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RESEARCH ARTICLE

Proline accumulation and antioxidant response are crucial for citrus tolerance to UV-B light-induced stress

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ABSTRACT

- Plants face a wide range of biotic and abiotic stress conditions, which are further intensified by climate change. Among these stressors, increased irradiation in terms of intensity and wavelength range can lead to detrimental effects, such as chlorophyll degradation, destruction of the PSII reaction center, generation of ROS, alterations to plant metabolism, and even plant death.
- Here, we investigated the responses of two citrus genotypes, *Citrus macrophylla* (CM), and Troyer citrange (TC) to UV-B light-induced stress, by growing plants of both genotypes under control and UV-B stress conditions for 5 days to evaluate their tolerance mechanisms.
- TC seedlings had higher sensitivity to UV-B light than CM seedlings, as they showed more damage and increased levels of oxidative harm (indicated by the accumulation of MDA). In contrast, CM seedlings exhibited specific adaptive mechanisms, including accumulation of higher levels of proline under stressful conditions, and enhanced antioxidant capacity, as evidenced by increased ascorbate peroxidase activity and upregulation of the *CsAPX2* gene. Phytohormone accumulation patterns were similar in both genotypes, with a decrease in ABA content in response to UV-B light. Furthermore, expression of genes involved in light perception and response was specifically affected in the tolerant CM seedlings, which exhibited higher expression of *CsHYH/CsHY5* and *CsRUP1-2* genes.
- These findings underscore the importance of the antioxidant system in citrus plants subjected to UV-B light-induced stress and suggest that *CsHYH/CsHY5* and *CsRUP1-2* could be considered genes associated with tolerance to such challenging conditions.

INTRODUCTION

Solar ultraviolet (UV) radiation has been extensively studied over the past decades because of its significant role in many natural processes. UV radiation contributes to decomposition of organic matter in soils and water, leading to the formation of smaller molecules and impacting the carbon cycle, ultimately resulting in increased emission of CO and CO_2. Additionally, UV radiation can damage living organisms and trigger the production of halocarbons in seawater, which act as atmospheric pollutants (Sulzberger *et al.* 2019). UV radiation is divided into wavelength bands; UV-A (315–400 nm), UV-B (280–320 nm), and UV-C (100–280 nm). Among these, UV-B and UV-C have higher energy levels and pose a greater threat to living organisms. Although a significant portion of these wavelengths is reflected by the ozone layer and atmospheric oxygen (Semenova *et al.* 2022), the remaining radiation can still damage plant cells, affecting various physiological processes.

The effects of different UV wavelengths and intensities can vary significantly depending on the specific plant species or genotype (Kurdziel *et al.* 2018; Priatama *et al.* 2022). UV negatively affects plants by inducing changes at morphological, physiological, and biochemical levels, causing chlorophyll degradation, destruction of the photosystem II (PSII) center, generation of reactive oxygen species (ROS) and othe rmetabolic alterations (Gudkov *et al.* 2019; Xu *et al.* 2022). However, some studies suggest that weak UV radiation may have positive impacts on plant growth, metabolism, and resistance to biotic stresses (Esnault *et al.* 2010; Gudkov *et al.* 2019).

The detrimental effects of UV radiation on plants are similar to those under high light intensity stress, although the shorter wavelengths of UV light make it more harmful to plants (Shi *et al.* 2022). Plants have developed a range of strategies to regulate their response to UV radiation and mitigate negative effects. First, plants perceive UV light through various photoreceptors, including phytochromes (involved in red and far-red light absorption), cryptochromes (mainly act as blue light photoreceptors, e.g., CRYPTOCHROME 1 (CRY1) and CRYPTOCHROME 2 (CRY2)), or UV Resistance Locus 8 (UVR8), which does not require chromophores to convert the light to biochemical signals (Yang *et al.* 2015). Activation of these signalling pathways leads to protein modifications, biochemical changes, such as activation of ROS scavenging enzymes, and physiological adaptations, such as etiolation repression, photomorphogenesis induction, or flowering regulation. These responses are regulated through genes such as *CONSTITUTIVE PHOTOMORPHOGENIC 1* (*COP1*), *ELONGATED HYPOCOTYL 5* (*HY5*) or *HOMOLOGUE* (*HYH*), as well as phytohormones including abscisic acid (ABA), auxins, or cytokinins (Escobar-Bravo *et al.* 2017; Sanchez *et al.* 2020).

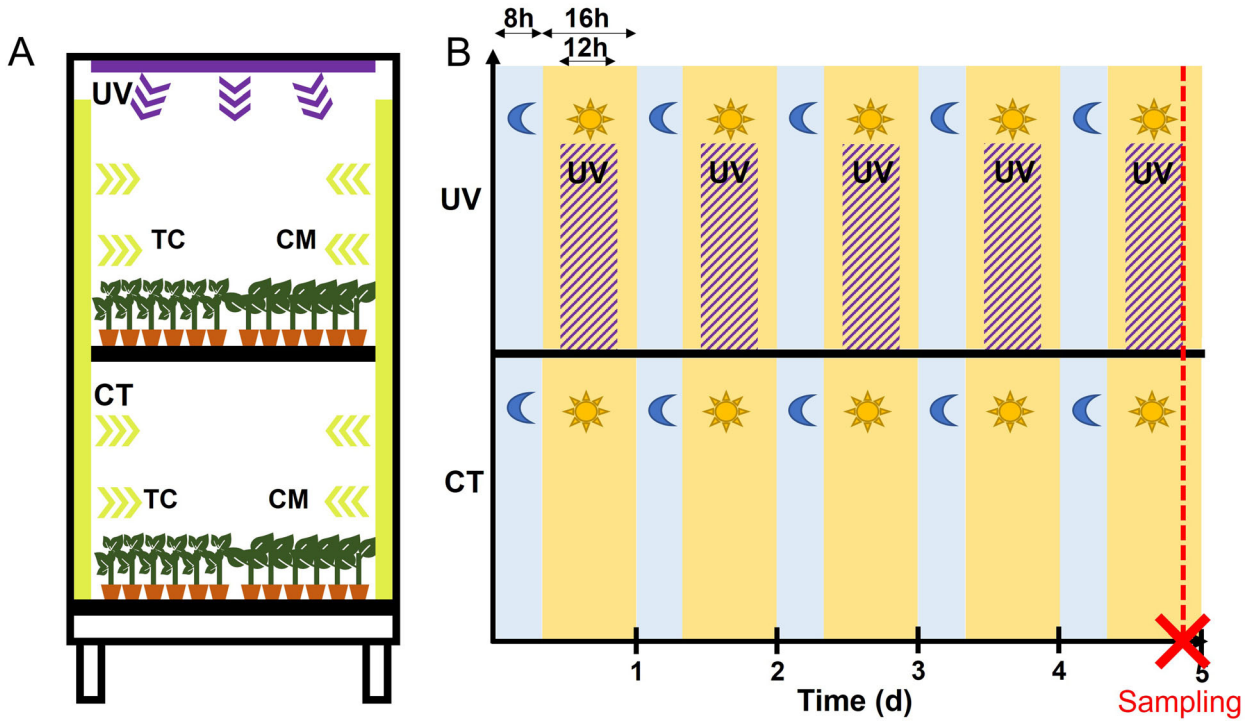

Fig. 1. Schematic representation of the experimental design. A: Distribution of control (lower side) and UV-B stressed (upper side) plants in the environment chamber. B: Representation of the used circadian cycle, based on a 16-h light period, which was supplemented with UV-B irradiation during the 12 central hours of the day in the stressed plants for 5 days.

Although there is limited information available regarding citrus responses to UV-induced stress, previous studies have highlighted the significance of antioxidant machinery in preserving the photosynthetic apparatus against oxidative damage induced by ROS generated under high light exposure (Santini *et al.* 2012; Balfagón *et al.* 2022a).

The aim of this study was to investigate the effects of UV-B-induced stress on citrus plants and examine the adaptive mechanisms that enable them to tolerate such challenging conditions. To achieve this objective, seedlings from two citrus genotypes, Troyer citrange (TC) and *Citrus macrophylla* (CM), were exposed to UV-B stress and morphological, biochemical (proline, chlorophylls and phytohormone content and oxidative status), and genetic responses evaluated to differentiate between common and genotype-specific tolerance mechanisms to UV-B stress.

MATERIAL AND METHODS

Experimental design

Certified citrus seeds from two different genotypes, *C. macrophylla* Wester (CM), and Troyer citrange (*C. sinensis* L. Osb. × *C. trifoliata* L. Raf.; TC) were used as plant material for this study. Although the behaviour of these two genotypes under UV-B induced stress has not previously been studied, other studies with CM and the Carrizo citrange (a hybrid close to TC, with the same parentals) have demonstrated that they have different tolerance to other abiotic stresses, e.g., high salinity or heat (Vives-Peris *et al.* 2017). As citrus seeds are parthenocarpic, clonal descent is guaranteed, allowing use of this plant material for experiments with citrus genotypes. Upon removal of seed coats, the seeds were surface sterilized by immersion in 2% sodium hypochlorite solution with 0.1% Tween 20 (v:v) for 10 min. Subsequently, the seeds were washed three times with sterile distilled water (Pérez-Clemente *et al.* 2012), and individually cultured *in vitro* in 15-cm glass tubes containing 25 mL culture medium. The culture medium was prepared by supplementing Murashige and Skoog salts (Murashige & Skoog 1962) with 0.55 mM myo-inositol, 4.86 μM pyridoxine–HCl, 0.59 μM thiamine-HCl, 8.12 μM nicotinic acid, and 87.64 mM sucrose. The pH of the medium was adjusted to 5.7 ± 0.1 using 0.1 N NaOH, and 0.9% agar was added (Vives-Peris *et al.* 2017). The tubes containing seeds were kept in an environment chamber in darkness at a temperature of 25 °C for 3 weeks. After which the seedlings were transferred to 72-cell trays filled with sterile vermiculite as substrate. They were further cultured for 4 weeks under controlled conditions of 25 °C with a 16-h day and 8-h night photoperiod, at 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The seedlings were watered twice a week with half-strength Hoagland solution (Manzi *et al.* 2015).

After the initial growth period, seedlings were divided into two groups in an environment chamber. The first group served as control and was maintained under the previous conditions, while the second group was subjected to UV-B-induced stress. To achieve this, four additional lighting tubes (Philips Narrowband TL 20W/01) that emit UV-B light at 305–315 nm (peak intensity 311 nm) were placed in the upper part of the climate chamber, positioned 30 cm above the seedlings. An opaque surface separated the control and stressed groups to prevent UV-B light exposure to control plants (Fig. 1A). The UV-B stress was applied for 5 days, maintaining both groups of

plants under 16-h/8-h day/night photoperiod, and adding UV-B light during the 12 central daylight hours (Fig. 1B). This harmful UV-B exposure of stressed plants provided a dose of around 537,000 $J \cdot cm^{-2}$ during the 5-day stress period.

After this stress period, plants were evaluated for phenotypic damage, and shoots from both genotypes were collected, immediately frozen in liquid nitrogen, ground to a fine powder, and stored at −80°C for subsequent analytical determinations. The experiment was conducted in triplicate to ensure reproducibility.

Proline and malondialdehyde determination

The proline content was determined following Bates *et al.* (1973). Briefly, 50 mg fresh ground frozen plant material were extracted with 5 ml 3% (v/v) sulfosalicylic acid through sonication for 30 min (Elma S30; Elma Schmidbauer, Singen, Germany). The extract was then centrifuged at 4800 rpm for 20 min at 4 °C (Digicen 21 R; Ortoalresa, Madrid, Spain), and 1 ml supernatant was mixed with glacial acetic acid and ninhydrin reagent, prepared by dissolving 6.25 g ninhydrin in 150 ml glacial acetic acid and 100 ml 6 M orthophosphoric acid (1:1:1; v:v:v). After which the samples were heated in a water bath for 1 h at 100 °C, followed by rapid cooling on ice, and centrifuged for 10 min at 2000 rpm and 4 °C. Finally, absorbance of the supernatant was measured at 520 nm using a spectrophotometer (Genesys 180; Thermo Fisher, Waltham, MA, USA). The obtained absorbance values were interpolated using a calibration curve prepared from a commercial proline standard.

The determination of malonaldehyde (MDA) content followed Hodges *et al.* (1999). An aliquot of 200 mg fresh pulverized frozen plant material was extracted through 30 min of sonication (Elma S30), in 2 ml 80% ethanol (v/v) as extraction solvent. After sonication, samples were centrifuged for 20 min at 4500 rpm (Digicen 21 R). Subsequently, 800 µl supernatant were mixed with 20% (v/v) trichloroacetic acid or a mixture of 20% (v/v) trichloroacetic acid and 0.5% (v/v) thiobarbituric acid in a 1:1 (v:v) ratio. The sample mixtures were heated at 90 °C for 1 h in a water bath and rapidly cooled in ice. Finally, the samples were centrifuged at 2000 rpm for 10 min (Digicen 21 R) and absorbance measured at 440, 532, and 600 nm. The quantification of MDA content was performed based on calculations described in Zandalinas *et al.* (2017).

Hydrogen peroxide determination

The content of hydrogen peroxide (H_2O_2) was determined with the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Waltham, MA, USA) following Chakraborty *et al.* (2016) with some modifications. Initially, 100 mg fresh plant material were extracted in 350 µl sodium phosphate buffer (50 mM, pH 7.4) by shaking samples at 450 rpm in a thermostated laboratory shaker (ThermoMixer F2.0; Eppendorf, Hamburg, Germany) at 25 °C for 30 min. After extraction, samples were centrifuged for 10 min at 14000 rpm. Next, 5 µl supernatant were mixed with 455 µl extraction buffer and 50 µl working solution. The working solution was prepared by supplementing the extraction buffer with 25 µM Amplex Red, and 1 U horseradish peroxidase. Samples were vortexed and incubated at 30 °C for 30 min in the dark. Finally, absorbance at 560 nm of 1 µl samples was measured in a Nanodrop spectrophotometer (Nanodrop 2000; Thermo Scientific, Wilmington, DE, USA). The final quantification was obtained by interpolating the obtained absorbance values from a standard curve prepared with H_2O_2 standard provided with the commercial kit.

Antioxidant enzyme activity

Activity of antioxidant enzymes catalase (CAT) and ascorbate peroxidase (APX) was analysed to assess the ability of CM and TC citrus genotypes to counteract the negative effects induced by ROS under UV stress. The enzyme extraction followed the method of Zandalinas *et al.* (2017) with some modifications. Briefly, 100 mg fresh frozen material was mixed with 1.8 ml 100 mM potassium phosphate buffer (pH 7.5) supplemented with 0.1% Triton X-100 (v/v) and 0.1% (w/v) polyvinylpyrrolidone, using a ball mill (MillMix 20; Domel, Železniki, Slovenija) for 10 min at 17 rps. Samples were centrifuged at 14,000 rpm for 10 min at 4 °C and the supernatant collected in new tubes. Both CAT and APX activities were normalized according to protein content of the extracts, and results expressed as units of enzyme per mg protein. The total protein content of the extracts was determined following Bradford (1976), and quantification was performed using a standard curve prepared with commercial bovine serum albumin.

For CAT activity, 100 µl extract was mixed with 900 µl reaction mixture prepared with 50 mM sodium phosphate buffer (pH 6.8) supplemented with 20 mM H_2O_2. This mixture was incubated at 30 °C, and 150 µl of the sample taken at 0, 7, 10, and 20 min, and exposed to 850 µl indicator mixture, prepared with 50 mM sodium phosphate buffer (pH 6.8) supplemented with $TiCl_4$. Finally, absorbance at 415 nm of samples was measured (Genesys 180), and data were interpolated from a standard curve prepared with commercial CAT (Brennan & Frenkel 1977).

Activity of APX was evaluated by adding 100 µl extract to 880 µl 50 mM sodium phosphate buffer (pH 6.8) supplemented with 9.7 mM EDTA disodium salt, 1 mM sodium ascorbate, and 20 µl 440 mM H_2O_2. The absorbance at 290 nm was recorded every 10 s for 1 min after adding the extract, by measuring the consumption of ascorbate. Total APX units were calculated based on the slope obtained during this period, with 1 unit APX defined as the amount of enzyme required to consume 1 $mmol \cdot sodium \cdot ascorbate \cdot min^{-1}$ (Nakano & Asada 1981).

Chlorophyll analysis

The extraction of chlorophyll *a* and *b* (Chl*a* and Chl*b*) and carotenoids from plant tissue was performed in glass tubes containing 20 mg finely ground fresh frozen tissue. The plant material was mixed with 2 ml dimethyl sulfoxide (DMSO) and extracted in an oven at 37 °C for 15 h. After extraction, samples were centrifuged, and absorbance of the supernatants measured at 665, 649 and 480 nm (Genesys 180). Finally, Chl*a*, Chl*b*, and carotenoid content in samples was calculated using the equations of Wellburn (1994).

Phytohormone quantification

The shoot content of abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and indole acetic acid (IAA) was determined using liquid chromatography coupled to mass spectrometry, following Durgbanshi *et al.* (2005) with some modifications.

Approximately 150 mg fresh ground frozen shoot was extracted in ultrapure water, with 25 ng [$^2H_6$]-ABA, [$^{13}C_6$]-SA, dihydrojasmonic acid (DHJA), and 2.5 ng [$^2H_5$]-IAA as internal standards. The extraction was performed using a ball mill for 10 min at 17 rps (MillMix 20). After centrifugation at 14,000 rpm for 10 min at 4 °C, the supernatants were recovered, and their pH adjusted within the range 2.8–3.2. They were then partitioned twice with diethyl ether, and the organic phase containing the extracted phytohormones transferred to new tubes. The samples were dried in a speed vacuum system (Speed Vac; Jouan, Saint Herblain Cedex, France). The dried samples were resuspended in 0.5 ml 90:10 (v:v) water:methanol through sonication (Elma S30), filtered through PTFE syringe filters with a 0.22 µm pore size, and diluted 1:3 (v:v) in liquid chromatography vials.

For analysis, a loop volume of 15 µl was injected into the UPLC system coupled to a triple quadrupole mass spectrometer (Xevo TQ-S; Waters, Milford, MA, USA). Separation was achieved using a C_{18} reverse phase column (50 × 2.1 mm, 1.6 µm particle size; Luna Omega, Phenomenex, Torrance, CA, USA) maintained at 40 °C. A gradient of ultrapure water and acetonitrile, both supplemented with 0.1% (v/v) formic acid, was used as mobile phase at a constant flow of 300 $\mu l \cdot min^{-1}$. The triple quadrupole mass spectrometer was operated in MRM mode, with a gas flow of 250 $l \cdot h^{-1}$ and nitrogen flow of 1200 $l \cdot h^{-1}$ at 650 °C. Quantification was performed by interpolating the obtained response in a standard curve prepared with commercial standards, together with the internal standards mentioned earlier. The data obtained from the analyses were processed using Masslynx 4.2 software (Waters, Milford, MA, USA).

Gene expression analysis

Total RNA was obtained from approximately 30 mg frozen grounded tissue with the RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quality of the extracted RNA was assessed using a Nanodrop spectrophotometer (Nanodrop 2000) by measuring the absorbance ratios at 260/280 and 260/230 nm. To remove any contaminating DNA, the extracted RNA was treated with DNase I (Fermentas, Waltham, MA, USA). Subsequently, 1 µg treated RNA was converted into cDNA using Primescript RT Reagent Kit (Takara, Shiga, Japan). RT-qPCR were performed on an ABI StepOne system (Applied Biosystems, Foster City, CA, USA). Each reaction consisted of 1 µg cDNA, 5 µl SYBR Green/ROX qPCR mix (Thermo Scientific, Wilmington, DE, USA), 1 µl of a mixture of forward and reverse primers, each at 10 µM concentration, and 3 µl water, resulting in a final reaction volume of 10 µl. The amplification protocol consisted of an initial preincubation step at 95 °C for 10 min, followed by 40 cycles of amplification, with each cycle consisting of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 20 s. The resulting data were analysed using StepOne Software version 2.3 and Relative Expression Software Tool version 2 (REST; Pfaffl 2001; Pfaffl *et al.* 2002). The expression levels of the target genes were normalized to expression levels of the housekeeping genes, actin and tubulin, as described by Vives-Peris *et al.* (2018).

Primers for the target genes were designed based on the protein sequences of *Arabidopsis* obtained from the TAIR database (Berardini *et al.* 2015), which were used in a TBLASTN search in the Phytozome genome database for *Citrus sinensis* version 1.1 (Goodstein *et al.* 2012). The corresponding coding sequences (CDS) were retrieved for primer design (Table S1).

Statistical analysis

A Student *t*-test was used to compare potential differences between UV-stressed and control seedlings within the same genotype. To visualize the multivariate data, a Principal Components Analysis (PCA) was performed using SigmaPlot version 14.0 software (Systat Software, Chicago, IL, USA).

RESULTS

Phenotypic damage

Different forms of damage were observed in citrus seedlings after 5 days of UV-B-induced stress (Fig. 2). Both citrus genotypes cultivated under UV light had retracted leaves, with 55.3% in CM and 82.5% in TC (Fig. 2A–C). Moreover, TC plants subjected to UV-B also had burnt apices (82.5% of seedlings), whereas those from CM did not (Fig. 2D). None of these symptoms were observed in the control seedlings.

Proline content

The endogenous content of proline was only affected by UV-induced stress in CM shoots, with a 25.9% increase compared to control plants, while there was a smaller increase in TC seedlings (1.16-fold above control), but this was not significant when compared with the enhanced accumulation of proline in CM seedlings subjected to UV light (Fig. 3). Additionally, when compared with the two control groups, CM control plants had a proline content 1.58-fold higher than TC control plants ($P = 0.0308$).

Oxidative status

After 5 days of UV-B treatment, TC seedlings sufferd oxidative damage, with higher MDA content (a product of membrane lipid peroxidation) in UV-stressed plants (1.2-fold higher than control). There were no differences in MDA content of CM seedlings under control or stress situations (Fig. 4A). However, there were no differences in H_2O_2 content in any of either genotype after application of UV-B stress compared to the controls (Fig. 4B).

Antioxidant enzyme activity evaluated through quantification of CAT and APX activity after UV-B stress revealed differences between control and UV-stressed seedlings, depending on the citrus genotype (Fig. 5). Thus, CAT activity was similar in CM and TC seedlings, with or without UV-B stress (Fig. 5A). In contrast, there was a significant increase of APX activity in CM seedlings subjected to UV-induced stress (1.4-fold increase compared to control seedlings), whereas there were no differences in TC seedlings (Fig. 5B). Additionally, under control conditions, higher basal activity of APX was found in CM seedlings compared to TC seedlings (4.9-fold higher; Fig. 5B). In parallel to enzyme activity, expression of genes coding for these two antioxidant enzymes, *CsCAT2* and *CsAPX2*m was analysed through RT-qPCR, revealing similar results to those obtained for CAT and APX antioxidant activity. Thus, although there were no differences in expression of *CsCAT2* in either citrus genotype (Fig. 5C), *CsAPX2* was exclusively upregulated in CM seedlings

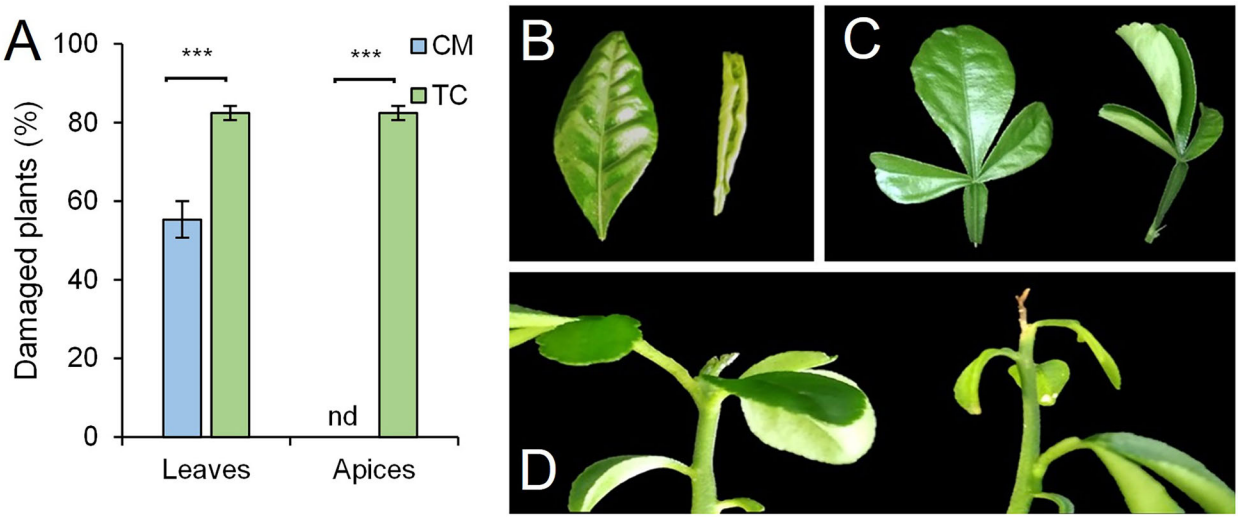

Fig. 2. Phenotypic damage. A: Quantification of the percentage of plants with affected leaves and burnt apices in *Citrus macrophylla* (CM; blue bars) and Troyer citrange (TC; green bars) after 5 days of UV-B induced stress (control plants did not exhibit any phenotypic damage and are not shown in the figure). Presented data refer to mean ± SE. Asterisks denote statistically significant differences between control and UV-B stressed plants after a Student's *t*-test at 0.01 (***), 0.05 (**) or 0.1 (*). B: Undamaged (left) and damaged leaves (right) of *C. macrophylla* plants; C: Undamaged (left) and damaged leaves (right) of Troyer citrange plants. D: Undamaged (left) and damaged apices (right) of *C. macrophylla*.

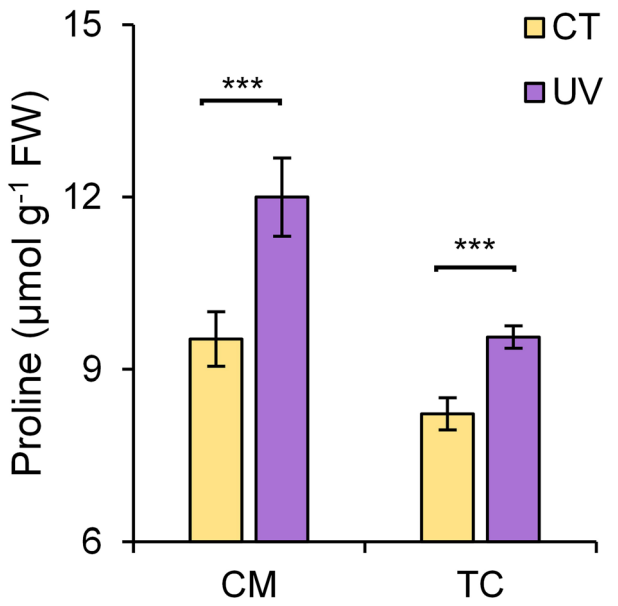

Fig. 3. Proline content in control (yellow bars) and UV-B stressed plants (purple bars) of *Citrus macrophylla* (CM) and Troyer citrange (TC) after 5 days. Presented data are mean ± SE. Asterisks denote statistically significant differences between control and UV-B stressed plants after a Student's *t*-test at 0.01 (***), 0.05 (**) or 0.1 (*).

exposed to UV-induced stress, with expression values 1.9-fold higher than in controls (Fig. 5D).

Chlorophyll and carotenoid content

No significant differences were observed in Chl*a* content (Figure S1A), Chl*b* content (Figure S1B), or in the Chl*a*/Chl*b* ratio and total chlorophyll content (Figure S1C, D) between control and UV-B-stressed seedlings of either genotype. Similarly, the total carotenoid content did not change in response to UV-B stress (Figure S1E).

Phytohormone content

The shoot phytohormone accumulation pattern was similar in both citrus genotypes, with a decrease in endogenous ABA content in both genotypes, being 42.9% and 28.5% lower than the controls in CM and TC UV-B-stressed plants, respectively (Fig. 6A). However, there were no differences in shoot content of the phytohormones SA, JA, or IAA in either of the two citrus seedlings types (Fig. 6B–D, respectively).

Relative expression of light responsive genes

The RT-qPCR analysis revealed differences in relative expression of certain genes involved in UV-induced stress tolerance depending on the plant genotype (Fig. 7). While expression of *CsUVR8*, *CsCRY1*, *CsCRY2* or *CsCOP1* remained unchanged in both citrus genotypes after 5 days of UV-B stress (Fig. 7A–C, F, respectively), expression of *CsHYH/CsHY5* and *CsRUP1-2* was exclusively induced in UV-B-stressed CM plants (5.0- and 5.5-fold increase in *CsHYH/CsHY5* and *CsRUP1-2*, respectively), compared to control seedlings. On the other hand, TC seedlings did not differ in relative expression of these genes in UV-B-stressed plants compared to controls (Fig. 7D, E).

Data integration

The PCA revealed that observed differences in physiological and biochemical parameters contributed to separation of control and UV-B-stressed groups in both citrus genotypes. The PCA explained total variability of 68.65%, with PC1 accounting for 49.90% and PC2 accounting for 18.75% of total variability

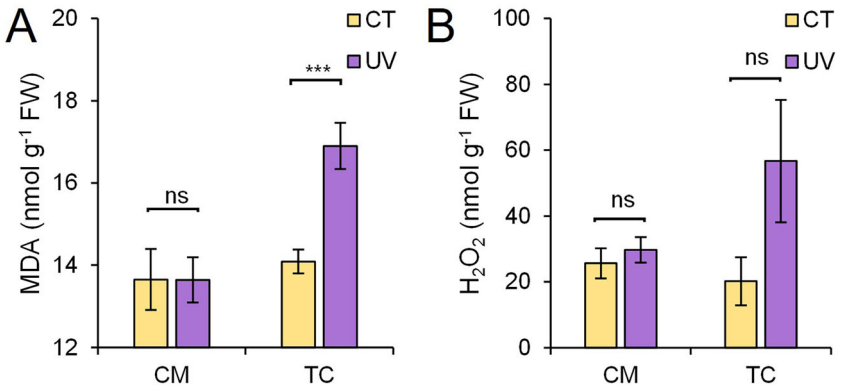

Fig. 4. Oxidative damage in control (yellow bars) and UV-B stressed plants (purple bars) of *Citrus macrophylla* (CM) and Troyer citrange (TC) after 5 days. A: Malondialdehyde; B: Hydrogen peroxide. Presented data are mean ± SE. Asterisks denote statistically significant differences between control and UV-B stressed plants after a Student's *t*-test at 0.01 (***), 0.05 (**) or 0.1 (*).

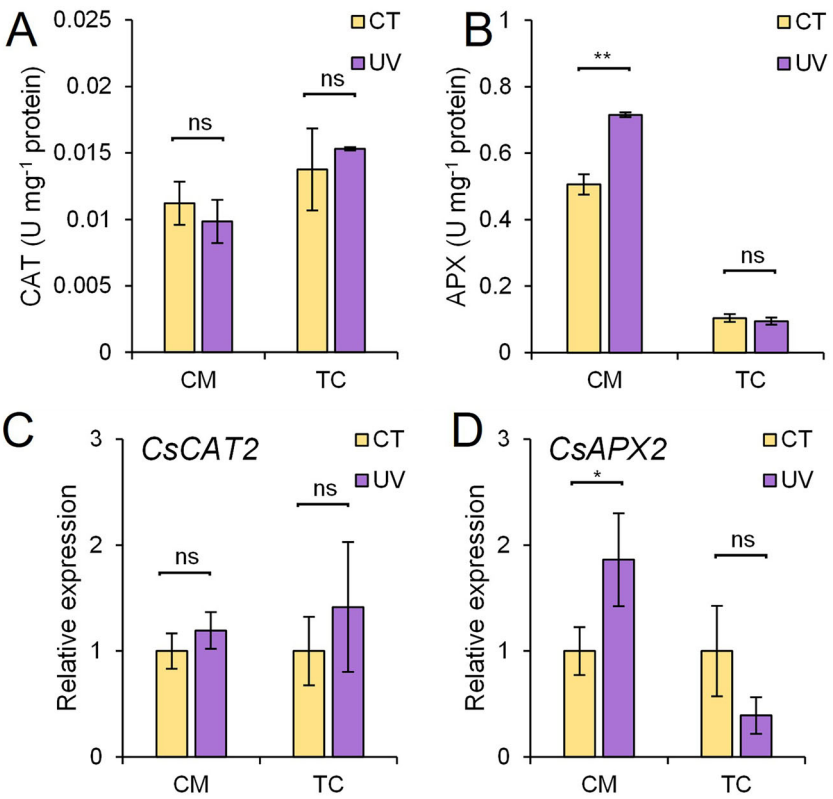

Fig. 5. Antioxidant status in control (yellow bars) and UV-B stressed plants (purple bars) of *Citrus macrophylla* (CM) and Troyer citrange (TC) after 5 days. A: Catalase activity; B: Ascorbate peroxidase activity; C: Relative expression of *CsCAT2*; D: Relative expression of *CsAPX2*. Presented data are mean ± SE. Asterisks denote statistically significant differences between control and UV-B stressed plants after a Student's *t*-test at 0.01 (***), 0.05 (**) or 0.1 (*).

(Fig. 8). By analysing the loading and score graphs in comparison to previous results, some parameters appeared to be particularly relevant in distinguishing between control and UV-stressed groups in both genotypes. In CM plants, these parameters included an increase in proline content and APX activity, as well as a reduction in ABA content, contributing to the separation of CM control and UV-stressed plants, positioned on the upper left side of the score plot. However, in TC seedlings, increased leaf and apex damage, together with reduced ABA levels, seemed to be the key variables influencing differences in distribution among control and UV-B-stressed plants.

DISCUSSION

Solar radiation provides the energy source needed by plants for photosynthesis but can also function as an abiotic stress factor. Climate change is also contributing to changes in UV radiation in the biosphere through impacts on cloud patterns, aerosols,

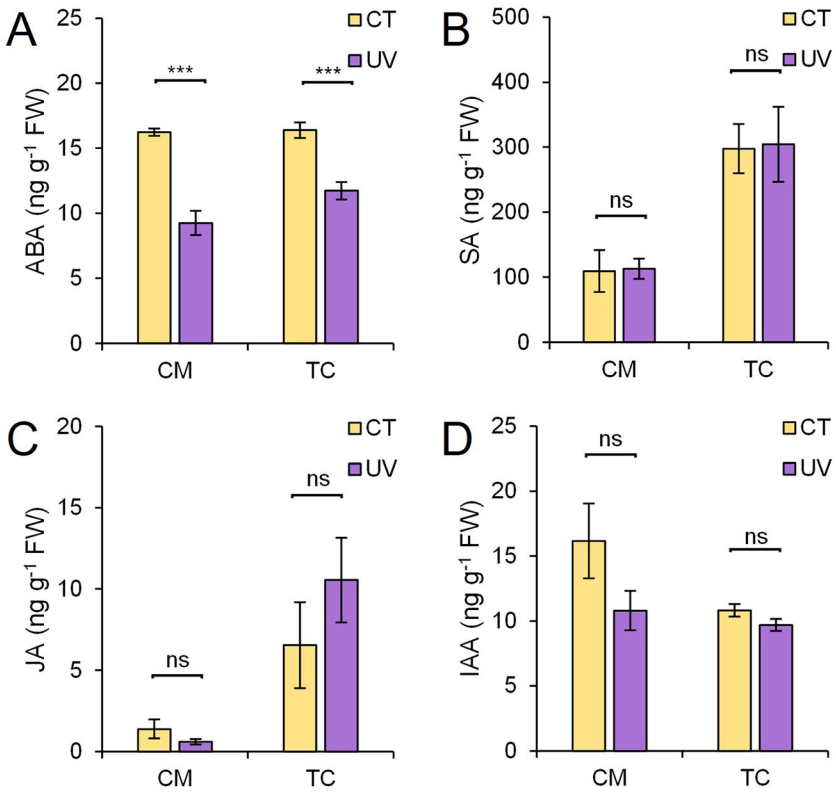

Fig. 6. Phytohormone content in control (yellow bars) and UV-B stressed (purple bars) plants of *Citrus macrophylla* (CM) and Troyer citrange (TC) after 5 days. A: Abscisic acid; B: Salicylic acid; C: Jasmonic acid; D: Indole acetic acid. Presented data are mean ± SE. Asterisks denote statistically significant differences between control and UV-B stressed plants after a Student's *t*-test at 0.01 (***), 0.05 (**) or 0.1 (*).

and surface reflectivity (Andrady *et al.* 2017). The stress induced by excess light intensities or increases in the incidence of radiation in the UV-B wavelength spectrum (280–315 nm), is one of the emerging abiotic stresses aggravated by climate change. Currently, understanding the effects of UV-B radiation on citrus plants remains limited. To address this knowledge gap, this study focused on evaluating the impact of UV-B radiation on two citrus genotypes, CM and TC, at several levels: physiological, biochemical, and genetic. The obtained results reveal the distinct responses to UV radiation exhibited by the genotypes. Specifically, CM plants demonstrated higher tolerance to the applied UV-B stress compared to TC plants. such.

The differences in genotype tolerance could be related to different adaption strategies, such as proline synthesis and/or accumulation. Proline is commonly accumulated under stress conditions, helps plant to avoid dehydration and turgor loss, and is well characterized as an osmoprotectant in citrus (Vives-Peris *et al.* 2017). Indeed, proline has also been reported as an antioxidant molecule capable of scavenging OH^- and O_2 (Das & Roychoudhury 2014). The detected proline accumulation under UV-B light-induced stress could be an adaptation mechanism to this adverse situation. Although both citrus genotypes exhibited a higher content of this amino acid under UV conditions, CM stressed seedlings accumulated higher quantities with respect to the control plants, which suggests its enhanced tolerance to this hazardous condition (Fig. 3). The overaccumulation of proline has been previously reported as beneficial against UV-induced stress in rice, mustard, mung bean, and grass species, not only because of its role as an osmoprotector but also as an antioxidant (Saradhi *et al.* 1995; Sarkar *et al.* 2011). In addition, the different basal proline concentration between different citrus genotypes has been previously described for CM and Carrizo citrange (a citrus hybrid very similar to TC, with the same parental) that have different proline contents (Vives-Peris *et al.* 2017). The high levels of proline recorded confirms its main role as an osmoprotectant, compared to other compounds, such as soluble sugars, trehalose, or glycine betaine, in citrus plants which accumulate more proline than other plant species (Nolte *et al.* 1997).

An alternative approach to mitigate UV-B light-induced damage involves maintaining the oxidative status by activating both enzymatic and non-enzymatic pathways. These mechanisms work together to reduce the production of ROS caused by such harmful environmental conditions. The higher oxidative damage and membrane degradation (quantified as shoot MDA content in Fig. 4) in the sensitive TC genotype, along with the enhanced APX gene expression and activity observed in the tolerant CM genotype (Fig. 5), suggest that APX activation plays a crucial role in conferring citrus tolerance to UV-B induced stress, as previously demonstrated in *A. thaliana*, where the ascorbate-deficient mutant *vtc1* has higher sensitivity to UV-B radiation compared to the wild type, resulting in higher production of ROS (Gao & Zhang 2008). However, no differences in CAT activity or *CsCAT2* expression between control and UV-stressed plants were recorded in either of the used genotypes, CM or TC. This is consistent with other studies

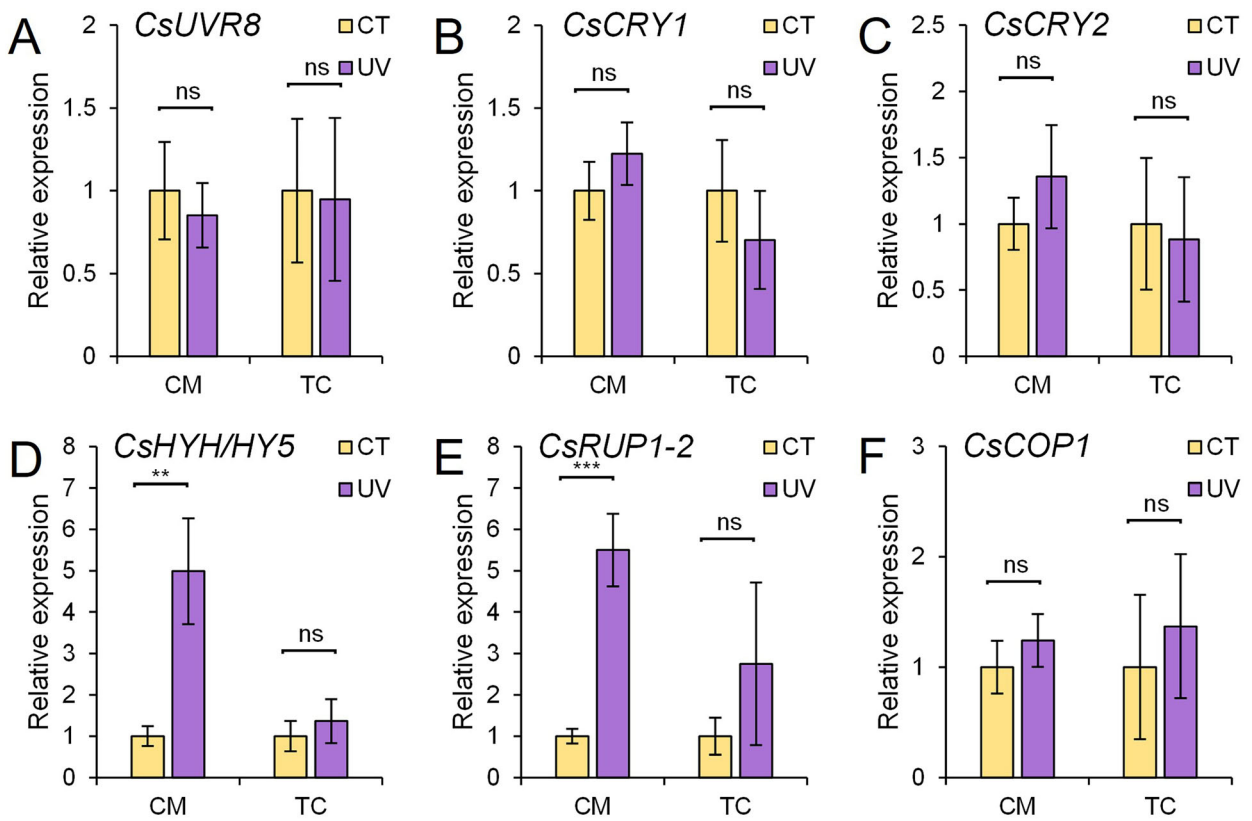

Fig. 7. Relative expression of genes involved in UV light perception and response in control (yellow bars) and UV-B stressed plants (purple bars) of *Citrus macrophylla* (CM) and Troyer citrange (TC) after 5 days. A: *CsUVR8*; B: *CsCRY1*; C: *CsCRY2*; D: *CsHYH/HY5*; E *CsRUP1-2*; F: *CsCOP1*. Presented data are mean ± SE. Asterisks denote statistically significant differences between control and UV-B stressed plants after a Student's *t*-test at 0.01 (***), 0.05 (**) or 0.1.

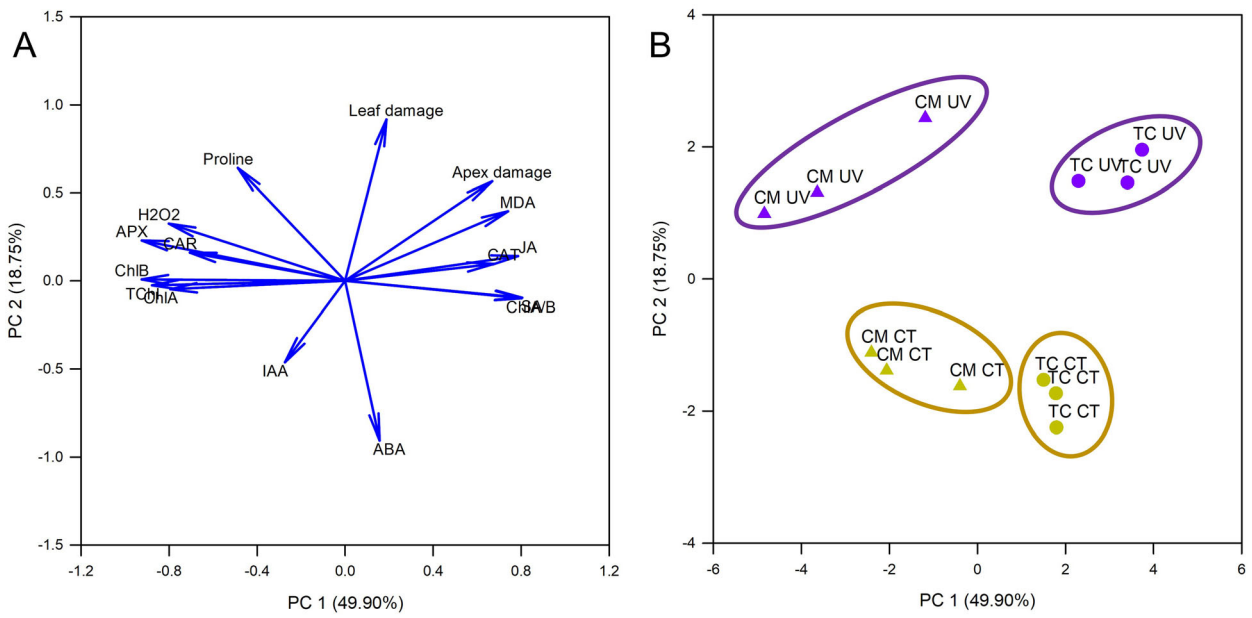

Fig. 8. Principal components analysis of the phenotypic and biochemical data. A: Loading plot; B: Score plot, where yellow and purple symbols refer to control and UV-B stressed plants, respectively, while circles and triangles refer to Troyer citrange and *Citrus macrophylla*, respectively.

conducted on *Nicotiana tabacum* calli (Zacchini & De Agazio 2004) and tomato fruits (Barka 2001), which experienced an increase of APX activity but not CAT activity in response to UV light induced stress. Additionally, although both APX and CAT reduce H_2O_2 to H_2O and O_2, they typically function in different cellular organelles. While APX is commonly found

in the cytosol and chloroplasts, CAT predominantly acts in peroxisomes. This leads to a higher scavenging capacity of APX and its activation at lower H_2O_2 concentrations (Das & Roychoudhury 2014). The importance of APX in citrus under abiotic stress conditions has also been also mentioned in previous works testing the response of this crop to abiotic stresses such as drought, heat stress and their combination, whereas CAT seems to be less relevant (Zandalinas *et al.* 2017; Balfagón *et al.* 2018). Thus, the absence of differences in H_2O_2 leaf content between the two citrus genotypes, as well as the relatively low H_2O_2 concentrations, in comparison with those described in other works performed with citrus plants (Balfagón *et al.* 2022b), suggests the lower severity of the oxidative stress after 5 days of UV-B-induced stress.

Phytohormones orchestrate various plant processes and play a pivotal role in plant signalling under both biotic and abiotic stress. ABA is a prominent hormone in plant defence mechanisms, influencing processes such as stomatal opening and stress-related gene regulation. The significance of ABA is also underscored through its intricate interplay with other hormones, such as jasmonates or salicylates (Gómez-Cadenas *et al.* 2015). In the context of the phytohormones examined in this study (ABA, SA, JA, and IAA), the endogenous ABA content emerged as the sole hormone affected by exposure to UV-B radiation, with a reduction in ABA content across both genotypes (Fig. 6). However, the role of ABA in plant responses and tolerance to UV-B irradiation remains contentious, with diverse outcomes reported for different plant species. For instance, in maize plants, exposure to 2–4 h of UV light led to an increase in endogenous ABA content, with this hormone implicated in initiating nitric oxide production to confer tolerance to UV stress (Tossi *et al.* 2009). In contrast, studies in other species, like pea, demonstrated decreases in ABA levels when subjected to various doses of UV-B and UV-C (Katerova *et al.* 2009). Similarly, no discernible differences in ABA concentration were observed in *Rumex patientia* exposed to UV-B for 1 to 5 days (Lindoo *et al.* 1979). This variability in ABA accumulation patterns among different plant species underscores the intricate relationship between ABA accumulation and UV exposure. Thus, the variation in the accumulation of this phytohormone seems to be influenced by factors such as plant species and age, light wavelength, as well as duration of the stress period (Vanhaelewyn *et al.* 2016). Moreover, there was low variability in JA levels when comparing control groups, TC, and CM ($P = 0.075$), but levels were not affected when applying UV-B-induced stress. These low variations could be related to natural variation among different citrus genotypes or culture conditions, and the detected JA concentrations were typical from control citrus plants, being similar to those previously described in the existing literature (Long *et al.* 2019; Terán *et al.* 2024).

Most genetic studies focused on the genetic response under UV-induced stress have reported UVR8 as the main photoreceptor regulating plant responses to UV-B. Thr UVR8 monomeric form (produced from degradation of the dimeric form due to UV light incidence) enhances and regulates the E3 ubiquitin ligase COP1 activity. *COP1* has also been suggested as one of the main genes converging the responses to different wavelengths of irradiation, whereas phytochromes (PHYs) and cryptochromes (CRYs) act as negative regulators (Yang *et al.* 2015). Although no differences have been detected in the expression of these genes in our study, the overexpression of *CsRUP1-2* in the tolerant genotype CM suggests a higher capacity to facilitate the re-dimerization and restoration of UVR8 protein. Thus, following the model proposed by Yang *et al.* (2015), the docking of COP1 with the UVR8 monomeric form would induce expression of *CsHYH/CsHY5*, as observed in CM plants. Finally, the complex of COP1 joined to the UVR8 monomer is responsible for induction of *CsHYH/CsHY5* homologues, which are the genes significantly involved in the photomorphogenesis process.

CONCLUSIONS

The data obtained from the present study highlight the divergent responses of seedlings from two citrus genotypes to UV-B-induced stress, with CM exhibiting enhanced tolerance compared to that of TC. Despite both genotypes sharing a common phytohormone response, some mechanisms, such as enhanced accumulation of proline and heightened antioxidant activity of APX, were observed in CM seedlings. These mechanisms appear to play a pivotal role in conferring tolerance to the adverse effects of UV-B in CM. Furthermore, activation of the genes *CsHYH/CsHY5* and *CsRUP1-2* also emerge as potentially significant contributors to the acclimation and survival of citrus plants under UV-induced stress. These findings collectively underline the multifaceted strategies employed by CM to withstand UV-B light-induced stress, setting it apart as a more resilient genotype in comparison to TC.

AUTHOR CONTRIBUTIONS

The work was devised by V. V.-P. and R. M. P.-C. and performed by V. V.-P. Analytical determinations were achieved by V. V.-P. The final version of the manuscript was written by V. V.-P. and revised by A. G.-C. and R. M. P.-C. A. G.-C. and R. M. P.-C. supervised the work and provided financial resources. All authors have read the final version of the manuscript and approved its publication.

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SUPPORTING INFORMATION

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

Table S1. Primers used for RT-qPCR analysis of gene expression.

Figure S1. Chlorophyll and carotenoid content in *Citrus macrophylla* (CM) Troyer citrange (TC) plants subjected to control (yellow bars) or UV-B induced stress (violet bars) for 5 days Asterisks denote statistically significant differences between control and UV-B stressed plants after a *t*-student test at 0.01 (***), 0.05 (**) or 0.1 (*).

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