafting combinations (Fig. 2). The principal component 1, accounting for a 27.4% of the total variability, grouped the samples by stress treatments. Stress combination was the most differentiated group from control ones, although both individual stresses were not differentiated by this component. In turn, PC2, explaining 15.2% of the variation, separated the samples based on the metabolic and hormonal profile of plants subjected to WS or HS. Therefore, taking into account PC1 and PC2, a 42.6% of the variation of the samples is based on the stress situation to which plants were subjected, regardless the genotypes of scion and rootstock. However, PC3 differentiated samples with the same scion genotype but different rootstock, while PC4 separated scion genotypes.

Hormonal leaf content was also affected by the stress conditions and the scion and rootstock genotypes. Under drought stress, ABA and PA levels increased in leaves of all plant groups whereas under drought and heat stress combination only $\frac{CC}{CM}$ plants showed an increase in leaf levels of these hormones. In Carrizo leaves, JA content increased under drought stress whereas in Cleopatra ones, this increase was detected only under stress combination. Leaf IAA levels increased in plants with Cleopatra as a rootstock ($\frac{CC}{CM}$ and $\frac{CM}{CM}$) under stress combination and decreased in leaves of plants with Carrizo as a rootstock ($\frac{CC}{CC}$ and $\frac{CM}{CC}$) (Table S1).

3.3. Rootstock affects scion sugar metabolism and accumulation of protective galactinol and raffinose under drought and heat stress conditions

In general, drought, heat and their combination significantly affected the accumulation of sugars such as sucrose, fructose, maltose, glucose, raffinose, galactinol and the polyalcohol myo-inositol (Fig. 3 and Supplementary Table 2). The variation of leaf sugar contents under stress was also affected by the rootstocks. All stress conditions caused a strong accumulation of leaf glucose in $\frac{CC}{CC}$ plants and in a lower extent in $\frac{CM}{CC}$ plants, whereas in plants that carried Cleopatra as a rootstock ($\frac{CC}{CM}$ and $\frac{CM}{CM}$), levels remained similar to those observed in plants subjected to control conditions or even decreased. Leaf fructose concentration also increased in $\frac{CC}{CC}$ plants under all stress conditions respect to control, whereas in the rest of plant groups its levels under stress combination were similar or lower than in control conditions. In general, leaf sucrose

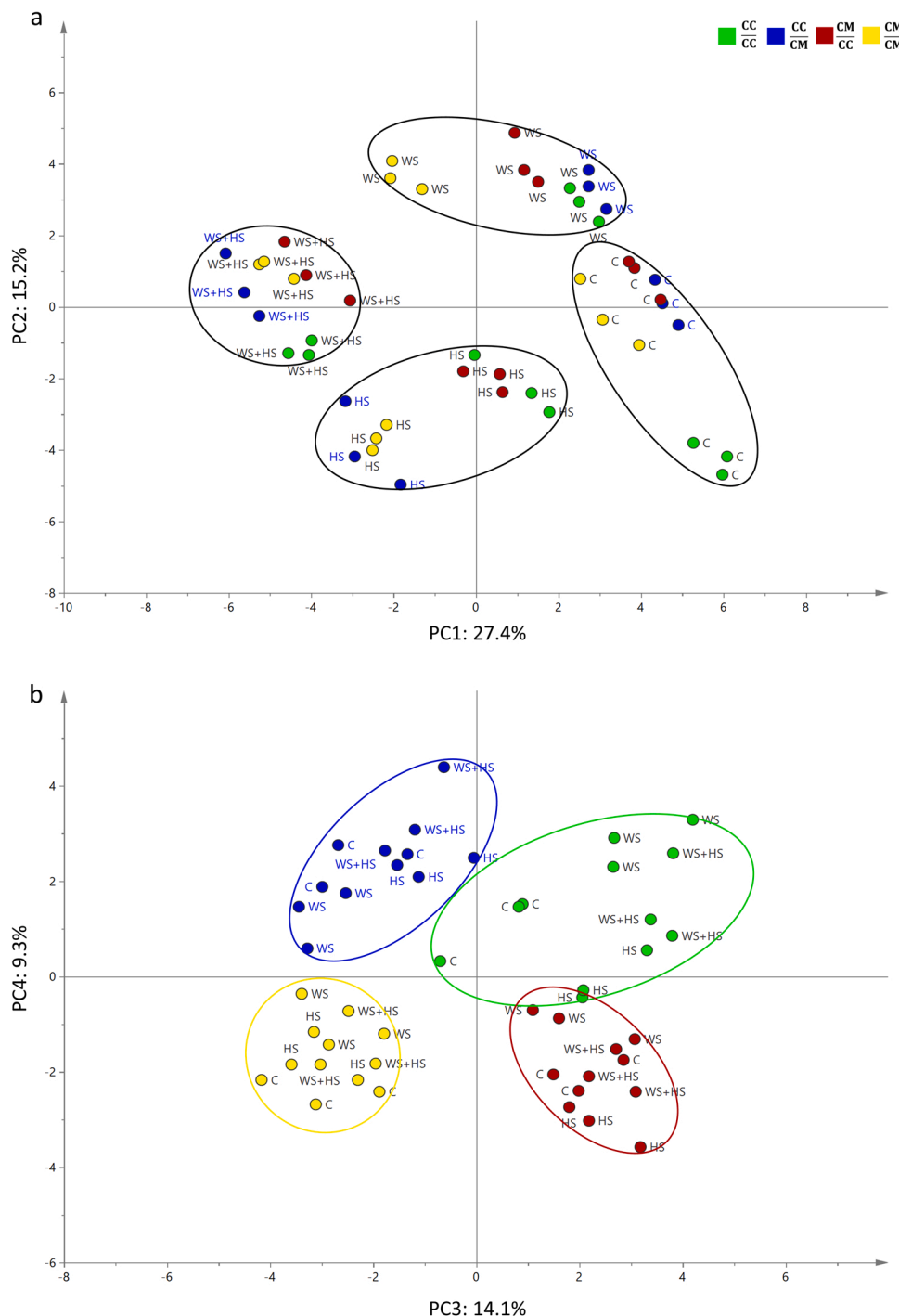


Fig. 2. Principal Component Analysis (PCA) score plot of metabolite and hormonal profiles obtained from Carrizo and Cleopatra plants self-grafted and grafted into each other subjected to control conditions (CT), drought (WS), heat stress (HS) and drought and heat stress combination (WS+HS).

levels increased under heat stress and stress combination in all plant groups. As an exception, in $\frac{CC}{CC}$ plants under water stress, leaf sucrose levels decreased importantly. Stress combination increased leaf maltose contents in all groups of plants, especially in $\frac{CC}{CC}$ plants. Raffinose and galactinol levels increased under individual stress conditions but these metabolites were particularly accumulated under stress combination in leaves of all plant groups. Despite these high increases, there were significant differences among plant groups. Contents of raffinose and galactinol were higher under combined stress conditions in leaves of plants with Carrizo as a rootstock (especially in Carrizo self-grafted plants), than in leaves of plants with Cleopatra as a rootstock (Fig. 3).

3.4. Differential accumulation of glutamate-derived metabolites was driven by the grafting combination

Glutamate metabolism diverts in numerous synthesis pathways of important compounds for stress responses. Here, we analyzed the accumulation of glutamate and its derived compounds putrescine, proline, 4-hydroxyproline, pyroglutamic acid and GABA in Carrizo and Cleopatra self- and reciprocal-grafts under single and combined stress conditions (Fig. 4 and Supplementary Table 2). Leaf glutamate content changed after the imposition of the different stress conditions. Drought stress induced a depletion of this metabolite concentration in Carrizo

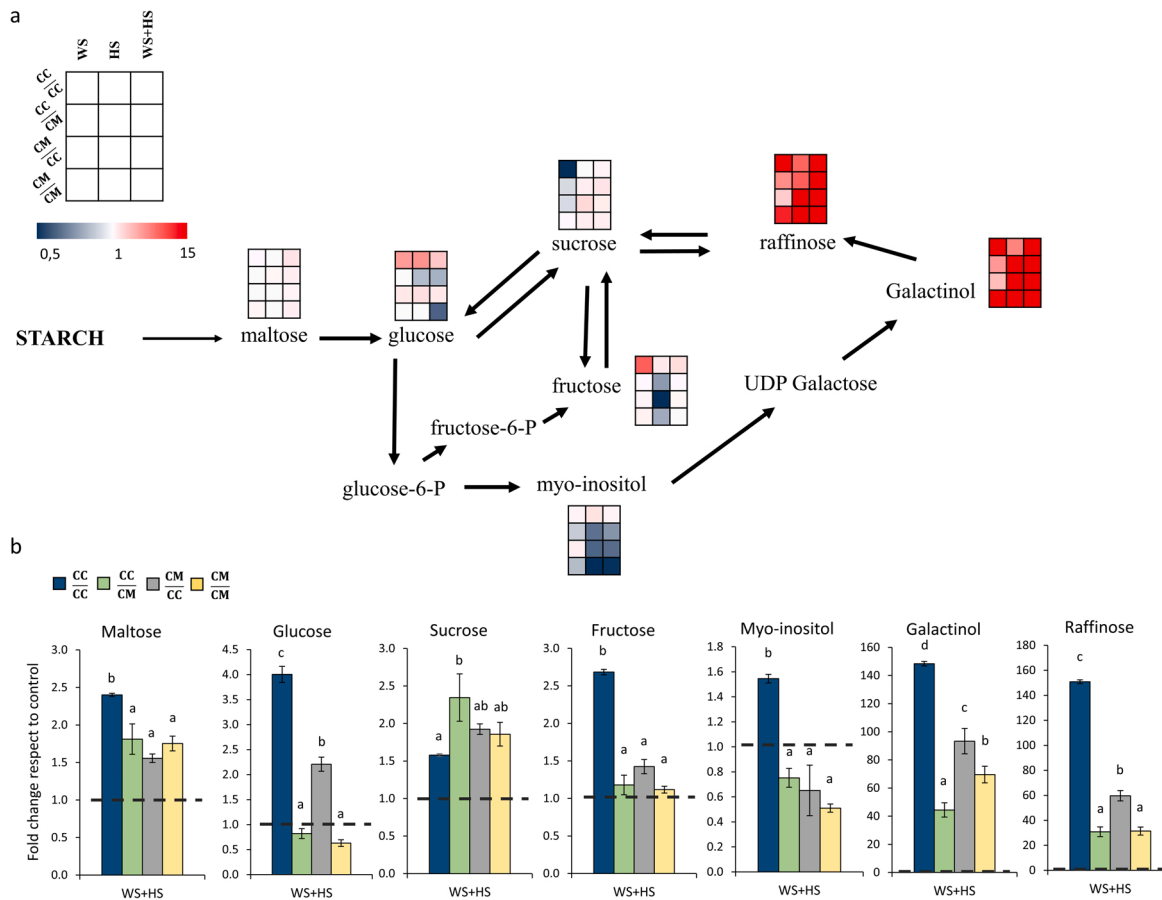


Fig. 3. Sugar levels (expressed as a color scale) in Carrizo and Cleopatra plants self-grafted and grafted into each other subjected to drought (WS), heat stress (HS) and drought and heat stress combination (WS+HS) (a). Sugar levels (expressed as fold change respect to control conditions) in Carrizo and Cleopatra plants self-grafted and grafted into each other subjected to drought and heat stress combination (WS+HS) (b). Dashed lines in the bar graphs indicate levels of each sugar in control plants. Data are mean values \pm standard errors. Different letters show statistical significance at $p \leq 0.05$.

leaves and a slight increase in Cleopatra ones. Under heat stress, leaves from all plant types showed accumulation of glutamate except those from self-grafted Carrizo plants which levels remained as control ones. Finally, under stress combination only $\frac{CM}{CM}$ plants showed a significant increase in glutamate leaf levels respect to control.

Under drought and heat stress combination, the pattern of accumulation for glutamate derived compounds differed between Carrizo and Cleopatra plants. $\frac{CC}{CC}$ plants strongly accumulated proline and 4-hydroxyproline in their leaves, whereas this response was not observed in $\frac{CC}{CM}$, $\frac{CM}{CC}$ and $\frac{CM}{CM}$ plants. Leaf levels of putrescine, pyroglutamic acid and GABA were lower in Carrizo self-grafted plants than in Cleopatra ones, showing that glutamate metabolism is directed to these metabolites in Cleopatra while it seems to be directed to proline in Carrizo. Levels of leaf putrescine in the reciprocal grafts were also higher than in Carrizo self-grafted plants, especially in $\frac{CM}{CC}$. Levels of leaf pyroglutamic acid increased significantly in $\frac{CM}{CM}$ plants after stress combination, whereas in $\frac{CC}{CC}$ remained as in control conditions. In the reciprocal grafts, $\frac{CC}{CM}$ and $\frac{CM}{CC}$, pyroglutamic acid accumulation in leaves was similar between them and lower than in $\frac{CM}{CM}$ plants. Finally, leaf GABA content increased significantly in $\frac{CC}{CC}$, $\frac{CM}{CC}$ and $\frac{CM}{CM}$ plants under stress combination although levels of this metabolite were significantly higher in $\frac{CM}{CM}$ plants than in the other groups.

3.5. Rootstock modified TCA cycle dynamics in Cleopatra leaves under drought and heat stress combination

Levels of metabolites α -ketoglutarate, succinate, fumarate, malate and the oxaloacetate derived metabolite, aspartate, were analyzed in leaves of Carrizo and Cleopatra self- and reciprocally grafted plants under the different stress conditions (Fig. 5). Under water stress condition, levels of these compounds either did not change or decreased with respect to controls in $\frac{CC}{CC}$, $\frac{CC}{CM}$ and $\frac{CM}{CC}$ plants, and slightly increased in $\frac{CM}{CM}$. Under heat stress, the accumulation pattern changed for each graft combination. In $\frac{CC}{CC}$ plants, there was only an increase of leaf α -ketoglutarate levels. In $\frac{CC}{CM}$, decreases in levels of fumarate, malate and aspartate respect to control were observed. Strong concentration reductions were observed for α -ketoglutarate, fumarate and malate in $\frac{CC}{CC}$ and $\frac{CM}{CM}$ plants under heat stress, whereas they accumulated succinate. Leaf aspartate levels increased in $\frac{CM}{CM}$ plants and decreased in $\frac{CC}{CC}$ ones. Finally, in $\frac{CC}{CC}$ and $\frac{CM}{CM}$ plants under drought and heat stress combination, increases in levels of foliar α -ketoglutarate and succinate, and decreases in the concentrations of fumarate and malate were observed. The content of leaf aspartate also decreased respect to control in $\frac{CC}{CC}$ plants but did not change in $\frac{CM}{CM}$. The accumulation pattern of these metabolites in Cleopatra leaves under stress combination was highly influenced by the rootstock. In Cleopatra self-grafted plants, the foliar content of α -ketoglutarate and succinate did not change with respect to control conditions. However, there was a strong accumulation of fumarate, malate and aspartate in leaves of these plants. On the contrary, in leaves of Cleopatra grafted onto Carrizo rootstock ($\frac{CM}{CC}$), levels of all metabolites

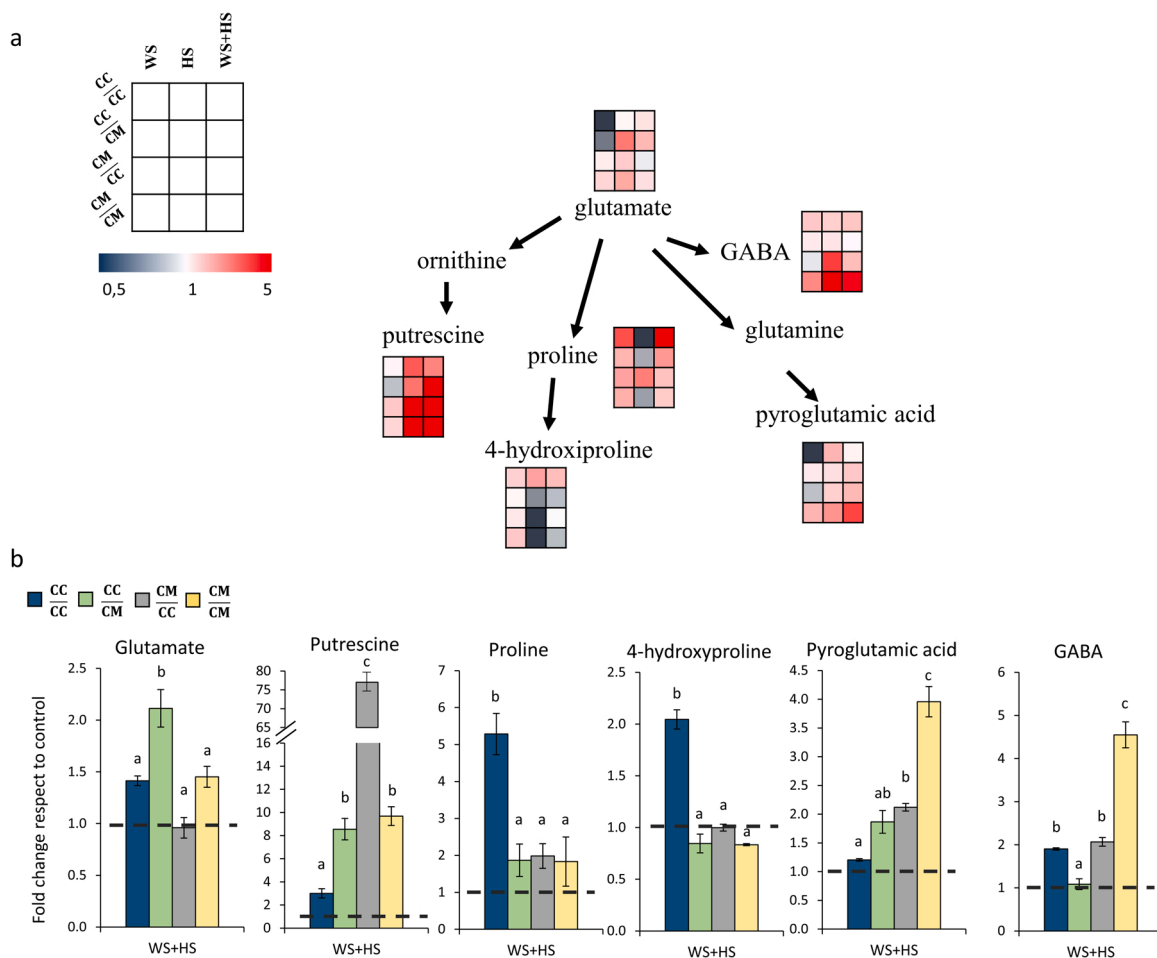


Fig. 4. Levels of glutamate and glutamate-derived compounds (expressed as a color scale) in Carrizo and Cleopatra plants self-grafted and grafted into each other subjected to drought (WS), heat stress (HS) and drought and heat stress combination (WS+HS) (a). Levels of glutamate and glutamate-derived compounds (expressed as fold change respect to control conditions) in Carrizo and Cleopatra plants self-grafted and grafted into each other subjected to drought and heat stress combination (WS+HS) (b). Dashed lines in the bar graphs indicate levels of each metabolite in control plants. Data are mean values \pm standard errors. Different letters show statistical significance at $p \leq 0.05$.

were highly diminished.

3.6. Shikimate derived metabolism in the scion is highly influenced by the rootstock

The shikimate pathway is the beginning of some of the most important secondary metabolism pathways. Fig. 6 shows relative levels of the shikimate derived compounds phenylalanine, salicylic acid (SA), tryptophan and tyramine under the different stress conditions. Phenylalanine was accumulated in Carrizo and Cleopatra leaves after the imposition of single and combined stress conditions, except for $\frac{CC}{CM}$ plants under heat stress and $\frac{CM}{CC}$ under drought stress. Under stress combination, the accumulation of phenylalanine in leaves of plants with Carrizo as a rootstock ($\frac{CC}{CC}$ and $\frac{CC}{CM}$) was higher than in those with Cleopatra as a rootstock ($\frac{CC}{CM}$ and $\frac{CM}{CM}$). The content of salicylic acid (SA), a hormone derived from phenylalanine, followed a similar pattern and increased under stress combination in leaves of plants with Carrizo as a rootstock, whereas it decreased in leaves of plants with Cleopatra as a rootstock. Similarly, the concentration of the amino acid tryptophan increased after single or combined stress conditions in leaves from all plant groups, except for $\frac{CM}{CC}$ and $\frac{CM}{CM}$ plants under heat stress that had diminished and similar levels than control, respectively. However, under heat stress, tryptophan foliar levels in plants with Carrizo as a rootstock were significantly higher than those of plants with Cleopatra as a rootstock.

Finally, tyramine (derived from shikimate through previous conversion to tyrosine), was accumulated in leaves of $\frac{CC}{CC}$ plants under heat stress, applied individually or combined with drought, and in leaves of $\frac{CC}{CM}$ plants under stress combination. Tyramine levels decreased respect to control in all stress conditions in Cleopatra leaves independently of the rootstock.

4. Discussion

In this study, leaves of plants of Carrizo citrange and Cleopatra mandarin subjected to individual or combined stress conditions showed differential and specific metabolic and hormonal profiles for each stress situation (Fig. 2). Moreover, drought and heat stress combination affected plants uniquely, in a way that could not be predicted from the study of individual stresses. Metabolic and hormonal profile changes of plants under combined conditions of drought and heat were more dramatic than those from plants under individual stress conditions (Fig. 2a). In addition, plant response to stress combination was neither an enhanced response to one of the individual stresses nor a combination of the responses to individual stresses. Therefore, drought and heat stress combination affected Carrizo and Cleopatra plants inducing specific metabolic changes to adapt to the stress conditions applied. This is in agreement with results obtained from other plant systems when studying the effect of stress combination on plant metabolome, transcriptome, proteome and physiology (Rizhsky et al., 2002; Pandey et al., 2015;

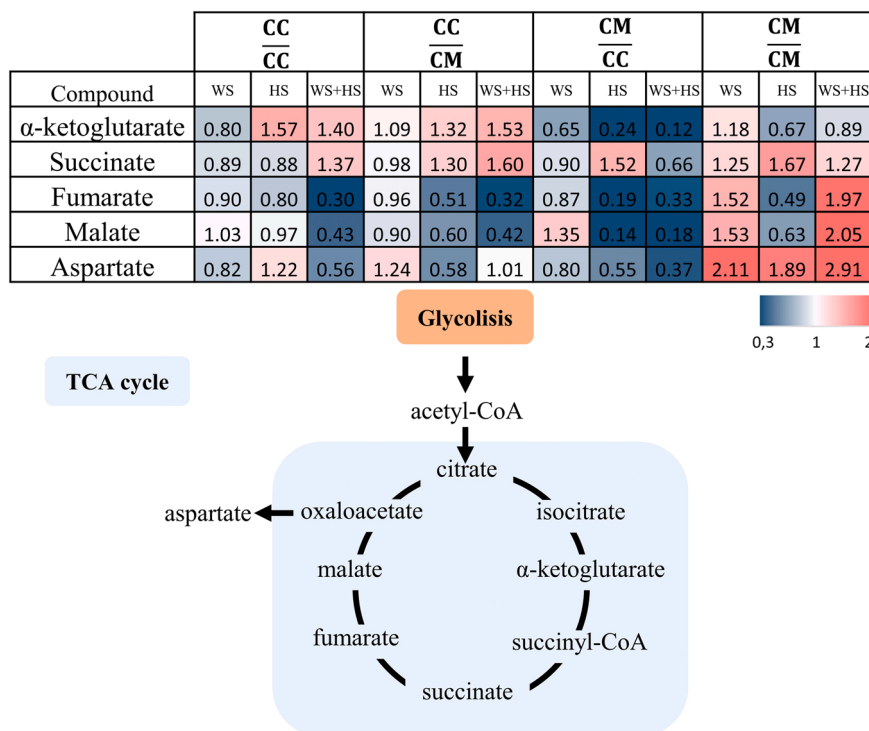


Fig. 5. Levels of metabolites in the TCA cycle in Carrizo and Cleopatra plants self-grafted and grafted into each other subjected to drought (WS), heat stress (HS) and drought and heat stress combination (WS+HS) expressed as fold change respect to control conditions and shown as a color scale.

Zandalinas et al., 2016, 2017b; Zhao et al., 2016; Balfagón et al., 2019; Lopez-Delacalle et al., 2021). However, not only the stress treatment conditioned the metabolic and hormonal profile of scion leaves. As it is showed in Fig. 2b, the scion genotype and, interestingly, the rootstock genotype employed, considerably affected the metabolism of plants under the different stress conditions. PCA showed that the rootstock significantly modified the metabolism of scion leaves under both control and stressing conditions. Similar results were observed in other plants (Tietel et al., 2020; Tedesco et al., 2021). In grapevine, the metabolic analyses of homografts and heterografts of five different genotypes determined that the use of a rootstock changed the metabolism of the phloem, leaves and stems of the scion (Tedesco et al., 2021). A metabolic analysis using grafted citrus found that sap of both parts of the plant and the composition of fruit juice changed either by the effect of the rootstock or by the interaction between the rootstock and the scion (Tietel et al., 2020).

The important differences in leaf damage and photosynthetic rate among the different plant groups ($\frac{CC}{CC}$, $\frac{CC}{CM}$, $\frac{CM}{CC}$ and $\frac{CM}{CM}$) caused by drought and heat stress combination are, in part, due to the different accumulation of metabolites in leaves. The higher tolerance of $\frac{CC}{CC}$ plants to stress combination respect to the rest of grafted plants correlated with higher leaf levels of sugars: maltose, glucose, fructose, and myo-inositol. In addition, galactinol and raffinose levels, that were highly increased under stress combination in leaves of all plant groups, were also significantly higher in $\frac{CC}{CC}$. Galactinol and raffinose levels increase under extreme environmental conditions and are considered as beneficial compounds in plants facing different kinds of abiotic stresses like heat, high light, cold, drought and oxidative stress (Taji et al., 2002; Panikulangara et al., 2004; Nishizawa et al., 2008). Under heat stress, galactinol and raffinose levels increase in vegetative tissues in a Heat Shock Factor 3 dependent manner (Panikulangara et al., 2004). Taji et al. (2002), demonstrated that the induction of galactinol synthase by stress led to a high accumulation of galactinol and raffinose, that functioned as osmoprotectants in drought- or cold-stressed plants. Also, a study with Arabidopsis mutants overexpressing the enzyme galactinol

synthase showed that its abnormally high accumulation of galactinol and raffinose conferred tolerance to different environmental stress due to the antioxidant and osmoprotectant capacity of these compounds (Nishizawa et al., 2008). Recently, it has been shown that in citrus grafted plants, rootstock influence in the antioxidant system of the scion is crucial for plant tolerance to stress combination (Balfagón et al., 2021). In this work, the reduction of A under stress combination in $\frac{CC}{CC}$ plants was much lower than in the rest of groups, and this ability to maintain relative higher photosynthetic rate could be a reason for the increased sugar content. Also, the higher levels of maltose could indicate that $\frac{CC}{CC}$ plants were using starch reserves to produce energy and metabolites needed under the stress combination. Nevertheless, the ability of $\frac{CC}{CC}$ plants to maintain or increase levels of sugars, precursors of galactinol and raffinose, may be a key trait that explains the higher tolerance of Carrizo self-grafted plants to drought and heat stress combination. Furthermore, galactinol and raffinose overaccumulation in response to stress combination was a rootstock dependent response. Leaves of Cleopatra plants grafted onto Carrizo had levels of both compounds higher than those in self-grafted plants. In contrast, leaves of Carrizo grafted onto Cleopatra plants accumulated considerably less galactinol and raffinose than Carrizo self-grafted plants. Both compounds have been described as protective metabolites (Nishizawa et al., 2008; Wulff-Zottele et al., 2010; Lauxmann et al., 2014). Differences in the content of these two protective metabolites may partially explain the higher tolerance to drought and heat stress combination of $\frac{CM}{CC}$ respect to $\frac{CM}{CM}$ plants and the lower tolerance of $\frac{CC}{CM}$ plants respect to $\frac{CC}{CC}$.

Glutamate has a central role in amino acid metabolism in plants, and it is the precursor for the synthesis of polyamines, GABA, proline, glutamine and pyroglutamic acid (Forde and Lea, 2007). Glutamate metabolism in Carrizo and Cleopatra plants seems to be conditioned by the genotype of both the scion and the rootstock. Profiles of glutamate-derived metabolites were different in leaves of Carrizo and Cleopatra self-grafted plants under drought and heat stress combination and, interestingly, metabolite levels were modified in each genotype when the rootstock changed. Glutamate metabolism in Carrizo under

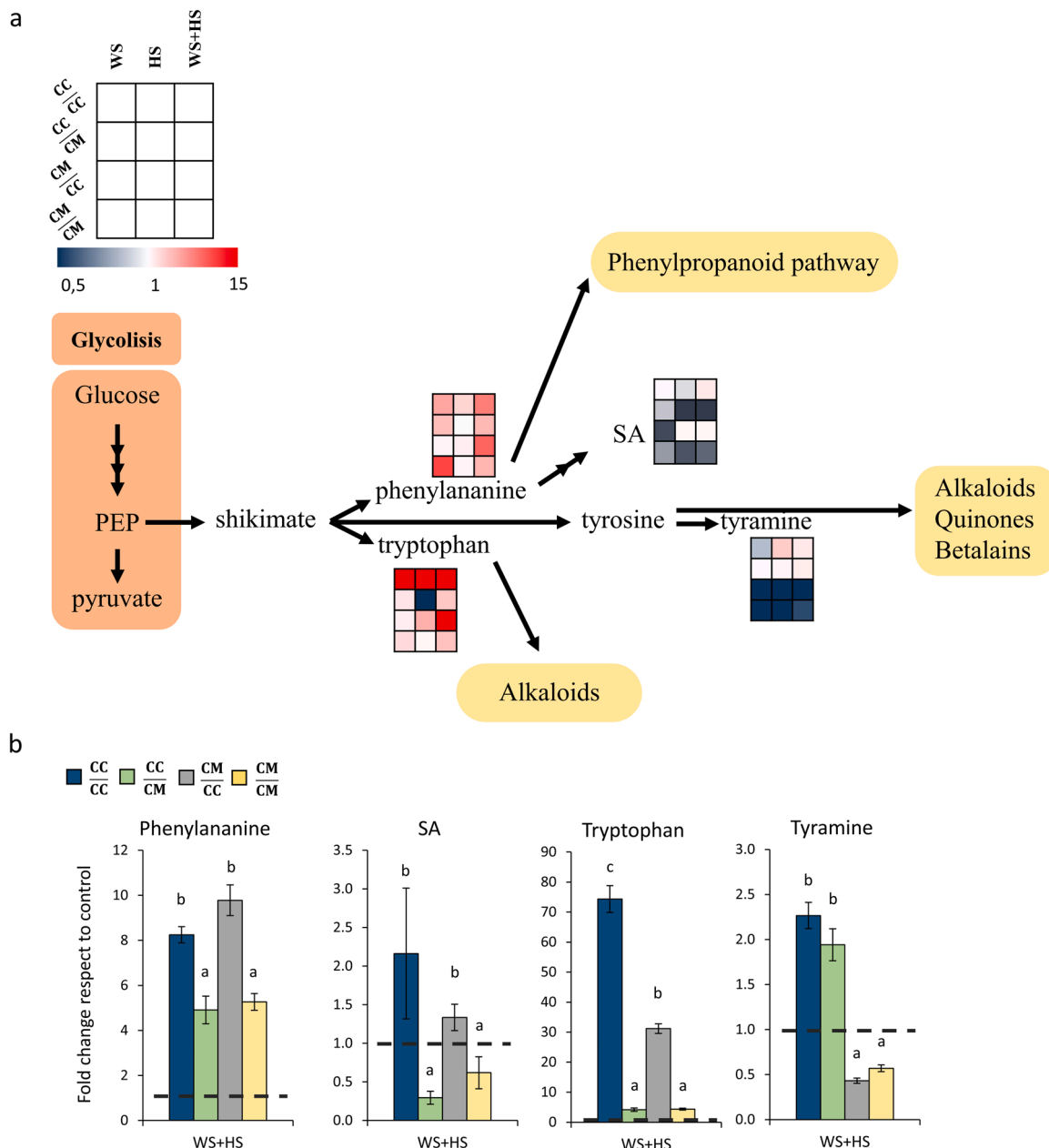


Fig. 6. Levels of phenylalanine, tryptophan, tyramine and salicylic acid (expressed as a color scale) in Carrizo and Cleopatra plants self-grafted and grafted into each other subjected to drought (WS), heat stress (HS) and drought and heat stress combination (WS+HS) (a). Levels of phenylalanine, tryptophan, tyramine and salicylic acid (expressed as fold change respect to control conditions) in Carrizo and Cleopatra plants self-grafted and grafted into each other subjected to drought and heat stress combination (WS+HS) (b). Dashed lines in the bar graphs indicate levels of each metabolite in control plants. Data are mean values \pm standard errors. Different letters show statistical significance at $p \leq 0.05$.

stress combination was directed to the production of proline, whereas in Cleopatra accumulations of putrescine, pyroglutamic acid and GABA were prioritized. The high levels of foliar proline in $\frac{CC}{CC}$ plants can be an additional factor that sum to achieve a higher ability for these plants to tolerate the adverse conditions. Proline is considered as a protective compound under diverse abiotic stress conditions. It has been shown to function as a chaperone to protect protein integrity and enhance enzymatic activity in the chloroplast and the cytosol, as an osmoprotectant, as a ROS scavenger and as a maintainer of cell redox balance (Szabados and Savouré, 2010; Krasensky and Jonak, 2012). Cleopatra as rootstock may induce a negative effect on the graft by preventing the accumulation of proline in response to combined stress, as it is observed in $\frac{CC}{CM}$ plants. Finally, although putrescine, pyroglutamic acid and GABA are considered beneficial compounds that can enhance tolerance to abiotic

stresses (Alcázar et al., 2010; Shelp et al., 2012; Shaar-Moshe et al., 2019; Jiménez-Arias et al., 2019; Fromm, 2021), proline role in citrus may be more important under drought and heat stress combination since $\frac{CC}{CC}$ plants were more tolerant than the rest.

The stress pressure caused by combined drought and high temperatures in $\frac{CM}{CM}$ plants led to a strong reduction of the photosynthetic rate. Thus, accumulation of sugars in leaves from these plants was lower than in $\frac{CC}{CC}$ ones. Particularly, glucose concentration was much lower under stress combination than under control conditions, which could be conditioning the glycolysis pathway and, therefore, limiting energy generation. During long periods of darkness, leaf senescence or stress situations, where photosynthesis and glycolysis are disturbed and carbon supply is limited, protein degradation and amino acid catabolism

may provide alternative energy sources for the plant. Amino acid carbon skeletons are generally converted into TCA cycle intermediates, which provide carbon for mitochondrial electron transport chain and produce ATP. In addition, the oxidation of some amino acids (both branched-chain and aromatic) can provide electrons directly to the electron transport chain (Obata et al., 2011; Araújo et al., 2011; Hildebrandt et al., 2015). Therefore, by means of these two mechanisms, amino acid catabolism renders comparable amounts of energy for ATP synthesis to those provided by glucose (Hildebrandt et al., 2015). $\frac{CC}{CM}$ plants may be using this alternative pathway to obtain energy, due to the low levels of glucose and the almost null photosynthesis rate. Therefore, the protein breakdown and the use of amino acid as carbon source may be increasing the levels of intermediates of the TCA cycle such as fumarate and malate. Foliar levels of leucine, isoleucine, proline and alanine (some of the amino acid that more energy can supply; Hildebrandt et al., 2015) under stress combination were lower in $\frac{CC}{CM}$ plants than in $\frac{CC}{CC}$ ones, what could indicate an enhanced amino acid catabolism. However, levels of other amino acids that also provide high-energy yields, such as lysine or valine, were similar or higher in leaves of these plants regarding the other groups (Supplementary Table 2). Further research addressing amino acid consumption in citrus leaves under drought and heat stress combination as an alternative carbon supply would be needed.

Shikimate is the precursor of the aromatic amino acids tyrosine, phenylalanine and tryptophan. All these three amino acids are produced from chorismate, the final product of the shikimate pathway, and they constitute the beginning of numerous routes of the secondary metabolism. While tryptophan is a precursor of alkaloids, tyrosine diverts in isoquinoline alkaloids, betalains, and quinones, and phenylalanine is a precursor of flavonoids, tannins, volatile and non-volatile phenylpropanoids, and the hormone salicylic acid. Secondary metabolites coming from these pathways have different roles protecting plant physiology under abiotic stress conditions, including ROS scavenging, enzyme activation, photoprotection and signal regulation (Yu et al., 2005; Oh et al., 2009; Arbona et al., 2013; Isah, 2019). In our results, leaf levels of phenylalanine and tryptophan increased in all plant groups after stress combination, suggesting the activation of biosynthesis routes of protective secondary metabolites. However, this increase was higher in plants with Carrizo as a rootstock, $\frac{CC}{CC}$ and $\frac{CM}{CC}$, than in those grafted onto Cleopatra. Thus, Carrizo rootstock may be improving Cleopatra tolerance to drought and heat stress combination by enhancing the production of secondary metabolites derived from phenylalanine and tryptophan. In addition, SA levels were correlated with the increase of the amino acid levels in $\frac{CC}{CC}$ and $\frac{CM}{CC}$ and it has been identified as an important compound in plant tolerance to multiple abiotic stresses (Khan et al., 2015). In turn, tyramine levels, an intermediate of many tyrosine metabolism pathways, were higher in leaves from $\frac{CC}{CC}$ and $\frac{CC}{CM}$ plants than in the control, whereas in $\frac{CM}{CC}$ and $\frac{CM}{CM}$ decreased respect to control conditions. In this case, the rootstock did not affect the metabolite accumulation in the scion.

Finally, apart from SA, the foliar accumulation pattern of other hormones also varied depending on the rootstock used, specially under stress combination conditions. The accumulation of ABA in $\frac{CC}{CM}$ plants under drought and heat stress combination could indicate a specific response of the scion-rootstock combination to the damaging condition (Table S1). Similarly, the increase in IAA concentration under stress combination in plants with Cleopatra as a rootstock ($\frac{CC}{CM}$ and $\frac{CM}{CM}$) could be another specific signal from the Cleopatra roots to the aerial part.

Results obtained in this work indicate that drought and heat stress combination caused a specific metabolic response differentiated from the responses to the individual stresses in citrus plants. In addition, we have demonstrated that the rootstock modifies the metabolic responses originated in the scion in response to abiotic stress combinations, which can improve or diminish the tolerance of the plant to these adverse

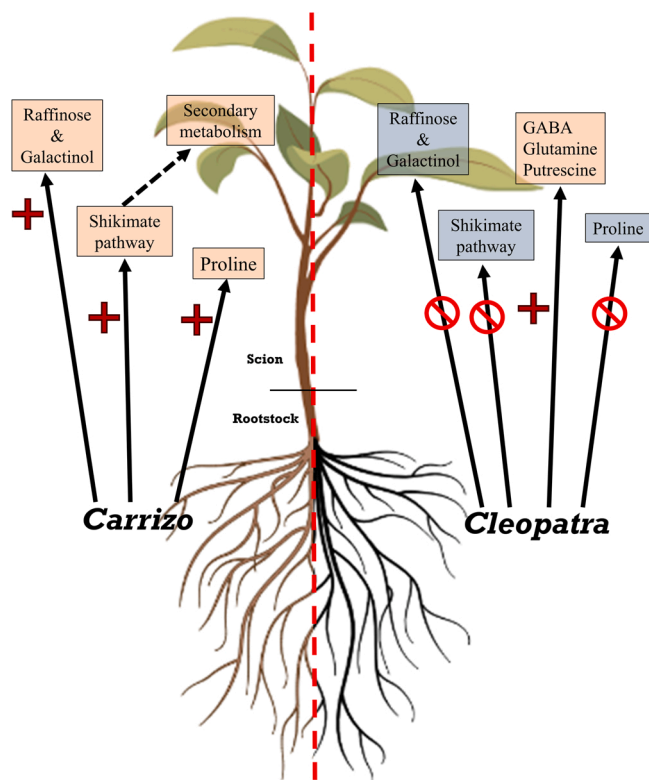


Fig. 7. Schematic representation of the effect of the tolerant (Carrizo citrange) and sensitive (Cleopatra mandarin) citrus rootstocks on scion metabolism in response to drought and heat stress combination. Carrizo improves scion tolerance to the stress combination by increasing levels of raffinose, galactinol, proline and shikimate derived compounds. Cleopatra has a negative effect on scion by reducing levels of galactinol, raffinose and shikimate derived compounds and modulating glutamate metabolism towards alternative routes to proline. Image created with BioRender.com.

conditions. In particular, when used as rootstock, Carrizo citrange positively influenced scions by inducing higher levels of key metabolic compounds in the response to the stress (galactinol, raffinose and SA). In turn, Cleopatra rootstock transferred negative traits to scions by modulating the metabolism to produce less galactinol, raffinose, proline, phenylalanine, or tryptophan. Finally, this research provides information about the importance of rootstocks to improve the tolerance of citrus plants to adverse environmental conditions that often occur due to the effects of Climate Change (Fig. 7).

CRediT authorship contribution statement

Damián Balfagón: Performed the experiments. **Damián Balfagón, Aurelio Gómez-Cadenas:** Designed and supervised the research. **Aurelio Gómez-Cadenas, Antonio Granell:** Provided laboratory infrastructure and funding. **José L. Rambla, Antonio Granell:** Performed the metabolomics analyses. **Damián Balfagón, Vicent Arbona, Aurelio Gómez-Cadenas:** Wrote the manuscript and prepared the figures. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.envexpbot.2022.104793](https://doi.org/10.1016/j.envexpbot.2022.104793).

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