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Alterations in Photosynthetic Apparatus, Antioxidant Systems, and Leaf Anatomical Structure of Some Citrus Genotypes Grafted on The Recently Introduced 'Macrophylla' Rootstock in Response to Chilling Stress

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Abstract: One-year-old transplants of 'Olinda Valencia orange', 'Murcott Tangerine', and 'Limoneira 8A Lisbon' genotypes were grafted on 'Macrophylla' rootstock and subjected, along with 'Macrophylla' rootstock, to 24 h of the night chilling (nocturnal) at 20/4 °C±1 (day/night) for three days, (8 h per day), followed by three days of recovery at 30/25 °C±1 (day/night). The results showed that leaf diffusion resistance (LDR), proline, malondialdehyde (MDA), peroxidase (POX), and superoxide dismutase (SOD) increased significantly in all genotypes, while there was a significant decrease in photosynthetic apparatus, polyphenol oxidase (PPO), and catalase (CAT) under nocturnal chilling stress. Under the chilling, Macrophylla rootstock had the highest concentrations of proline and SOD, whereas it had the lowest MDA and total chlorophyll concentrations. Noticeably, the enzymatic antioxidant profiles were enhanced after being recovered. Moreover, 'Murcott' had the highest LDR, MDA concentration, thickness of lamina, midvein, palisade tissue, spongy tissue, and dimension of midvein vascular bundle. Meanwhile, it had the lowest stomatal conductance (GS) and transpiration rate (E). Similar results were obtained with 'Valencia' genotype. In contrast, the 'Limoneira 8A' genotype under the chilling had the lowest levels of LDR, antioxidant enzymes (POX, CAT, and PPO), thickness of lamina, midvein, palisade tissue, spongy tissue, and dimension of midvein vascular bundle.

Index Terms: Anatomy, Antioxidant enzymes, Chilling stress, Citrus, Macrophylla rootstock.

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Alterações no aparelho fotossintético, sistemas antioxidantes e estrutura anatômica das folhas de alguns genótipos cítricos enxertados no recém-introduzido porta-enxerto 'Macrophylla' em resposta ao estresse por resfriamento.

Resumo: Transplantes de um ano de idade de genótipos 'Laranja Olinda Valência', 'Tangerina Murcott' e 'Limoneira 8A Lisbon' foram enxertados em porta-enxertos 'Macrophylla' e submetidos, juntamente com porta-enxertos 'Macrophylla', a 24 horas de resfriamento noturno (noturno) a 20/4 °C±1 (dia/noite), durante três dias (8 horas por dia), seguidos de três dias de recuperação a 30/25 °C±1 (dia/noite). Os resultados mostraram que a resistência à difusão foliar (RDF), prolina, malondialdeído (MDA), peroxidase (POX) e superóxido dismutase (SOD) aumentaram significativamente, em todos os genótipos, enquanto houve diminuição significativa no aparelho fotossintético, polifenol oxidase (PPO) e catalase (CAT) sob estresse de resfriamento noturno. Sob o resfriamento, o porta-enxerto 'Macrophylla' apresentou as maiores concentrações de prolina e SOD, enquanto apresentou as menores concentrações de MDA e clorofila total. Notavelmente, os perfis antioxidantes enzimáticos foram melhorados após serem restaurados. Além disso, a Tangerina 'Murcott' apresentou a maior RDF, concentração de MDA, espessura da lâmina, veia mediana, tecido paliçado, tecido esponjoso e dimensão do feixe de veia mediana. Enquanto isso, teve os menores teores de condutância estomática (GS) e taxa de transpiração (E). Resultados semelhantes foram obtidos com o genótipo 'Valência'. Em contraste, o genótipo 'Limoneira 8A', sob o resfriamento, apresentou os menores níveis de RDF, enzimas antioxidantes (POX, CAT e PPO), espessura da lâmina, veia mediana, tecido paliçado, tecido esponjoso e dimensão do feixe de veia mediana.

Termos para indexação: Anatomia, Enzimas antioxidantes, Estresse de resfriamento, Citrus, porta-enxerto Macrophylla.

Introduction

Citrus is a popular fruit that originated in the tropical and sub-tropical regions of the world. Citrus fruits are rich in vitamin C, carotenoids, riboflavin, calcium, folate, phytochemicals, and polyphenols, all of which are essential nutrients (LIU et al. 2012). According to FAOSTAT, 2022, the total world production of citrus was estimated at (158,490 million tons). Egypt ranked first in exports of citrus (1,450 million tons), and sixth in production (3,000 million tons) (USDA, 2022).

Citrus needs an optimum temperature range of 20 °C to 30 °C (KRIEDEMANN; BARRS, 1981), and the main climatic constraints on citrus production are low temperatures (below 13 °C), which cause chilling or freezing damage to the plants and may result in plant death (SAQIB et al. 2022). Data provided by the Central Laboratory for Agricultural Climate (CLAC) show that the weather has changed in the last ten years in Egypt and is expected to continue throughout the century.

Chilling stress affects geographic range, photosynthesis, and respiration. It can also result in physiological and biochemical changes that will reduce growth or even cause permanent damage to the trees (HMMAM et al., 2022; SAQIB et al., 2022). Some physiological and biochemical changes, like the formation of malondialdehyde (MDA), are

signs of chilling injury (TAJVAR et al., 2011; MACHADO et al., 2013; OUSTRIC et al., 2017). These changes are caused by an increase in reactive oxygen species (ROS), which changes antioxidant enzymes like peroxidase (POX), superoxide dismutase (SOD), polyphenol oxidase (PPO), and catalase (CAT) (SYROS et al., 2005; TAJVAR et al., 2011; MACHADO et al., 2013; OUSTRIC et al., 2017). Changes were also seen in antioxidants not made by enzymes, such as proline (KUSHAD, YELENOSKY, 1987; OUSTRIC et al., 2017). All these changes help plants avoid the adverse effects of ROS. Chilling injury is most common in plants that grow in tropical or subtropical climates, such as citrus (MUKHOPADHYAY; ROYCHOUDHURY, 2018). Additionally, plants adapt their anatomy to deal with low temperatures (CAO et al., 2014; ZHANG et al., 2015; XU et al., 2022). However, no research has been done on the leaf anatomical structure of citrus seedling leaves that are acclimated to cold stress.

Citrus trees are one of the most chilling tender crops, sensitive to fluctuant and low-temperature stress. Generally, citrus growers use rootstock/scion combinations to enhance fruit yield, quality, and resistance to biotic and abiotic stresses (TAJVAR et al., 2011). The rootstocks are selected based on their capacity to tolerate a wide range of biotic and abiotic stresses, such as soil-borne pests and diseases, salinity, drought, floods, and cold (OUSTRIC et al., 2017).

Alemow]*Citrus macrophylla* (L.(Wester] originating on the island of Cebu, Philippine Islands (SAUNT, 2000) and it is a seedy hybrid that may have *Citrus celebica* koord or another species of papeda as one of its original parents and *Citrus grandis* (L) Osbeck (pummelo) as a second parent (BARRETT; RHODES, 1976), but Curk et al. (2016) revealed that it resulted from hybridization between *Citrus micrantha* Wester or a close papeda species and citron. '*C. macrophylla*' is morphologically and genetically very similar to lemons and limes. Recently, 'Macrophylla' has been used as a new alternative rootstock in Egypt to expand the cultivation of citrus in the reclaimed lands since the scions budded on it grow well on both sandy and high pH calcareous soils (CASTLE, 1987). However, several studies have classified it as cold-sensitive rootstocks (IKEDA et al., 1980; CASTLE, 1987). Moreover, Inch et al. (2014) classified it as freeze-tender rootstocks.

Despite the freeze-tender nature of '*C. macrophylla*', very few studies have focused on its response to low temperatures (PRIMO-CAPELLA et al., 2022). However, the physiological, biochemical, and anatomical responses and the growth behaviour of scions grafted onto 'Macrophylla' rootstock have not received much attention in Egypt. The null hypothesis was assumed that all scions grafted on the newly introduced 'Macrophylla' rootstock are equal in their degree of response to chilling stress. Therefore, the current study evaluated the physiological, biochemical, and anatomical responses of the newly introduced '*C. macrophylla*' rootstock and different scions budded on it to chilling stress.

Material and methods

Plant material and growth conditions

This study was carried out at the experimental research farm of the Faculty of Agriculture of Cairo University (31° 12' 32 E, 30° 00' 44 N), Giza, Egypt. This study included one-year-old seedlings of three main citrus genotypes: 'Olinda Valencia orange' [*Citrus sinensis* (L.) Osbeck], 'Murcott Tangerine']*Citrus reticulata*) L.(Blanco[, and 'Limoneira 8A Lisbon']*Citrus limon*) L.(Burm.[grafted on '*Citrus macrophylla*' rootstock (MR), grown in six-liter plastic bags in a mixture of silt and sand soil under natural light with an average temperature of 30/25 °C (day/night). '*Citrus macrophylla*' rootstock (MR) was also studied individually to investigate its responses as a rootstock. All citrus genotypes were obtained from a private company in the Nobariya district and were selected based on their growth uniformity. All citrus genotypes were irrigated at two intervals with tap water to prevent the development of a water deficit, treated with suitable pesticides to

avoid pathological stress, and all other recommended culture operations were applied at an appropriate stage.

Experimental treatments

For the nocturnal chilling and recovery treatments, two groups of each studied citrus genotype were divided randomly. The first group was kept under greenhouse conditions (30/25 °C, day/night) throughout the experiment (control). The second group was transferred to the growth chamber at 20/4 °C±1 (day/night) for three days (eight-night hours of dark chilling/day), and then the same group was transferred to the greenhouse conditions (30/25 °C, day/night) for three days of recovery after being stressed. For each genotype, 54 plants were used, 27 of which were used as control treatments, and the other 27 were used for stress and recovery treatments. Leaf samples were taken immediately after each time point (control, three days of chilling, and recovery) from the 3rd to 5th leaf from the top of the plant from each genotype. These samples were then transported to the laboratory, where the following measurements were carried out:

Leaf gas exchange parameters

Leaf gas exchange parameters, including leaf diffusion resistance (LDR), leaf stomatal conductance (GS), and transpiration rate (E), were measured in sun-exposed and fully expanded leaves using a portable photosynthesis system (Steady State Porometer, LI-1600, LI-COR, Inc., Lincoln, Nebraska, USA).

Chlorophyll (a, b, and total) concentration

Extraction of chlorophyll was carried out from 0.25 g leaf material with 80% (*v/v*) of acetone (20 ml). The absorbance was detected at 663, and 646 nm, with a spectrophotometer (JANEWAY 6300, Staffordshire, UK), against a solvent (acetone) blank, and the chlorophyll concentration was calculated according to Lichtenthaler and Wellburn (1983) using the following equations:

Chl *a* (mg g^{-1} fw) = 12.21 × A_{663} – 2.81 × A_{646}

Chl *b* (mg g^{-1} fw) = 20.13 × A_{646} – 5.03 × A_{663}

Total chlorophyll concentration (mg g^{-1} fw) = Chl *a* + Chl *b*

Proline concentration

Proline concentration was determined in 0.5 g leaf material according to Bates et al. (1973) using a standard curve and calculated on a fresh weight basis as follows: µmoles proline/g of fresh weight = [(µg proline/mL× mL toluene)/115.5 µg/µmole]/ [(g sample)/5].

Malondialdehyde (MDA) concentration

The MDA content was calculated according to Draper et al. (1990) using its absorption coefficient of 155 nmol^{-1} cm^{-1} and expressed as nmol (MDA) g^{-1} fresh weight.

Extraction and determination of antioxidant enzyme activity

Two grams of citrus leaves were homogenized with 10 mL of phosphate buffer pH 6.8 (0.1 M), then centrifuged in a refrigerated centrifuge at 2 °C for 20 minutes at 20000 rpm. The clear supernatant (containing the enzymes) was taken as the source of the enzymes (MUKHERJEE; CHOUDHURI, 1983). Superoxide dismutase (SOD; E.C.1.15.1.1) activity was determined by measuring the inhibition of the auto-oxidation of pyrogallol using a method described by Marklund and Marklund (1974). The rate of pyrogallol reduction was measured at 325 nm with a UV-spectrophotometer (Jenway). Catalase (CAT; E.C.1.11.1.6) activity was determined by measuring the rate change of H_2o_2 absorbance in 60 seconds with a UV spectrophotometer (Jenway) at 250 nm, according to the method of Chen et al. (2000). Peroxidase (POX; EC 1.11.1.7) activity was assayed using a solution containing 5.8 mL of 50 mM phosphate buffer pH 7.0, 0.2 mL of the enzyme extract, and 2 mL of 20 mM H_2O_2. After the addition of 2 mL of 20 mM pyrogallol, the rate of increase in absorbance as pyrogallol was determined spectrophotometrically by UV-spectrophotometer (Jenway) within 60 seconds at 470 nm and 25 °C (Bergmeyer and Bernt 1974). The blank sample was made by using buffer instead of enzyme ex-

tract. Polyphenol oxidase (PPO; EC 1.10.3.1) activity was assayed using 125 µmol phosphate buffer (pH 6.8), 100 µmol of pyrogallols, and 2 mL of enzyme extract. After the incubation period of 5 minutes at 25 °C, the reaction was stopped by adding 1 mL of 5% H_2SO_4. The blank sample was made by using very well-boiled enzyme extract, the developed color was read at 430 nm and the enzyme activity was expressed as v the changes in the optical density/gram fresh weight/hour (KAR; MISHRA, 1976). In the case of enzyme assay, volume at zero time was taken as blank and the activity of the enzyme / g fresh weight/hour was expressed as (Δ × T v × 60 min) / (t × v × fw) where, Δ is the absorbance of the sample after incubation minus the absorbance at zero time, T v is the total volume of filtrate, t is the time (minutes) of incubation with substrate and v is the total volume of filtrate taken for incubation and fw is the fresh weight used (FICK; QUALSETT, 1975).

Anatomical studies

Tested material included the fourth leaf developed on the top of the main stem from three main citrus genotypes: 'Valencia orange' [*Citrus sinensis* (L.) Osbeck], 'Murcott Tangerine' [*Citrus reticulata* (L.(Blanco[, and 'Limoneira 8A Lisbon']*Citrus limon*) L.(Burm[grafted on 'Macrophylla', and 'Macrophylla' rootstock (MR), exposed to chilling and recovery treatment. Approximately 1.0 cm of the specimens were killed and fixed in FAA solution (5 mL glacial acetic acid, 10 mL formalin, 35 mL water, and 50 mL ethyl alcohol 70%) for at least 48 h. The selected materials were washed in 30% ethyl alcohol, dehydrated in normal ethanol and butyl alcohol, embedded in paraffin wax with a melting point of 56 °C, sectioned to a thickness of 15 µm stained with crystal violet-erythrosin, cleared in xylene, and mounted in Canada balsam in accordance with Mohammed and Guma (2015). Transverse sections were done with a Leica Microtome RM 2125. Then, they micrographed and measured using a Leica Light Image Analysis System DM 750 at the Faculty of Agriculture, Cairo University-Research Park (CURP). The following parameters were recorded: the thickness of the midvein (µm), lamina (µm), palisade tissue (µm), spongy tissue (µm), the upper epidermis (µm), the lower epidermis (µm), and the dimension of the vascular bundle of the midvein (µm).

Statistical analysis

The statistical analysis of the data comprised completely randomized block design (RCBD) in 3×4 factorial scheme (three conditions and four genotypes), with three replicates (n=9) using the online studio of SAS® OnDemand for Academics (*https://www.sas.com/en_us/software/on-demand-for-academics.html*) followed by the Duncan's multiple range test (DUNCAN, 1955) for all measurements except the anatomical measurements, followed by the t-test (LSD) as given by Snedecor and Cochran (1989) at a significance level of 0.05. Regression analysis was done between total chlorophyll and all other parameters. Pearson's simple correlation was used to determine the relationships between total chlorophyll and different physiological and biochemical parameters for each of the three temperature treatments (control, stress, and recovery).

Results and Discussion

Leaf gas exchange parameters

It is clear from the results in Table 1 that leaf diffusion resistance (LDR) following the nocturnal chilling treatment of all studied genotypes showed a significant increase, whereas the recovery and control treatments recorded the lowest significant values. The 'Murcott' genotype appeared to be the most significant ($P \leq 0.05$) genotype that was affected by the nocturnal chilling with a value equal to 26.000 s cm^{-1}, whereas the control transplants of the 'Valencia' genotype recorded the lowest significant ($P \leq 0.05$) value (2.070 s cm^{-1}). The results of Table 1 also showed that the control and recovery treatments of the four studied genotypes had the highest significant values of

stomatal conductance (GS), while the nocturnal chilling (stress) treatment had the lowest values. In details, 'Macrophylla' and 'Limoneira 8A' recorded the highest values of stomatal conductance without any significant difference between them (0.046 and 0.045 mol m^{-2} s^{-1}, respectively), while the lowest significant ($P \leq 0.05$) value was noticed with 'Murcott' genotype under stress conditions compared to the other studied genotypes and other treatments. Also, data tabulated in Table 1 showed that the transpiration rate was influenced by the nocturnal chilling to record a considerable decrease, while the recovery treatment recovered this parameter at its normal level compared to the control treatment in all studied citrus genotypes. 'Valencia' genotype following the recovery treatment showed the most significant ($P \leq 0.05$) increase in transpiration rate (7.559 mmol m^{-2} s^{-1}). In contrast, the least significant ($P \leq 0.05$) value was detected in 'Limoneira 8A' and 'Murcott' genotypes (0.574 and 0.543 mmol m^{-2} s^{-1}, respectively) following the nocturnal chilling treatment, sharing the same significance level.

Table 1. Changes in leaf diffusion resistance (LDR), stomatal conductance (GS), and transpiration ratio (E) of some citrus genotypes in response to nocturnal chilling stress and recovery. Different upper-case letters represent statistical differences ($P \leq 0.05$, Duncan) among the four genotypes within each treatment (each row). Different lower-case letters represent statistical differences ($P \leq 0.05$, Duncan) among the three treatments within each genotype (each column).

Genotype	Treatmen	'Macrophylla' (MR)	'Valencia'/MR	'Murcot'/MR	'Limoneira 8A'/MR
Leaf diffusion resistance (LDR) (s cm^{-1})	Control - 30/25 °C±1	3.175 Ab	2.070 Db	2.680 Cc	2.910 Bb
	Stress - 20/4 °C±1	21.800 Ca	23.850 Ba	26.000 Aa	22.350 Ca
	Recovery - 30/25 °C±1	3.030 Bb	3.350 ABb	3.697 Ab	2.555 Cc
Stomatal conductance (GS) (mol m^{-2} s^{-1})	Control - 30/25 °C±1	0.315 Db	0.483 Aa	0.373 Ba	0.344 Cb
	Stress - 20/4 °C±1	0.046 Ac	0.042 Bc	0.038 Cc	0.045 Ac
	Recovery - 30/25 °C±1	0.330 Aa	0.300 BCb	0.271 Cb	0.392 Aa
Transpiration ratio (E) (mmol m^{-2} s^{-1})	Control - 30/25 °C±1	4.566 Cb	7.012 Ab	5.802 Ba	7.122 Aa
	Stress - 20/4 °C±1	0.643 Ac	0.585 ABc	0.543 Bb	0.574 Bc
	Recovery - 30/25 °C±1	6.047 Ba	7.559 Aa	5.983 Ba	4.660 Cb

Chlorophyll (a, b, and total) concentration

Plant pigments (Chlorophyll *a*, *b*, and total) were significantly ($P \leq 0.05$) affected by the nocturnal chilling treatment (Table 2). Chlorophyll *a* recorded a significant decrease following the nocturnal chilling, and then increased after the recovery treatment to a value close to the normal level in all genotypes. The most significant ($P \leq 0.05$) value was noticed in 'Murcott' under the recovery conditions (0.968 mg g^{-1} fw). Meanwhile, the most significant increase in chlorophyll *a* was observed in 'Valencia' (0.442 mg g^{-1} fw) following the nocturnal chilling. In contrast, the least significant ($P \leq 0.05$) value was observed in 'Macrophylla' following the nocturnal chilling, with value equal to 0.324 mg g^{-1} fw (Table 2). Similarly, chlorophyll *b* decreased significantly in the four studied genotypes following the nocturnal chilling sharing the same significance level and recovered again after the recovery period as compared to the control. However, 'Murcott' genotype showed the most significant ($P \leq 0.05$) increase in chlorophyll *b* (0.266 mg g^{-1} fw) under the recovery conditions with no significant variance among the other three genotypes (Table 2).

Table 2. Changes in leaf chlorophyll *a*, *b*, and total chlorophyll concentration of some citrus genotypes in response to nocturnal chilling stress and recovery. Different upper-case letters represent statistical differences ($P \leq 0.05$, Duncan) among the four genotypes within each treatment (each row). Different lower-case letters represent statistical differences ($P \leq 0.05$, Duncan) among the three treatments within each genotype (each column).

Genotype	Treatmen	'Macrophylla' (MR)	'Valencia'/MR	'Murcot'/MR	'Limoneira 8A'/MR
Chlorophyll a concentration (mg g^{-1} fw)	Control - 30/25 °C±1	0.809 Ca	0.936 ABa	0.964 Aa	0.901 Ba
	Stress - 20/4 °C±1	0.324 Cb	0.442 Ac	0.363 Bb	0.365 Bc
	Recovery - 30/25 °C±1	0.785 Ca	0.888 Bb	0.968 Aa	0.475 Db
Chlorophyll b concentration (mg g^{-1} fw)	Control - 30/25 °C±1	0.201 Da	0.224 Ca	0.262 Aa	0.231 Ba
	Stress - 20/4 °C±1	0.138 Ac	0.150 Ac	0.148 Ab	0.139 Ab
	Recovery - 30/25 °C±1	0.181 Bb	0.207 Bb	0.266 Aa	0.198 Ba
Total chlorophyll concentration (mg g^{-1} fw)	Control - 30/25 °C±1	1.010 Ca	1.160 Ba	1.226 Aa	1.132 Ba
	Stress - 20/4 °C±1	0.462 Cb	0.592 Ac	0.511 Bb	0.504 Bc
	Recovery - 30/25 °C±1	0.967 Ca	1.095 Bb	1.234 Aa	0.672 Db

Consequently, total chlorophyll was affected by the nocturnal chilling to show a marked decrease in all genotypes as compared to the control, while the recovery period raised it also to a value greater than the stressed plants. At stress treatment, 'Macrophylla' detected the least significant ($P \leq 0.05$) concentration (0.462 mg g^{-1} fw) compared to all genotypes within the stress treatment, and all treatments within 'Macrophylla' rootstock. Meanwhile, 'Valencia' genotype gained the highest significant ($P \leq 0.05$) concentration under the same treatment (stress) equal to 0.592 mg g^{-1} fw. Moreover, 'Murcott' genotype at recovery treatment noticed the highest concentration of total chlorophyll (1.234 mg g^{-1} fw) compared to other genotypes and other treatments as well (Table 2). A significant positive correlation was found for total chlorophyll concentration with transpiration (E) and stomatal conductance (GS), while it was negative for leaf diffusion resistance (LDR) (Figure 1).

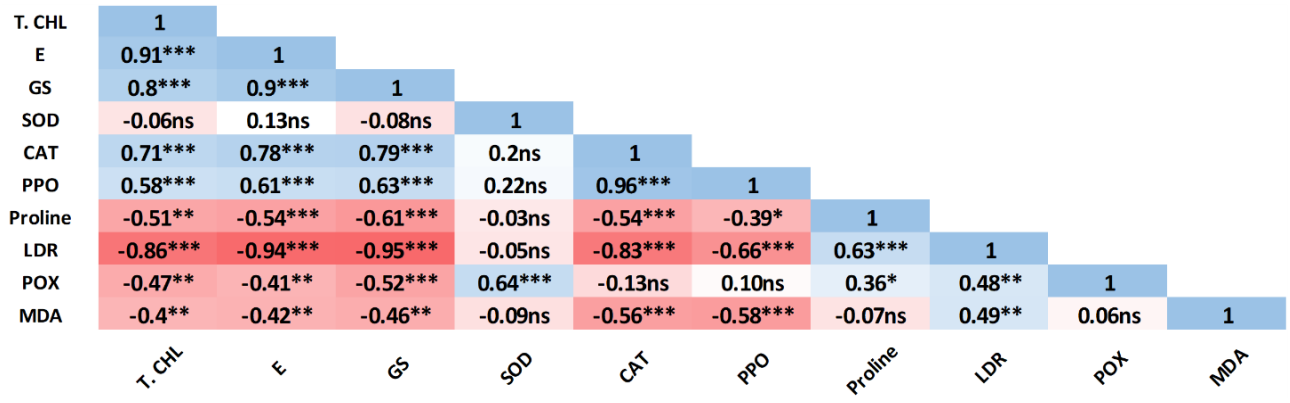
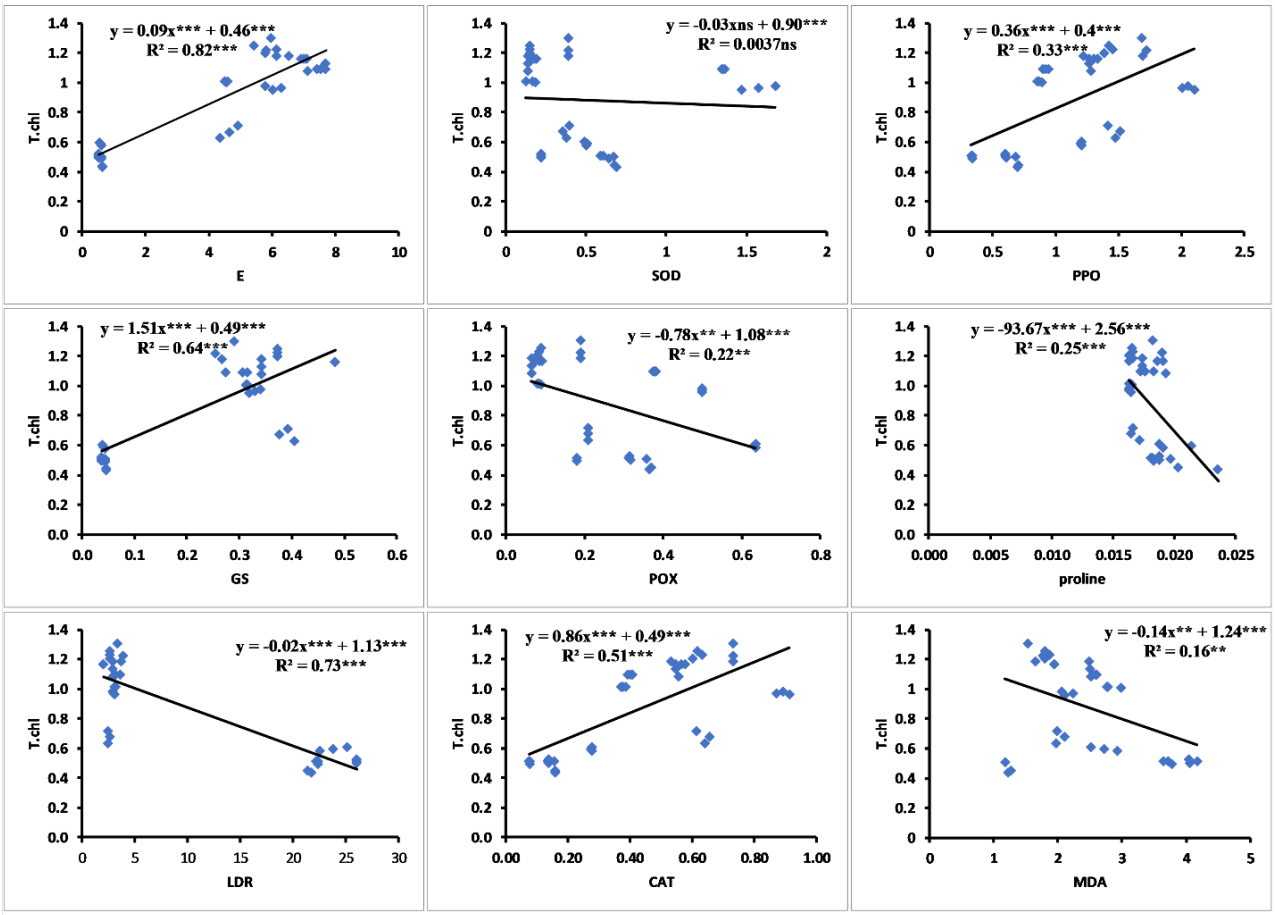


Figure 1 - Pearson's correlation coefficient analysis of total chlorophyll (T.CHL) with different physiological and biochemical parameters as per the treatment means of all temperature regimes. These parameters include transpiration rate (E), leaf stomatal conductance (GS), superoxide dismutase (SOD), catalase (CAT), polyphenol oxidase (PPO), proline, leaf diffusion resistance (LDR), peroxidase (POX), and malondialdehyde (MDA). R is presented in different colors; the blue color refers to the positive correlation, while the red color indicates the negative correlation. R values with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ as indicators of statistical significance.

Regarding the simple linear regression, positive values were found for the coefficient of X_E ($0.09X_E$***; R^2 = 0.82***) and X_{GS} ($1.51X_{GS}$***; R^2 = 0.64***) variables, while the coefficient of X_{LDR} recorded a negative value ($-0.02X_{LDR}$***; R^2 = 0.73***) (Figure 2).



$$Y=+0.12X_E^{***}-1.09X_{GS}^{**}-0.01X_{LDR}^{ns}+0.12X_{SOD}^{ns}-1.30X_{POX}^{ns}-3.23X_{CAT}^{*}+1.58X_{PPO}^{*}-29.96X_{proline}^{ns}+0.009X_{MDA}^{ns}+1.13^{**}$$

$$R^2 = 0.90^{***}$$

Figure 2 - Simple linear regressions (within boxes) and multiple regressions (below the boxes) were illustrated in some citrus genotypes between the total chlorophyll as dependent variable and transpiration rate (E), leaf stomatal conductance (GS), leaf diffusion resistance (LDR), superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), polyphenol oxidase (PPO), proline, and malondialdehyde (MDA) as independent variables.

Our results revealed that citrus genotypes showed marked and different changes in physiological, biochemical, and anatomical behaviour in response to nocturnal chilling. The increase in leaf diffusion resistance (LDR) observed following the nocturnal chilling periods for all genotypes led to the decline in stomatal conductance (GS) and transpiration rate (E) (Table 1). Furthermore, 'Murcott' cultivar (cold tolerant) under the chilling stress showed the most increase in LDR versus 'Limoneira 8A' and 'Macrophylla' rootstock (cold sensitive), which recorded the lowest LDR under the stress conditions with no significant differences between them. Also, GS and E were significantly different among the cultivars under the chilling conditions, especially in 'Murcott' cultivar, which showed a significant decrease in GS and E. These results were clearly confirmed by the correlation test, which showed that E and GS were positively correlated with each other but negatively correlated with LDR (Figure 1). Plants can preserve their water potential in response to nocturnal chilling by closing their stomata to avoid water loss through transpiration, which affects gas exchange and CO_2 fixation (VU; YELENOSKY, 1987; ALLEN; ORT,

2001; RIBEIRO et al., 2009; SANTINI et al., 2013; OUSTRIC et al., 2017). One of the most important reasons for limiting photosynthetic activity is low temperature, and chlorotic leaves are a common symptom that a plant is stressed. Chlorosis can occur for one of two reasons: either the rate of synthesis of chlorophyll precursors is slowed, or the rate of photodestruction of chlorophyll and associated pigments exceeds the rate of synthesis (MCWILLIAM; NAYLOR, 1967). Total chlorophyll concentration had a significant positive correlation with transpiration (E) and stomatal conductance (GS), but it had a negative correlation with leaf diffusion resistance (LDR) and MDA (Figure 1). The coefficient of X_{LDR} and X_{MDA} recorded negative values (-0.02 X_{LDR} ***; R^2 = 0.73***) (-0.14x**X_{MDA}; R^2 = 0.16**), respectively, according to the simple linear regression (Figure 2). These results indicated that the decrease in chlorophyll *a*, *b*, and total chlorophyll concentration (Table 2) is due to the increase in leaf diffusion resistance and the accumulation of ROS in leaf cells, which led to chlorophyll degradation and disruption of carbon assimilation, consequently photosynthesis and plant growth. Regarding the cultivars under chilling stress, 'Valencia' (moderately cold-tolerant) recorded the most significant concentration of total chlorophyll, followed by 'Murcott' and 'Limoneira 8A' without any significant differences between them, while the lowest concentration was found in 'Macrophylla' rootstock. A similar decrease in chlorophyll concentration was observed in different plant species under chilling stress such as citrus (YELENOSKY; GUY, 1977; VU; YELENOSKY, 1987; OUSTRIC et al., 2017; HMMAM et al., 2023), mango (SAYED et al., 2020; HMMAM et al., 2022), papaya (PRADHAN et al., 2019), and rice (AGHAEE et al., 2011).

Proline concentration

Compared with the control, stress treatment recorded the highest significant ($P \leq 0.05$) proline concentrations only in 'Macrophylla' rootstock and 'Murcott' genotype (0.021 and 0.019 µmoles g^{-1} fw). Meanwhile within 'Valencia' and 'Limoneira 8A' there was no significant ($P \leq 0.05$) differences in proline concentration under the chilling conditions compared with the control. Within stress treatment, the 'Macrophylla' rootstock and 'Valencia' genotype detected the most significant ($P \leq 0.05$) proline concentrations (0.021 and 0.020 µmoles g^{-1} fw) without any significant differences between them, whereas 'Murcott' and 'Limoneira 8A' showed the lowest significant ($P \leq 0.05$) concentration (0.019 and 0.018 µmoles g^{-1} fw) with no significant differences between them (Table 3).

Table 3. Changes in leaf proline concentration and malondialdehyde (MDA) concentration some citrus genotypes in response to nocturnal chilling stress and recovery. Different upper-case letters represent statistical differences ($P \leq 0.05$, Duncan) among the four genotypes within each treatment (each row). Different lower-case letters represent statistical differences ($P \leq 0.05$, Duncan) among the three treatments within each genotype (each column).

Genotypes	Treatments	Macrophylla (MR)	Valencia/MR	Murcott/MR	Limoneira 8A/MR
Proline concentration (µmoles g^{-1} fw)	Control - 30/25 °C±1	0.016 Ab	0.018 Aa	0.017 Ab	0.018 Aa
	Stress - 20/4 °C±1	0.021 Aa	0.020 ABa	0.019 Ba	0.018 Ba
	Recovery - - 30/25 °C±1	0.016 Bb	0.018 Aa	0.018 Aa	0.017 Ba
Malondialdehyde (MDA) concentration (nmol g^{-1} fw)	Control - 30/25 °C±1	2.835 Aa	1.938 Cb	1.818 Cb	2.491 Bb
	Stress - 20/4 °C±1	1.220 Dc	2.710 Ca	4.083 Aa	3.709 Ba
	Recovery - 30/25 °C±1	2.128 Bb	2.574 Aa	1.646 Cc	2.019 Bc

Malondialdehyde (MDA) concentration

After the exposure to the nocturnal chilling, a significant ($P \leq 0.05$) increase in malondialdehyde (MDA) concentration was observed in all studied genotypes compared to normal conditions (control and recovery treat-

ments) except 'Macrophylla' rootstock. Also, 'Murcott' genotype disclosed the highest significant ($P \leq 0.05$) value of MDA (4.083 nmol g^{-1} fw) at stress treatment (Table 3).

Proline is an amino acid that acts as an osmoprotector. Proline can regulate many functions in the cell, particularly in osmotic adjustment by increasing the ability to resist cell dehydration. Also, at the cellular level, by promoting the stabilization of subcellular structures and membranes, the stabilization of proteins upon denaturation, and the detoxification of reactive oxygen species (ROS) (PRIMO-CAPELLA et al., 2021). In this study, only for Macrophylla' and 'Murcott/MR' there was an increase in the proline content in the stress (Table 3). These results are in line with previous reports on diverse plants such as Citrus (YELENOSKY, 1979; KUSHAD; YELENOSKY, 1987; OUSTRIC et al., 2017; PRIMO-CAPELLA et al., 2022) *Brassica napus* (LEI et al., 2019), and papaya (PRADHAN et al., 2019). One of the end products of the process of stress-induced lipid peroxidation of polyunsaturated fatty acids is MDA (LESHEM, 1987). MDA acts as a biomarker for oxidative damage that occurs during chilling stress (MUKHOPADHYAY; ROYCHOUDHURY, 2018). Alternately, it might indicate the activation of defence responses (KU´ZNIAK; URBANEK, 2000; RAZA et al., 2021). An increase in MDA concentration was observed following the chilling stress. Moreover, 'Murcott' cultivar (cold tolerant) noticed the highest significant concentration compared to other genotypes within chilling stress, whereas 'Macrophylla' rootstock (cold sensitive) recorded the lowest amount under the same condition (Table 3). These findings are consistent with earlier research on many plants, including citrus (TAJVAR et al., 2011; PRIMO-CAPELLA et al., 2022; HMMAM et al., 2023), coffee (CAMPOS et al., 2003), and papaya (PRADHAN et al., 2019).

Antioxidant enzyme activity

Nocturnal chilling significantly ($P \leq 0.05$) increased superoxide dismutase (SOD) enzyme activity compared to the control. The effects of nocturnal chilling and genotypes on SOD activity were significant ($P \leq 0.05$), and the highest and lowest activities of SOD were observed in 'Macrophylla' and 'Murcott' genotypes (0.680 and 0.219 U g^{-1} fw, respectively). 'Macrophylla' recorded the highest significant ($P \leq 0.05$) value (1.575 U g^{-1} fw) compared with other genotypes within recovery treatment, while all genotypes under the control treatment recorded the least values of SOD with the same significance level (Figure 3A). Also, data presented in Figure 3B showed that nocturnal chilling caused a significant decrease in catalase (CAT) activity, while the normal conditions (control and recovery treatments) showed a marked increase in CAT activity. Regarding the genotypes within each treatment, 'Macrophylla' under recovery treatment recorded the highest significant ($P \leq 0.05$) value (0.891 U g^{-1} fw) compared with the other genotypes under the same treatment, whereas 'Limoneira 8A' under stress treatment had the lowest significant ($P \leq 0.05$) value (0.077 U g^{-1} fw). In contrast, the highest significant ($P \leq 0.05$) activity within this treatment (stress) was observed in 'Valencia' with a value equal to 0.275 U g^{-1} fw. Moreover, the effects of nocturnal chilling and genotypes on peroxidase (POX) activity were significant ($P \leq 0.05$). The nocturnal chilling increased the POX activity compared to the control. Concerning genotypes, 'Valencia' genotype at stress treatment obtained the most significant increase in POX activity (0.633 U g^{-1} fw), and the least significant ($P \leq 0.05$) value (0.180 U g^{-1} fw) was recorded with the 'Limoneira 8A' (Figure 3C). Furthermore, polyphenol oxidase (PPO) showed a significant reduction ($P \leq 0.05$) following the nocturnal chilling treatments, while the normal conditions (control and recovery) recorded a significant increase in PPO activity (Figure 3D). 'Macrophylla' at recovery treatment had the most significant ($P \leq 0.05$) value (2.050 U g^{-1} fw), however the lowest significant ($P \leq 0.05$) value under the same treatment was recorded in 'Valencia' genotype. Whereas under stress treatment, the highest significant ($P \leq 0.05$) value was detected in 'Valencia' (1.203 U g^{-1} fw), while 'Limoneira 8A' recorded the least significant value (0.336 U g^{-1} fw).

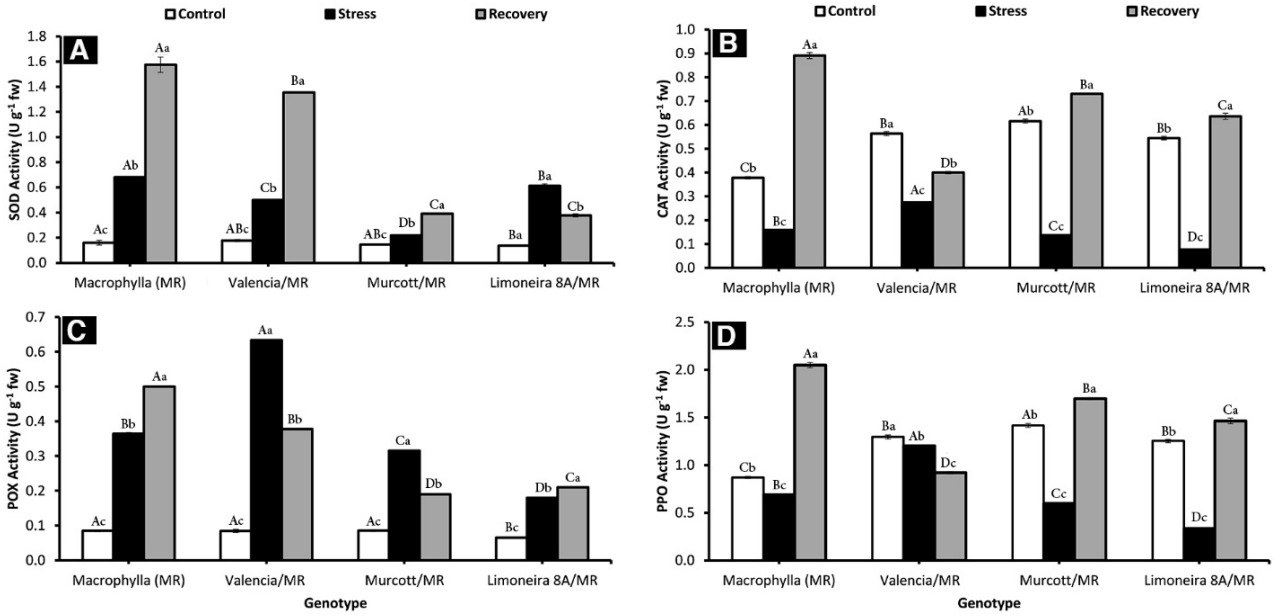


Figure 3 - Changes in leaf Antioxidant enzymatic activities. A) superoxide dismutase (SOD); B) catalase (CAT); C) peroxidase (POX), and D) polyphenol oxidase (PPO) of some citrus genotypes in response to nocturnal chilling stress and recovery. Different upper-case letters represent statistical differences ($P \leq 0.05$, Duncan) among the four genotypes within each treatment. Different lower-case letters represent statistical differences ($P \leq 0.05$, Duncan) among the three treatments within each genotype. Error bars represent stander error (SE).

The most common ROS-scavenging mechanisms in plant systems are antioxidant enzymes (SOD, POX, CAT, and PPO) (KAPOOR et al., 2019; HUCHZERMEYER et al., 2022). The increase of SOD activity (Figure 3A) by nocturnal chilling may have contributed to the protection of chloroplasts and peroxisomes by removing excess superoxide and H_2O_2 radicals and mitigating oxidative damage caused by the imbalance between the photochemical and biochemical phases of CO_2 uptake (MITTLER, 2002). Also, Tajvar (2011) found that the SOD level significantly increased in Thomson Navel Orange (*Citrus sinensis*) under low-temperature stress. But despite this increase, 'Macrophylla' rootstock (cold-sensitive) under chilling showed the highest activity of SOD, whereas the 'Murcott' cultivar recorded the lowest activity at the same time point. This result shed light on the fact that grafting 'Murcott' cultivar on 'Macrophylla' rootstock affected the performance of the cultivar and led to a decrease in enzyme activity under stress conditions. Chen et al. (2006) suggested that POX enzyme activity plays a crucial role in plant growth processes by scavenging generated H_2O_2 in plant cells. It has been reported that POX increases in plants when plants are subjected to chilling (MONERRI; GUARDIOLA, 2001; TAJVAR et al., 2011; MACHADO et al., 2013). Our results confirmed that the nocturnal chilling treatment increased the POX activity of citrus leaves (Figure 3B). As regards the genotypes under the chilling, the most significant increase in POX was found in 'Valencia' while the lowest activity was detected in 'Limoneira 8A'.

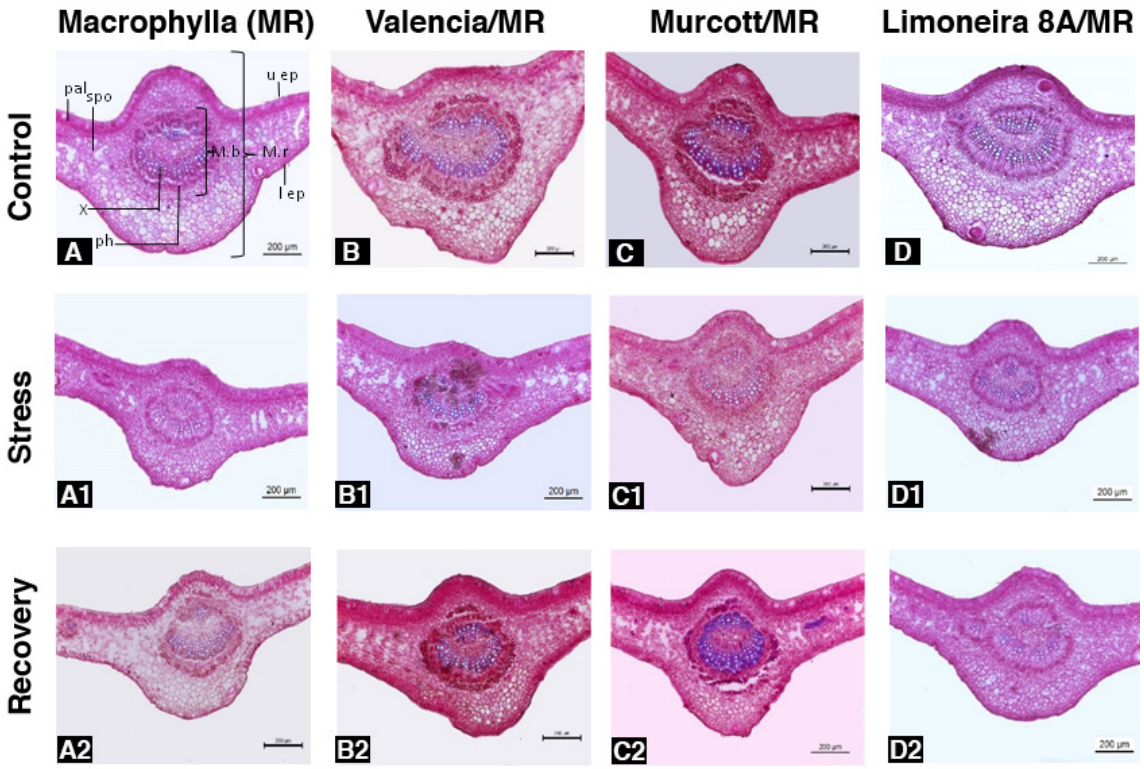
Oxidative enzymes like polyphenol oxidase (PPO), which catalyze the oxidation of phenols to quinones, play a role in the metabolism of phenolic compounds (JUKANTI, 2017). Some studies have reported that PPO and CAT enzyme activities increase in response to different types of both biotic and abiotic stresses (ORTEGA-GARCÍA; PERAGÓN, 2009; RUIZ et al., 1999). PPO activities were lower in all genotypes under the nocturnal chilling treatments in this study. In contrast, the PPO enzyme has been related to the appearance of physiological injuries caused by thermal stress (MARTINEZ-TELLEZ; LAFUENTE, 1997; THIPYAPONG et al., 2007). The inhibition of the phenolic oxidation could be due to the

acclimation mechanism of the plant against nocturnal chilling stress as found in watermelon (RIVERO et al., 2001). In this study, nocturnal chilling caused a significant increase in lipid peroxidation, which may arise from ROS accumulation more than the capacity of anti-oxidative systems to scavenge ROS. Therefore, there was a remarkable decrease in the activities of some antioxidant enzymes (CAT and PPO) assayed following the nocturnal chilling treatments (Figure 3. C and D). These results were confirmed by correlation analysis, which revealed that CAT and PPO were positively correlated but negatively correlated with MDA (Figure 1). Regarding the genotypes under stress, the most increase in CAT and PPO activities was recorded in 'Valencia' while the lowest activities was in 'Limoneira 8A'. Chen et al. (2006) observed a similar decrease in CAT activity in *Sabina* seedlings following exposure to chilling. Furthermore, CAT activity in mulberry seedling leaves reduced at 3 °C and rose during the 25 °C recovery period (LIU et al., 2019). The simple linear regression (Figure 2) for the predictor variables (CAT and PPO) with the dependent variable (total chlorophyll) showed a positive coefficient of X_{CAT} ($0.86X_{CAT}$***; R^2 = 0.51***) and X_{PPO} ($0.36X_{PPO}$***; R^2 = 0.33***). For multiple regression, total chlorophyll was the dependent variable and the variables (E, GS, LDR, SOD, POX, CAT, PPO, proline, and MDA) were used as predictors, with an R^2= 0.90. The anatomical study of the plant tissues is crucial to clarify any internal structure changes.

Data in Table 4 and Figure 4 demonstrated that citrus leaves are sensitive to the effects of nocturnal chilling stress as they are the main organs for photosynthesis and transpiration. This information will contribute to understanding the degree of tolerance of this new rootstock and taking the appropriate decision regarding the feasibility of expanding its cultivation in the future.

Table 4. Measurements (µm) and counts of some histological aspects in the cross sections of forth leaf developed on the median portion of the main stem for genotypes 'Valencia', 'Murcott Tangerine', and 'Limoneira 8A Lisbon' grafted on 'Citrus macrophylla', and 'C. macrophylla' rootstock (MR), aged one year, exposed to chilling and recovery treatments (means of three sections)

Treatments	Genotypes	Histological aspects						
		Thickness of midvein	Thickness of lamina	Thickness of palisade tissue	Thickness of spongy tissue	Dimension of midvein vascular bundle	Thickness of upper epidermis	Thickness of lower epidermis
Control	'Macrophylla' (MR)	865.40	265.01	50.80	214.21	476.57	11.53	10.23
	'Valencia'/MR	911.89	320.81	73.03	246.78	610.93	12.95	12.09
	'Murcott'/MR	890.37	301.25	70.86	234.39	544.94	12.70	11.93
	'Limoneira 8A' /MR	876.10	273.09	64.14	208.95	506.50	12.67	11.65
Stress	'Macrophylla' (MR)	642.67	190.19	35.32	155.87	341.69	12.44	10.74
	'Valencia'/MR	760.52	263.83	57.38	210.45	441.22	13.71	12.12
	'Murcott'/MR	810.24	250.28	60.25	200.03	453.22	13.47	12.15
	'Limoneira 8A' /MR	661.03	210.47	41.81	169.31	377.12	13.08	12.10
Recovery	'Macrophylla' (MR)	670.13	220.35	46.87	174.45	382.42	12.22	10.24
	'Valencia'/MR	800.33	280.96	67.96	217.00	487.05	13.60	12.05
	'Murcott'/MR	851.84	270.58	67.07	203.51	495.86	13.16	12.00
	'Limoneira 8A' /MR	710.33	231.10	50.17	181.93	430.61	12.90	11.90
LSD (P ≤ 0.05)								
Temperature (T)		8.36	26.27	8.03	8.43	8.33	0.79	0.73
Genotype (G)		9.66	30.33	9.28	9.73	9.62	0.91	0.84
G×T		16.74	52.54	16.07	16.86	16.67	1.58	1.46



A = Control of Macrophylla (MR); B = Control of Valencia/MR; C = Control of Murcott/MR; D = Control of Limoneira 8A/MR
A1 = Stress of Macrophylla (MR); B1 = Stress of Valencia/MR; C1 = Stress of Murcott/MR; D1 = Stress of Limoneira 8A/MR
A2 = Recovery of Macrophylla (MR); B2 = Recovery of Valencia/MR; C2 = Recovery of Murcott/MR; D2 = Recovery of Limoneira 8A/MR.

Figure 4 - Microphotographs of cross sections through the fourth leaf developed on the median portion of the main stem for genotypes 'Valencia', 'Murcott Tangerine', and 'Limoneira 8A Lisbon' grafted on 'Citrus macrophylla', and 'C macrophylla' rootstock (MR), aged one year, exposed to chilling and recovery treatments. Scale bars = 200 µm. A- From Macrophylla plants, A1- From Macrophylla plants exposed to stress treatment, A2- From Macrophylla plants exposed to recovery treatment, B- From Valencia plants, B1- From Valencia plants exposed to stress treatment, B2- From Valencia plants exposed to recovery treatment, C-From Murcott plants, C1- From Murcott plants exposed to stress treatment, C2- From Murcott plants exposed to recovery treatment, D- From Limoneira plants, D1- From Limoneira plants exposed to stress treatment, D2- From Limoneira plants exposed to recovery treatment Details: mid b, midvein bundle; mid r, midvein region; ph, phloem; x, xylem; l ep, lower epidermis; spo, spongy tissue; pal, palisade tissue and u ep, upper epidermis.

Leaf anatomical studies

The data in Table 4 and Figure 4 show that the control plants gave the best results for all anatomical aspects under the study, followed by the recovery treatment, while the nocturnal chilling stress treatment had the lowest value. Concerning the evaluated genotypes, the 'Valencia' genotype recorded the greatest value in the thickness of the midvein, lamina, palisade tissue, spongy tissue, upper epidermis, and lower epidermis by 5.37%, 21.05%, 43.75%, 15.20%, 12.31%, and 18.18%, respectively, when compared with the 'Macrophylla' rootstock (Figure 4. A and B). The dimension of the vascular bundle of the midvein also recorded the highest value of 28.19% when compared with the 'Macrophylla' rootstock. Concerning the chilling stress treatment, the plants of the 'Limoneira 8A' genotype were the most sensitive to the effect of nocturnal chilling stress, followed by the 'Valencia' genotype, followed by the 'Murcott' (being a chilling tolerant variety). 'Murcott' cultivar plants affected by nocturnal chilling stress treatment reduced in the thickness of both lamina and midvein by 16.91% and 9.05%, respectively, less than the control plants of the 'Murcott' cultivar. As well as the thickness of palisade tissue, spongy tissue, and the dimension of midvein vascular bundle by 14.97%, 14.65% and 16.83%, respectively, less than the con-

trol plants of the 'Murcott' cultivar. The upper and lower epidermis increased by 6.06% and 1.84% compared to the control plants of the 'Murcott' genotype (Figure 4. C and C1). The recovery treatment caused enhancements in the anatomical structure of leaves for all genotypes due to the increase in their thickness. Concerning the recovery treatment, 'Murcott' genotype plants were the best in the recovery, followed by 'Valencia', then 'Limoneira 8A' genotype. The recovery treatment of 'Murcott' genotype caused a slight decrease in the thickness of both midvein and lamina of leaves by 4.32% and 10.18% less than the control plants of the 'Murcott' genotype, respectively. The thickness of palisade tissues, spongy tissues, and the dimension of the midvein vascular bundle was slightly decreased by 5.34%, 13.17%, and 9.00%, respectively, less than the control plants of the 'Murcott' genotype. The effect of such treatment is also evident in the upper and lower epidermis by 3.62% and 0.58% more than in the control plants of the 'Murcott' genotype (Figure 4. C and C2). It is worthwhile to note that the values of all tissues in the leaves of 'Murcott' plants that were recovered after stress were decidedly higher than those of stressed conditions, the thickness of midvein and lamina were increased by 5.13% and 8.11%, respectively, over those of stressed conditions due to increments in all included tissues by 11.31%, 1.73%, and 9.40% for the thickness of palisade and spongy tissues, and the dimension of midvein vascular bundle, respectively (Figure 4. C1 and C2).

In this study, nocturnal chilling stress caused a reduction in the leaf midvein of all genotypes compared to the control. This stenosis could be due to decreases in the various tissues that contain the leaf, including lamina, palisade tissue, spongy tissue, and midvein bundles. On the contrary, the thickness of both the upper and lower epidermis increased. This may be attributable to the increase in cell wall thickness. The present results follow those of Xu et al. (2022). They found that leaves of *Camellia weiningensis* seedlings had greater leaf and palisade thicknesses and tissue structure tightness for chilling stress at 4 °C but freezing stress (−4 °C) caused loosening of the leaf tissue structure in both *Camellia weiningensis* and *Camellia oleifera* seedlings. Contrary to the obtained results, Wu et al. (2020) found that leaf thickness of *Camellia oleifera* Abel increased in the low-temperature treatment (6 °C) compared to the normal temperature (25 °C). Also, Hajihashemi et al. (2018) found that cold stress increased the epidermal cell density, stem diameter, number of sclerenchyma, xylem vessel width, and phloem tissue width in all genotypes of *Stevia rebaudiana.* Zhang et al. (2015) found that the arrangement of mesophyll cells in *Triticum* L. cv. Zhengmai 9023 (poor cold tolerance) was more irregular than in cv. Yannong 19, being a hard-tolerant variety after exposure to low temperatures. Mesophyll cells shrank, and their vessels and sieve tubes ruptured at the tillering stage.

Conclusion

Our results revealed that nocturnal chilling significantly changed the physiological, biochemical, and anatomical behaviour of the studied genotypes and the null hypothesis was rejected. The exposure of citrus seedlings to chilling increased the diffusion resistance of the leaf, which led to the reduction of transpiration and stomatal conductance. Consequently, the accumulation of reactive oxygen, which is indicated by the higher value of MDA, results in chlorophyll degradation and an increase in non-enzymatic antioxidants (proline) and enzymatic antioxidants (POX, and SOD). Our results also suggested that only three days of normal conditions after the chilling could help citrus genotypes return to their normal physiological, biochemical, and anatomical behaviour. Amongst the genotypes, 'Murcott' and 'Valencia' were the most tolerant genotypes since they maintained the antioxidative and osmotic activity along with the photosynthetic system, whereas 'Limoneira 8A' genotype and 'Macrophylla' rootstock were

the most affected by the nocturnal chilling. Before citrus cultivation can potentially rely on 'Macrophylla' rootstock under Egyptian conditions, more research is needed to determine the scions/rootstock's resilience to other abiotic stresses. Moreover, the same assessment protocol used in this study can be applied to other tropical fruit crops, which have been greatly affected by the recent chilling stress in Egypt.

Author contributions IH: Conceptualization, Methodology, Experimental design, Investigation, Resources, Reviewing and Editing. RAA: Investigation, Data curation, Formal analysis, Writing-original draft preparation. SNA: Anatomy, Data curation, Writing. AHG: Supervision, Discussion, Revision, Reviewing and Editing. The final manuscript has been read and approved by all authors.

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