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Research Article

Effects of bioactive compounds on chlorophyll fluorescence parameters of Mexican lime seedlings (*Citrus aurantifolia* Swingle) under cold stress

Alireza Sanie Khatam[1], Somayeh Rastegar *[1], Abdolhossein Aboutalebi Jahromi[2], Hamed Hassanzadeh Khankahdani[3] and Seyed Ali Akbar Bagherian[4]

[1] Department of Horticultural Science, Agriculture and Natural Resource College, University of Hormozgan, Bandar Abbas, Iran
[2] Department of Horticultural Science, Agriculture College, Islamic Azad University, Jahrom Branch, Jahrom, Iran
[3] Horticulture Crops Research Department, Hormozgan Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education and Extension Organization (AREEO), Bandar Abbas, Iran
[4] Department of Horticultural Science, Faculty of Agriculture, Jahrom University, Jahrom, Iran
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Abstract

Cold stress is one of the abiotic stresses that leads to most losses in citrus. Mexican lime is one of the commercial citrus cultivars in the world, which is commonly used as a citrus rootstock in the southern regions of Iran. That is one of the most sensitive citrus cultivars to cold stress. In order to investigate the ameliorating effect of some bioactive compounds on chlorophyll fluorescence parameters and electrolyte leakage of Mexican lime seedlings under cold stress, a split-factorial experiment was conducted in the form of the randomized complete block design. The experimental factors included temperature (0 and -6 °C) as a main factor and foliar application of melatonin (0 and 500 µM), mannitol (0 and 50 µM) and acetic acid (0 and 15 µM) as sub-factors. According to the results, the combined application of melatonin + mannitol + and acetic acid, by improving the photosynthetic parameters, reduced the destructive effects of cold stress (0 and -6 ºC) on photosynthetic machinery in Mexican lime seedlings. The highest Area was observed in the treatment of melatonin + mannitol + acetic acid. The treatments of melatonin and melatonin + acetic acid had the highest values of F_v/F_m and F_v/F_0 and the lowest values of F_0/F_m at both temperatures compared to the control. In this study, the most appropriate parameters to identify the best treatments included Area (total area between initial fluorescence curve and maximal fluorescence), maximum quantum yield of PSII photochemistry (F_v/F_m), thermal dissipation quantum yield (F_0/F_m), and an indicator of the activity of the oxygen-evolving complex on the donor side of PSII (F_v/F_0). Therefore, the mentioned bioactive compounds, especially melatonin as well as acetic acid, are proposed to manage cold stress in Mexican lime seedlings.

Keywords: Chilling, Freezing, Citrus, Photosynthesis, Melatonin, Mannitol, Acetic acid

Abbreviations: ABS/RC (absorption flux per active reaction center); Area (total area between initial fluorescence curve and maximal fluorescence); DI_0/RC (dissipated energy flux per active reaction center); ET_0/RC (electron transport flux per active reaction center); F_0 (initial fluorescence intensity when all PSII reaction centers are open); F_m (maximal fluorescence intensity when all PSII reaction centers are closed); F_0/F_m (thermal dissipation quantum yield); F_v (variable fluorescence); F_v/F_m (maximum quantum yield of PSII photochemistry); F_v/F_0 (activity of the oxygen-evolving complex on the donor side of PSII); OEC (oxygen-evolving complex); PI (performance index); PI_{ABS} (Performance index of PSII based to absorption); PI_{total} (performance index electron flux to the final PSI electron acceptors); RC (reaction center); φE_0 (quantum yield of electron transport).

Introduction

On a global scale, citrus is one of the fruit culture sectors with the highest production .The citrus market is generally divided into the production of oranges, mandarins (tangerine, mandarin, clementine, satsuma), lemons, and limes (Primo-Capella *et al.*, 2021). Mexican lime (*Citrus aurantifolia* Swingle cv. Mexican lime) is one of the commercial citrus cultivars in the world (Ladaniya *et al.*, 2020; Romero-romero *et al.*, 2019). However, Mexican lime is one of the most sensitive citrus cultivars to cold stress, so it is used in many studies as a cold-sensitive criterion plant (control) compared to the other citrus cultivars and species (Abouzari *et al.*, 2020).

*Corresponding Author, Email: rastegarhort@gmail.com

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Cold stress, which includes chilling stress (0-15 °C) and freezing stress (<0 °C), is an important factor in geographical distribution, reduced growth and development, and reduced plant productivity (Maleki and Ghorbanpour, 2018). Low-temperature stress is one of the abiotic stresses that leads to most losses in citrus. Citrus is considered a tropical and subtropical crop that is generally vulnerable to chilling and freezing (Primo-Capella *et al.*, 2021). Plants are affected by cold stress in a variety of physiological activities, including photosynthesis. Reactive oxygen species (ROS) are formed in response to abiotic stress, such as a cold. Also, lipids, proteins, carbohydrates, and DNA may all be destroyed by ROS molecules. The major cause of ROS formation in chloroplasts is a CO_2 fixation restriction combined with an electron transport chain over reduction (Maleki and Ghorbanpour, 2018). During cold stress, the absorption of light by chlorophyll molecules exceeds the photosynthetic capacity of the organism. Therefore, the plant, dissipates the absorbed energy through various means (heat and fluorescence) (Hu *et al.*, 2016; Mishra 2018).

Melatonin is a multifunctional hormone that regulates several physiological mechanisms in response to stress (Li *et al.*, 2019). Melatonin delays protein degradation and plays an important role in maintaining the chlorophyll content and photosynthetic capacity of plants under various stress factors (Bose and Howlader, 2020). Changes in chlorophyll fluorescence parameters after melatonin treatment have shown the participation of this substance in improving the response of the photosystem to cold stress (Hu *et al.*, 2016).

Mannitol is an alcoholic sugar and one of the most important products of photosynthesis in some plants and its concentration increases due to abiotic stresses. The role of mannitol as an osmoprotectant, protein and membrane structure stabilizer, ROS scavenger and photosynthetic apparatus protector under stress environments is well known in some plant species (Franzoni *et al.*, 2019; Jawahar *et al.*, 2019). The biosynthesis of compounds such as mannitol is an endergonic reaction and requires large amounts of ATP molecules (Jawahar *et al.*, 2019). Therefore, in situations where the plant is stressed and the supply of ATP molecules is reduced, the use of exogenous osmoprotectants can be promising and increase the tolerance to abiotic stresses in plants (Semida *et al.*, 2020). Mannitol has been shown to ramp up cold tolerance of edible mushrooms, *Volvariella volvacea* (Zhao *et al.*, 2019), and petunia (Chiang *et al*., 2005).

Doses of acetic acid less than 50 mM have been recently targeted as a biostimulant under drought stress conditions for a variety of major crops such as mung bean (Rahman *et al.*, 2019), corn (Allen and Allen, 2020), and begonia (Allen and Allen, 2021). In addition, acetic acid has been shown to increase salinity tolerance of lemon balm (*Melissa officinalis* L.) (Dahajipour Heidarabadi *et al.*, 2021). Acetic acid (CH_3COOH) acts as a mediator in many central metabolic pathways. Acetic acid is a fat-soluble compound and therefore, can cross cell membranes (Lawford and Rousseau, 1993). It seems that acetic acid can be used as an energy source to conserve cellular energy. (Rasheed *et al.*, 2018). Acetic acid has been shown to have antioxidant properties and reduces stress-induced damage by protecting chlorophyll a, chlorophyll b, and carotenoid and reducing ROS (Dahjipour Heidarabadi *et al.*, 2021).

Citrus breeding programs against abiotic stress are very complex as they involve many genes. Hence the importance of new improvement programs to obtain new crops that are more tolerant to abiotic stresses (Primo-Capella *et al.*, 2021). Therefore, foliar application of acetic acid, mannitol and melatonin may improve the photosynthetic system under cold stress. Although in recent years, the use of acetic acid on the response of plants to abiotic stresses has increased, there is no information on the effect of this compound on the response of plants to cold stress. In addition, little is known about the effect of melatonin on chlorophyll fluorescence parameters in citrus. Meanwhile, there is little information about the use of mannitol on the response of plants to stress. Therefore, the main purpose of this study was to investigate the effect of foliar application of melatonin, mannitol, and acetic acid and their combined effects on the fluorescence parameters of citrus seedlings (Mexican lime) under cold stress. In addition, the most appropriate fluorescence parameters were identified among the studied parameters to distinguish treatments effective in reducing the effect of cold stress.

Materials and methods

Treatments and experimental design: Two-year seedlings of Mexican lime were selected based on uniform crown diameter, height, and the number of lateral branches from a commercial and standard nursery (Kargar-fard Nursery, Iran, Jahrom). The seedlings were planted in 9 kg polyethylene bags containing a mixture of sand, soil, and leaf mold in equal volumes. All horticultural care was carried out uniformly for all seedlings. Three months after the complete establishment of seedlings, a foliar application was performed. Materials and concentrations used in this study included melatonin (Sigma-Aldrich, St. Louis, USA) at 0 and 500 µM, mannitol (Merck, Darmstadt, Germany) at 0 and 50 µM, and acetic acid (Kimia Exir, Iran) at 0 and 15 mM. The experiment was carried out as a factorial (Table 1). The first foliar application was performed one week before cold stress and the second foliar application was performed one day before cold stress.

Cold stress was applied based on a 10-year absolute minimum temperature and similar to natural stresses. Refrigeration temperature monitoring was performed using an operator panel (Touch Panel, Weintek, Taiwan) and programmed logic controllers (PLC, Siemens, South Korea). The temperature was programmed to decrease to reach the desired

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Table 1. List of treatments obtained from the factorial composition of the studied agents

Treatment	Main factor (0 and -6 °C)	Sub-factor (Factorial 2×2×2)		
		Melatonin (Me)	Mannitol (Ma)	Acetic acid (AA)
A		0	0	0
B		0	0	15
C		0	50	0
D		0	50	15
E		500	0	0
F		500	0	15
G		500	50	0
H		500	50	15

temperatures (0 and -6 °C) at a rate of 2 °C decrements per hour. The seedlings were subjected to for 6 hours at each of the target temperatures. To create relative cold tolerance and prevent cold shock (Lang *et al.*, 2005), the seedlings were kept at a day/night temperature of 22/10°C for one week and then 18/6 °C for one week. Sixty-four seedlings (group 1) were exposed to 0 ºC for 6 hours to apply chilling stress. At the end of the stress, the chlorophyll fluorescence of the first group was measured. Another 64 seedlings (group 2) were kept at a day/night temperature of 10/-2 °C and 6/-6 °C for one day more to apply freezing stress. The seedlings were placed under -6 °C for 6 hrs. At the end of stress, their chlorophyll fluorescence was measured. Using the timer (Theben, Germany), the photoperiod was set to 12/12 h light/dark, according to the natural rhythm. Lighting was provided by LED linear lamps [light output 80 watts, 6500 lumens flux, 6500 Kelvin color temperature, color rendering index; above 80, and photosynthetic photon flux density (PPFD); 100 µmol m^{-2} s^{-1}].

Chlorophyll fluorescence measurement: The plants' youngest, completely grown, and connected leaves were fixed on a leaf-clip holder in the dark for 20 minutes before the fluorescence experiment. A portable fluorimeter (Pocket PEA, Hansatech Instruments Ltd., Kings Lynn, U.K.) was used to measure chlorophyll fluorescence. Chlorophyll fluorescence parameters were measured and recorded by emitting a light pulse with a wavelength of 627 nm and an intensity of 3500 µmol photons per square meter per second. These parameters included initial fluorescence intensity when all PSII reaction centers (RCs) are open (F_0), maximal fluorescence intensity when all PSII RCs are closed (F_m), the total area between F_0 curve and F_m (Area), variable fluorescence (F_v), thermal dissipation quantum yield (F_0/F_m), the maximum quantum yield of PSII photochemistry (F_v/F_m), the activity of the oxygen-evolving complex (OEC) on the donor side of PSII (F_v/F_0), absorption flux per active RC (ABS/RC), dissipated energy flux per active RC (DI_0/RC), electron transport flux per active RC (ET_0/RC), quantum yield of electron transport (φE_0), Performance index of PSII based to absorption (PI_{ABS}) and performance index electron flux to the final PSI electron acceptors (PI_{total}) (Plich *et al.*, 2020; Strasser *et al.*, 2004).

Measurement of electrolyte leakage (EL) and lipid peroxidation (malondialdehyde (MDA) content): Immediately after stress, Electrolyte leakage was measured using the method described by Lutts *et al.* (1996). Leaf segments (10 mm in size) of plants were put in 10 ml deionized water and incubated at 25°C on a rotational shaker for 24 hours, after which the solution's electrical conductivity (EC_1) was evaluated using an EC meter (Portable Conductivity Meter, 8301, China). Then, the samples were autoclaved at 120°C for 20 min to achieve the final electrical conductivity (EC_2). Finally, electrolyte leakage was determined as follows:

$EL\ (\%) = (EC_1 / EC_2) \times 100$

Lipid peroxidation was measured according to the method of Hodges *et al.* (1999) and was estimated by the thiobarbituric acid (TBA) reaction method. Leaf sample (0.1 g) was homogenized in 2 ml of trichloroacetic acid (5%). The homogenate was centrifuged at 15,000 × g for 15 min. In 500 µL of suspension, trichloroacetic acid (20%) and 2-thiobarbituric acid (0.5%) were added and heated to 95°C for 30 min. The cooled mixture was centrifuged for 10 min at 10,000 × g. The absorbance at 600 nm was subtracted from the data obtained at 532 nm using a spectrophotometer (Germany, Biotech Epoch). The malondialdehyde (MDA) content per gram of fresh weight (FW) was calculated using the MDA extinction coefficient (0.155 mM^{-1} cm^{-1}) and was expressed as µmol MDA g^{-1} fresh weight.

Statistical analysis: This study was performed as a split-factorial experiment in the form of the randomized complete block design with 8 replications. The temperature as a main factor included 0 and -6 °C. In addition, melatonin, mannitol and acetic acid were as sub-factors (a total of 8 treatments). The data were analyzed using SAS 9.1 software. The means were compared with the protected least significant difference test (LSD) and the charts were drawn using Microsoft Excel 2016. Correlation coefficient was conducted based on Pearson method using R. Principal component analysis (PCA) was performed using SPSS version 22.0.

Results

Area and F_o: Temperature had a significant effect on all fluorescence parameters except ET_0/RC and φE_0 (Table 2). The area parameter decreased (37%) with increasing stress from 0° C (520884) to -6° C (328424) (Figure 1a). The highest area was observed in treatment

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Table 2. ANOVA for depentent variables for melatonin, mannitol, acetic acid, temperature and thire interactions for Mexican lime seedling

Parameter	T	Me	Ma	AA	T×Me	T×Ma	T×AA	Me×Ma	Me×AA	Ma×AA	T×Me ×Ma	T×Me × AA	T×Ma × AA	Me×M a× AA	T×Me× Ma×AA
Area	**	**	*	**	NS	NS	NS	*	NS	**	NS	NS	*	**	NS
F_0	**	NS	NS	**	**	**	*	NS	**	NS	NS	NS	NS	**	NS
F_m	**	**	NS	**	NS	NS	*	NS	**	NS	*	NS	*	**	**
F_v	**	**	NS	**	NS	NS	*	NS	**	NS	*	NS	*	**	**
F_0/F_m	**	**	NS	**	**	NS	**	NS	NS	**	**	NS	NS	**	*
F_v/F_m	**	**	NS	**	**	NS	**	NS	NS	**	**	NS	NS	**	*
F_v/F_0	**	**	NS	**	*	NS	**	NS	NS	NS	*	NS	NS	**	**
ABS/RC	**	**	NS	**	*	NS	**	NS	NS	*	NS	NS	NS	**	NS
DI_0/RC	**	**	NS	**	**	NS	**	NS	NS	**	**	NS	NS	**	NS
ET_0/RC	NS	**	**	**	NS	NS	NS	*	**	**	NS	NS	NS	**	**
φE_0	NS	NS	NS	**	NS	**	*	NS	**	**	NS	NS	NS	**	NS
PI_{ABS}	**	**	NS	**	NS	**	**	**	**	**	**	**	**	**	**
PI_{total}	**	**	*	**	**	**	NS	**	**	**	**	NS	NS	**	**
EL	**	*	**	NS	NS	NS	NS	**	NS	NS	NS	NS	NS	**	NS
MDA	**	**	**	NS	NS	NS	NS	NS	**	**	**	NS	NS	**	NS

^{NS} Represent non-significance; * Represents significance at the 0.05 level ** Represents significance at the 0.01 level
Treatments are represented as 'Temperature'(T), 'Melatonin' (Me), 'Mannitol' (Ma) and 'Acetic acid' (AA).

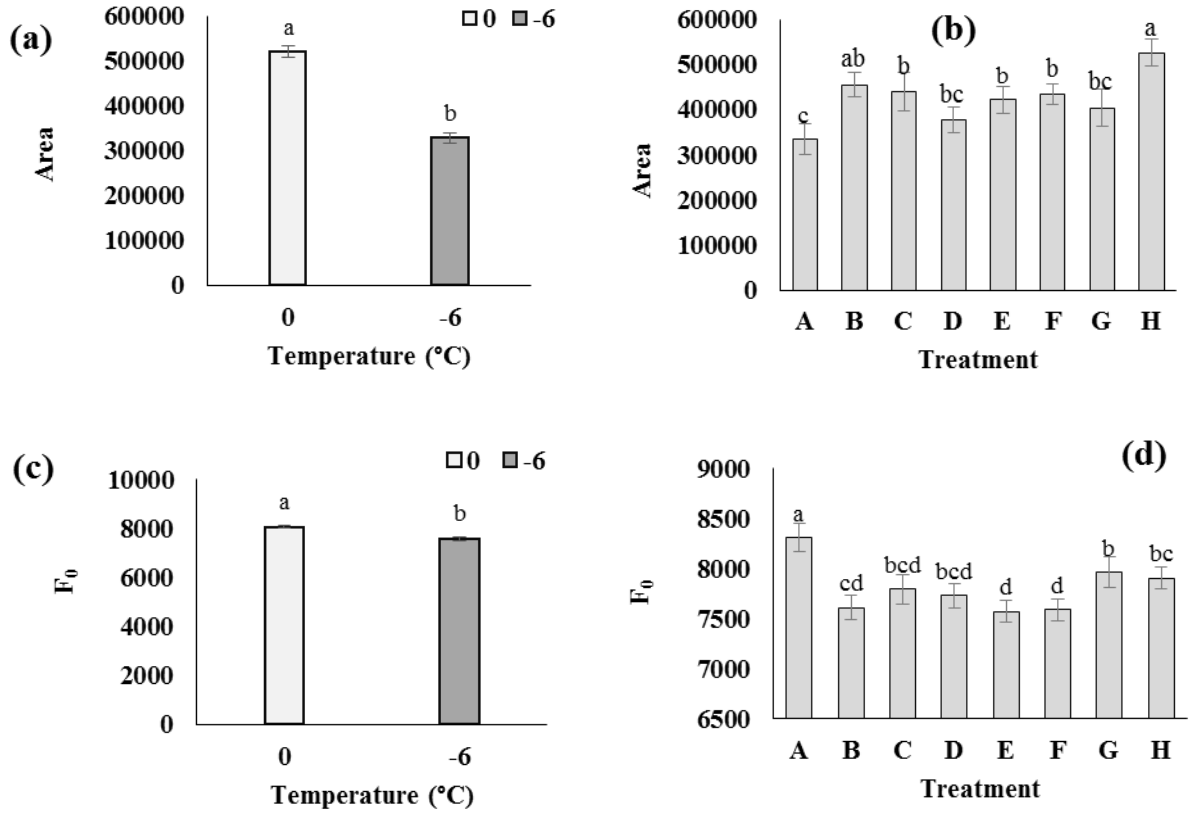

Fig. 1- Comparison of the effect of temperature (0 and -6 °C) on the parameters of Area (a) and F_0 (c); Comparison of the interaction of melatonin, mannitol and acetic acid on the parameters of Area (b) and F_0 (d); Treatments are represented as control (A), acetic acid (B), mannitol (C), mannitol + acetic acid (D), melatonin (E), melatonin + acetic acid (F), melatonin + mannitol (G) and melatonin + mannitol + acetic acid (H); Columns with common letters are not significantly different at the 5% level of PLSD test.

H (526446), followed by treatment B (455823), both of which were significantly different from A (334389) (Figure 1b). The area parameter in treatments D and G, although greater than A, did not significantly differ from it.

The F_0 parameter decreased by 6% with increasing the stress intensity from 0 temperature (8054) to -6°C (7566) (Figure 1c). F_0 was higher in treatment A (8309) with significant differences with all treatments. The lowest F_0 belonged to the treatments E, F, and B (7573,

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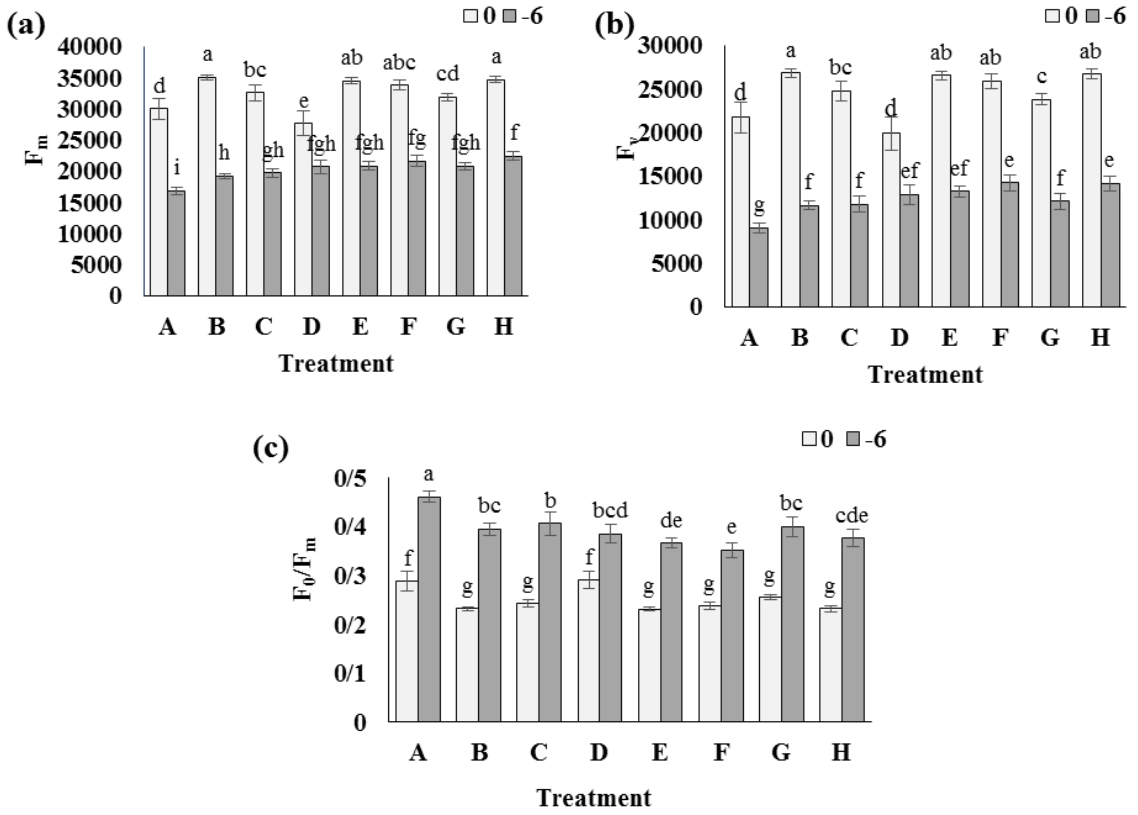

Fig. 2- Comparison of the interaction of temperature, melatonin, mannitol and acetic acid on the parameters of F_m (a), F_v (b) and F_0/F_m (c); Treatments are represented as control (A), acetic acid (B), mannitol (C), mannitol + acetic acid (D), melatonin (E), melatonin + acetic acid (F), melatonin + mannitol (G) and melatonin + mannitol + acetic acid (H); Columns with common letters are not significantly different at the 5% level of PLSD test.

7589, and 7613, respectively) (Figure 1d).

F_m, F_v and F_0/F_m: The highest F_m with significant differences compared to A at 0°C was observed under treatments B, H, and E (35134, 34799, and 34526, respectively), and at -6°C under treatments H, F, E (22491, 21679, and 20888, respectively). The lowest F_m was recorded for treatment A, at both temperatures of 0°C (30073) and -6°C (16885) (Figure 2a).

The highest F_v with significant differences compared to A was recorded for treatments B, H, and E (26876, 26722, and 26578, respectively) at 0°C, and for treatments F, H, and E (14244, 14154, and 13253, respectively) at -6°C. The lowest F_v was recorded for treatment A at both temperatures of 0 and -6°C (Figure 2b).

The highest F_0/F_m was related to treatment A (0.461) at -6°C. At this temperature, the lowest values of F_0/F_m was observed under treatments F, E, and H (0.352, 0.357, and 0.376, respectively). At 0°C, the lowest F_0/F_m was related to treatments E, H, and B (0.231, 0.232, and 0.232, respectively), with significant differences with A (0.288) (Figure 2c).

F_v/F_m and F_v/F_0: The F_v/F_m parameter had a significant increase in all treatments at 0 °C compared to the treatments D and A (0.709 and 0.712). The lowest F_v/F_m was observed in the treatments A and C (0.539 and 0.594, respectively) at -6°C. At this temperature, the highest F_v/F_m was related to the treatments F, E, and H (0.648, 0.633, and 0.624, respectively), which had significant differences with A (Figure 3a).

The highest values of F_v/F_0 was related to the treatments E, H, and B (3.35, 3.33, and 3.31, respectively) at 0°C, which had significant differences with A (2.65). The lowest F_v/F_0 was related to the treatment A (1.20) at -6°C. At this temperature, the highest F_v/F_0 was allocated to the treatments F, E, and H (1.93, 1.77, and 1.76, respectively), with significant differences with A (Figure 3b).

ABS/RC and DI_0/RC: The ABS/RC parameter decreased by 10% with increasing the stress intensity from 0°C (2.09) to -6°C (1.88) (Figure 4a). The highest ABS/RC was observed under the treatment A (2.33). The lowest ABS/RC (1.78) was seen in the treatments B, H, F, and E with a significant difference with A Figure 4b).

The DI_0/RC parameter ramped up by 42% with increasing the stress from 0°C (0.539) to -6°C (0.767) (Figure 4c). The highest DI_0/RC was observed in the treatment A with a significant difference compared to the other treatments. Treatments F, H, and E had the lowest values of DI_0/RC (0.524, 0.554, and 0.564, respectively) (Figure 4d).

ET_0/RC, φE_0, PI_{ABS} and PI_{total}: Regarding the ET_0/RC parameter, treatments H, D, and G were significantly different from A and increased at 0 °C. The highest ET_0/RC was related to treatments H, B, and E at

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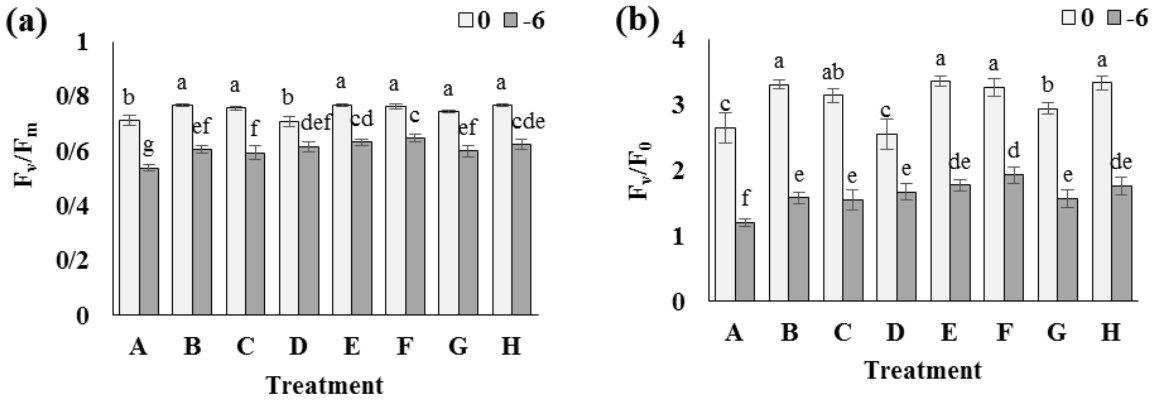

Fig. 3- Comparison of the interaction of temperature, melatonin, mannitol and acetic acid on the parameters of F_v/F_m (a) and F_v/F_0 (b); Treatments are represented as control (A), acetic acid (B), mannitol (C), mannitol + acetic acid (D), melatonin (E), melatonin + acetic acid (F), melatonin + mannitol (G) and melatonin + mannitol + acetic acid (H); Columns with common letters are not significantly different at the 5% level of PLSD test.

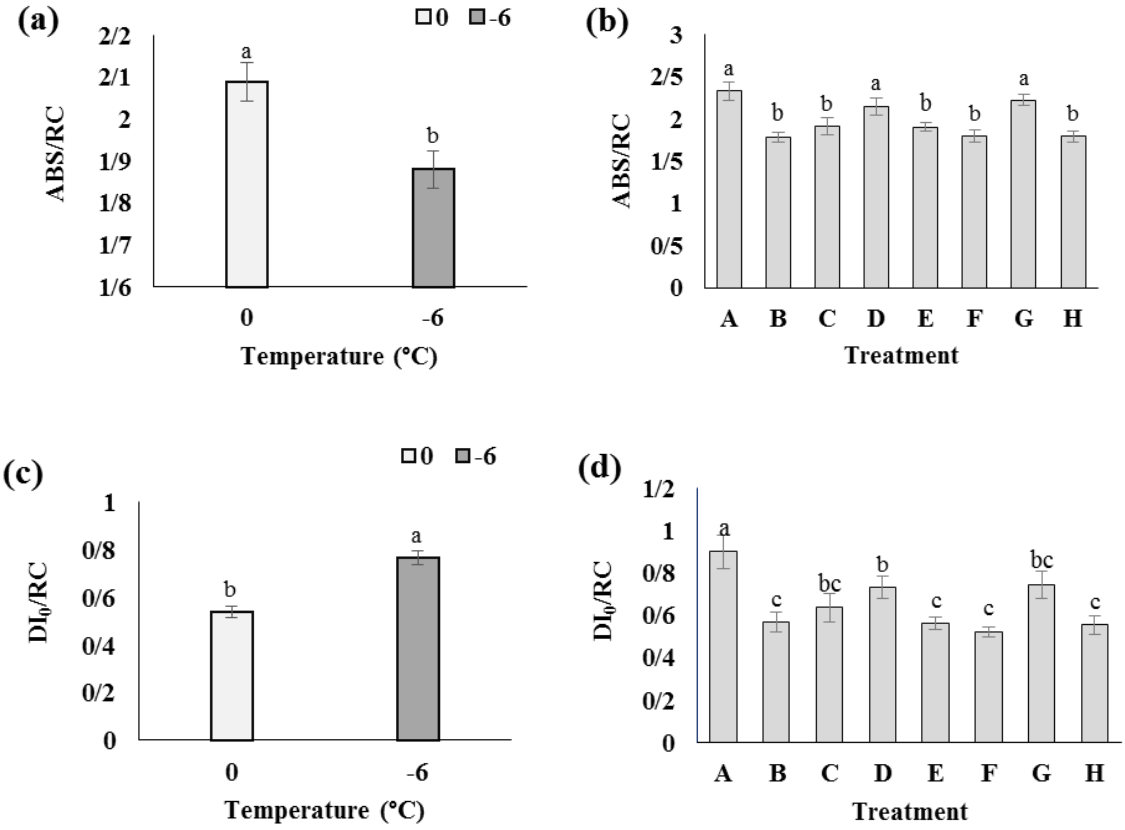

Fig. 3- Comparison of the effect of temperature (0 and -6 °C) on the parameters of ABS/RC (a) and DI_0/RC (c); Comparison of the interaction of melatonin, mannitol and acetic acid on the parameters of ABS/RC (b) and DI_0/RC (d); Treatments are represented as Control (A), acetic acid (B), mannitol (C), mannitol + acetic acid (D), melatonin (E), melatonin + acetic acid (F), melatonin + mannitol (G) and melatonin + mannitol + acetic acid (H); Columns with common letters are not significantly different at the 5% level of LSD test.

-6°C (0.395, 0.372, and 0.347), which had significant differences with A (0.248). It is noteworthy that with increasing the stress, ET_0/RC increased in treatments B, C, E, F, and H (Figure 5a).

The highest values of φE_0 was observed in the treatments H, B, and F (0.169, 0.163, and 0.157, respectively), with significant differences with A. The lowest φE_0 was recorded in the treatments G and A (0.129 and 0.130, respectively) (Figure 5b).

The highest PI_{ABS} was observed in the treatments C, H, and B at 0°C (0.524, 0.511, and 0.474, respectively), which were significantly different from A (0.296). The lowest PI_{ABS} was related to the treatments G and A (0.168 and 0.199) at -6°C. At this temperature, the

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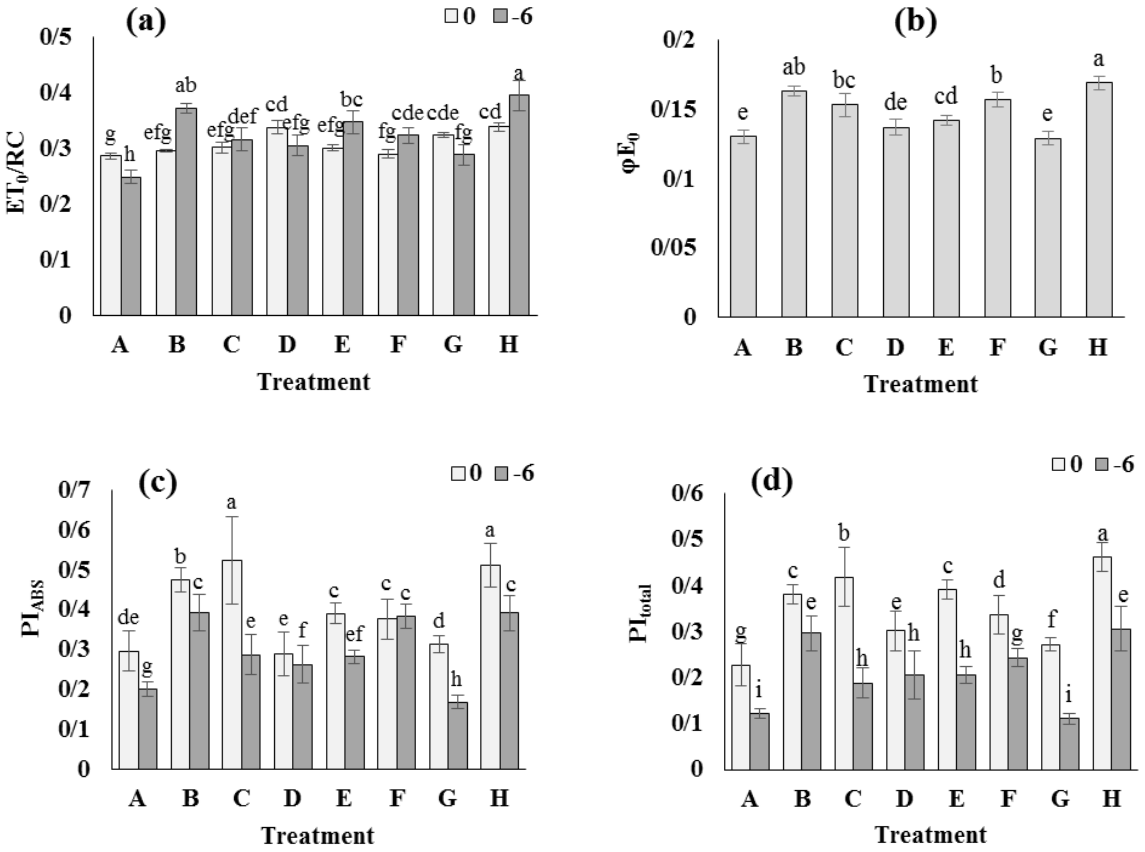

Fig. 5- Comparison of the interaction of temperature, melatonin, mannitol and acetic acid on the parameters of ET0/RC (a), PIABS (c) and PItotal (d); Comparison of the interaction of melatonin, mannitol and acetic acid on the parameter of φE0 (b); Treatments are represented as control (A), acetic acid (B), mannitol (C), mannitol + acetic acid (D), melatonin (E), melatonin + acetic acid (F), melatonin + mannitol (G) and melatonin + mannitol + acetic acid (H); Columns with common letters are not significantly different at the 5% level of LSD test.

treatments B, H, and F (0.392, 0.392, and 0.384) had highest PI_{ABS}, which had the significant differences with A (Figure 5c).

The highest PI_{total} was observed in the treatments H, C, and E at 0 °C (0.462, 0.418, and 0.391, respectively), which were significantly different from A (0.227). The lowest PI_{total} was related to the treatments G and A (0.110 and 0.121, respectively) at -6°C. At this temperature, the treatments H, B, and F (0.305, 0.296, and 0.243) caused highest PI_{total}, which had the significant differences with A (Figure 5d).

Electrolyte leakage (EL) and MDA content: The EL and MDA ramped up (58% and 50%, respectively), with increasing the stress intensity from 0°C to -6°C (Figure 6 a and c). Treatments E, F, and B (54.0%, 63.6%, 65.3%, respectively) significantly reduced ion leakage compared to A (76.7%). (Figure 6b). However, no significant difference was observed in the MDA content of the majority of the treatments (Figure 6d).

All fluorescence parameters were measured regardless of stress levels (0 and -6 °C) are shown in Figure 7 (a). In the charts, the differences between the control treatment and other ones in parameters such as PI_{ABS} and PI_{total} are more clearly seen, while in some parameters such as F_0, the differences among the treatments are less clearly seen (Figure 7, a and b).

Correlation analysis: The relationships between chlorophyll fluorescence parameters in seedlings were examined by correlation analysis. PI_{ABS} and PI_{total} had a negative correlation with F_0/F_m and DI_0/RC while showing a positive correlation with other treatments. PI_{ABS} and PI_{total} showed a slight positive correlation with F_0, ABS/RC and ET_0/RC. A positive correlation of DI_0/RC was observed with F_0, F_0/F_m and ABS/RC. In addition, EL and MDA, while having a positive correlation with each other, also showed a positive correlation with F_0/F_m and DI_0/RC (Figure 8).

Principal Component Analysis (PCA): Data were subjected to PCA to determine the relationship between fluorescence parameters. Data processing with PCA showed that the two main components explained 87.94% of the total changes. Accordingly, PC1 was accounted for 61.54% of the total changes and was mainly related to Area, F_v/F_m, F_0/F_m, and F_v/F_0. The major contributions to PC2, which were accounted for 26.4% of the total changes, were mostly ABS/RC, φE_0, F_0, and ET_0/RC (Figure 9a). The PCA could suitably show the differences among treatments. At 0°C, treatments E and F showed the highest values of F_v/F_0, F_v, F_v/F_m, and F_m, and treatments H, and B showed the

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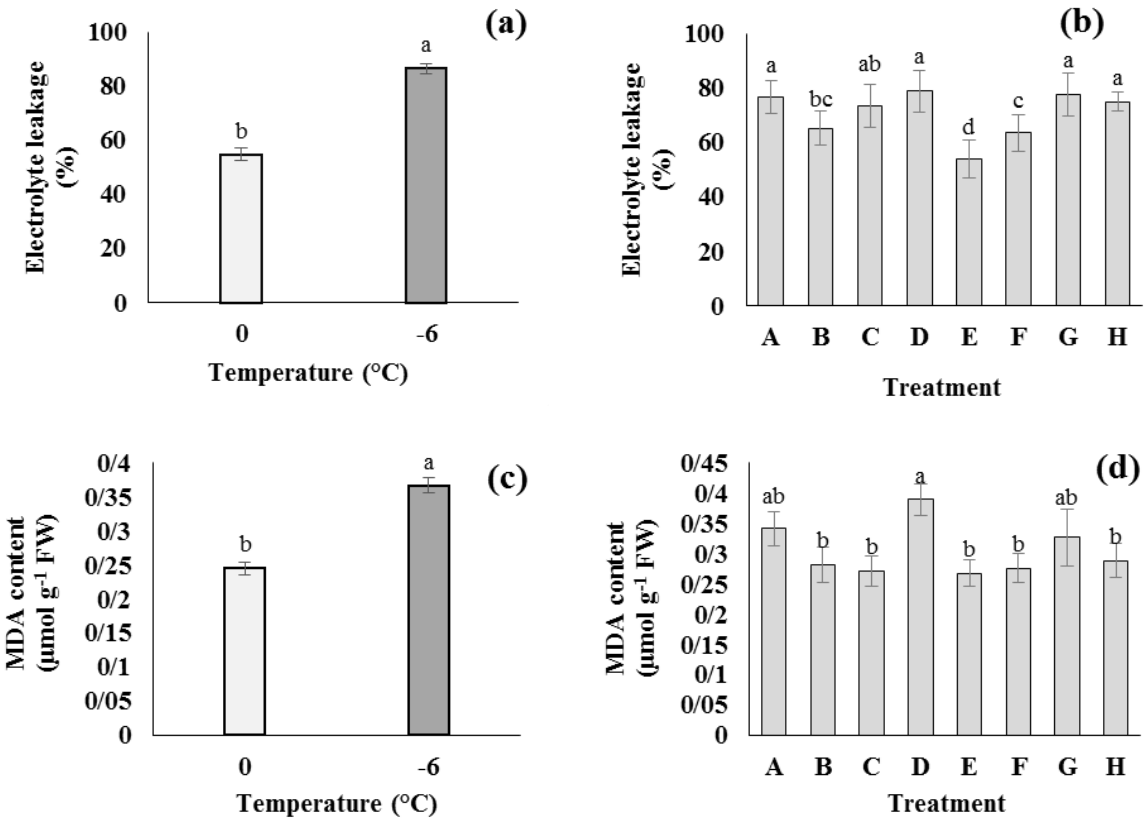

Fig. 6- Comparison of the effect of temperature (0 and -6 °C) on the electrolyte leakage (a) and MDA (c); Comparison of the interaction of melatonin, mannitol and acetic acid on the parameters of electrolyte leakage (b) and MDA (d); Treatments are represented as Control (A), acetic acid (B), mannitol (C), mannitol + acetic acid (D), melatonin (E), melatonin + acetic acid (F), melatonin + mannitol (G) and melatonin + mannitol + acetic acid (H); Columns with common letters are not significantly different at the 5% level of LSD test.

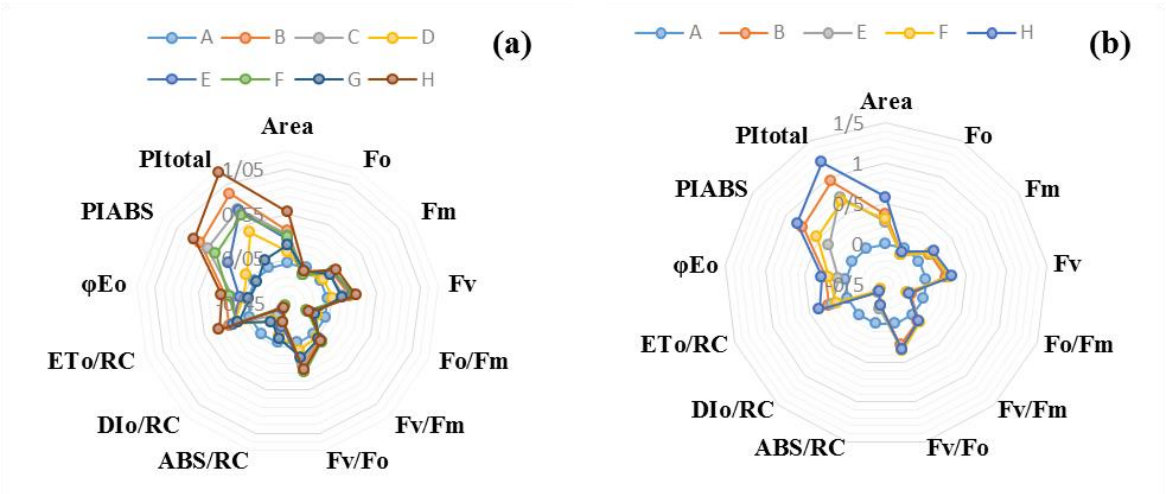

Fig. 7- Radar diagram of chlorophyll fluorescence parameters (a) and (b); Treatments are represented as control (A), acetic acid (B), mannitol (C), mannitol + acetic acid (D), melatonin (E), melatonin + acetic acid (F), melatonin + mannitol (G) and melatonin + mannitol + acetic acid (H).

highest values of PI_{ABS}, PI_{total}, and Area. Treatments H, F, and B provided the highest values of PI_{ABS}, PI_{total}, and φE_0 at -6°C (Figure 9b).

Discussion

As the temperature decreases, it occurs some changes in the structure of chloroplast thylakoids, chlorophyll content, photosynthetic enzyme activity, and electronic transport (Adam and Murthy, 2014). It has been shown the expression of genes encoding core PSII proteins and

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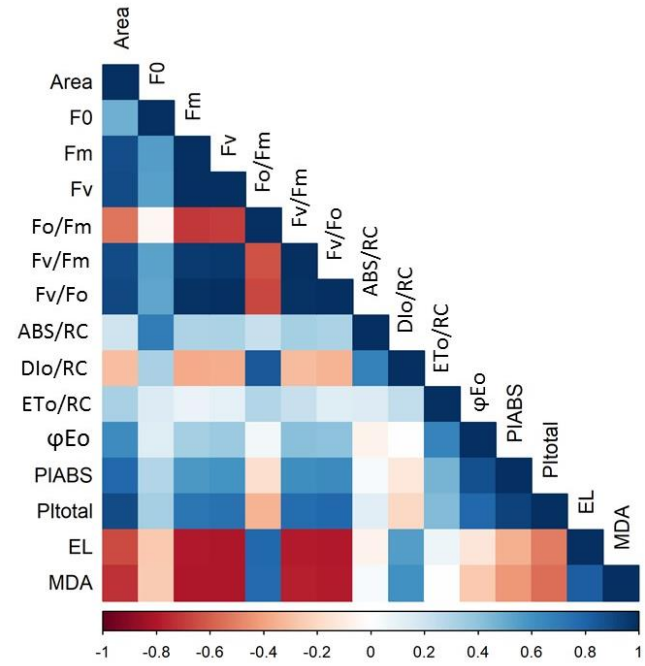

Fig. 8- Pearson's correlation coefficients among chlorophyll fluorescence parameters, electrolyte leakage (EL) and MDA content in leaves of Mexican lime under cold stress

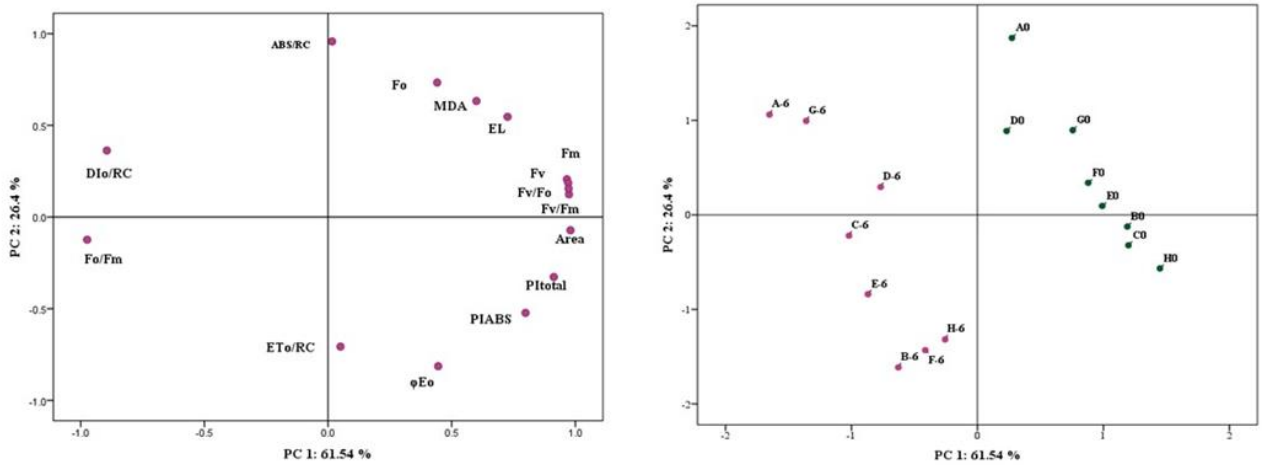

Fig. 9- Principal Component Analysis (PCA) of chlorophyll fluorescence parameters (a) and treatments (b); Sample signature: Treatments are represented as Control (A), acetic acid (B), mannitol (C), mannitol + acetic acid (D), melatonin (E), melatonin + acetic acid (F), melatonin + mannitol (G) and melatonin + mannitol + acetic acid (H).

ROS-scavenging enzymes was changed after melatonin application (Alyammahi and Gururani, 2020). Exogenous melatonin has been proven to protect cells by maintaining chlorophyll levels (Shomali *et al.*, 2021). Melatonin reduced the stress effects on the plant and kept the volume of the electron-receiving quinones at a higher level (Ayyaz *et al.*, 2020). The use of melatonin or acetic acid alone could keep the Area parameter higher than the use of any of them in combination with mannitol. The function of mannitol in combination with melatonin (G) was better than the function of mannitol combined with acetic acid (D). It is recommended that lower concentrations of mannitol be used, especially in the combined treatments.

Studies have shown that exogenous melatonin increased the resistance of bermudagrass by increasing the content of organic acids (Hu, *et al.*, 2016). Therefore, in addition to melatonin's direct role in inhibiting free radicals and increasing the activity of enzymatic and non-enzymatic antioxidants, melatonin also reduces the effects of cold stress through the production of some organic acids. Organic acids play an important role in tricarboxylic acid cycle metabolism (TCA), photosynthesis, respiration, and nutrient uptake in plants (Araujo *et al.*, 2012; Szabados and Savoure, 2010). In addition, organic acids reduce oxidative stress-induced damage and increase photosynthesis through regulations on enzymatic and non-enzymatic

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antioxidants (Guo *et al.*, 2017).

Inhibition of the PSII reaction center, which restricts electron transit from QA to QB and reduces the effectiveness of energy trapping at PSII, is most likely the reason for the rise in F_0 (Sengar and Singh, 2018). In the present study, all treatments reduced this parameter by increasing the electron transfer efficiency, with significant differences compared to the control. The variable fluorescence (F_v) is obtained from the difference between F_m and F_0. Higher F_v indicates the good performance of chlorophyll fluorescence mechanism under stress conditions and of the decrease in the rate of photochemical reactions (Mishra, 2018).

The decrease in F_v/F_m indicates a decrease in PSII performance, which may be due to the imbalanced energy transfer from the light-absorbing complex from the photosystem to the chlorophyll reaction center (Jahan *et al.*, 2021). F_v/F_m is often regarded as a sensitive environmental stress indicator. Under stress conditions, as indicated by the variable DI_0/RC values, the absorbed energy by the photosynthetic system was released as heat. Considering the electron transfer losses and the greater diversion of absorbed light energy as heat or fluorescence, these results indicate stronger photoinhibition in control plants. Furthermore, the photoinhibition for the control (A) might be attributable to lower quantities of photoprotective carotenoid pigments in its leaves (Falqueto *et al.*, 2017; Mishra, 2018). Melatonin and acetic acid can prevent the destruction of pigments and improve the overall process of photosynthesis (Ahmad *et al.*, 2021; Rahman, *et al.*, 2021).

All treatments except mannitol + acetic acid (D) treatment at 0°C significantly reduced the thermal dissipation quantum yield (F_0/F_m) and increased the F_v/F_m. Melatonin acts as an antioxidant, protecting electron mediators from damage and increasing electron transfer by removing stress-induced ROS. It, therefore, increases photochemical efficiency (F_v/F_m) (Liang *et al.*, 2019).

Acetic acid may involve in the synthesis and /or slowing down the breakdown of photosynthetic pigments (Rahman *et al.*, 2021). In the present study, acetic acid increased the F_v/F_m parameter under both stress levels compared to the control. Our results are similar to the results (Rahman *et al.*, 2019), he also showed that the application of exogenous acetic acid on mung bean plants under salinity stress by preserving the plant pigments (chlorophyll a, chlorophyll b, and carotenoids), increased the photosynthetic efficiency. The use of acetic acid increases the content of K^+, Ca^{2+} and Mg^{2+}. The amplified divalent cations Ca^{2+}, and Mg^{2+} may contribute to chlorophyll synthesis, protein synthesis, enzyme activation, membrane structure protection, and signal transduction (Rahman *et al.*, 2021; Rahman *et al.*, 2019).

The decrease in F_v/F_0 was accompanied by a higher inhibition of PSI and PSII owing to disruption of electron transport at their oxidation sides, according to one study (Strasser *et al.*, 2004; Umar *et al.*, 2019). Stress causes an electron flow imbalance from the electron donor (oxygen-releasing complex or OEC) to the electron acceptor (reaction center of photosystem II). Melatonin and acetic acid reduced this imbalance (Ayyaz *et al.*, 2020).

The ABS/RC is calculated by dividing the total quantity of photons absorbed by chlorophyll molecules across all RCs by the total number of active RCs. As the number of active centers grew, the ABS/RC ratio fell (Kumar *et al.*, 2020). Plants have evolved a system that allows absorbed energy to be dissipated as heat. DI_0/ RC is a metric that refers to the rate at which PSII dissipates energy (Kalaji *et al.*, 2018). The inactivation of reaction centers, which causes them to become heat sinks, might be connected to an increase in DI_0/RC (Plich *et al.*, 2020). The results showed a reduction in heat dissipation (DI_0/RC) in seedlings treated with melatonin and acetic acid. Low temperature increases the values of ABS/RC and DI_0/RC. The increase in ABS/RC was inhibited in all treatments except mannitol + acetic acid (D) and melatonin + mannitol (G) treatments while all treatments inhibited DI_0/RC increase (Hu *et al.*, 2016). Our outcomes were comparable to those of others. Following the application of melatonin, they observed a decrease in ABS/RC in *Chara australis*. Furthermore, as compared to the control, DI_0/RC and ABS/RC dropped in primed plants, but ET_0/RC increased (Shomali *et al*., 2021).

The absence of significant differences between melatonin (E) and acetic acid (B) treatments with the control (A) at 0 °C indicates that ET_0/RC alone is not a suitable parameter for detecting the cold resistance at 0°C. All treatments except mannitol + acetic acid (D) and melatonin + mannitol (G) treatments significantly increased the φE_0. Previous studies have shown similar increases in φE_0 in melatonin-treated plants (Alyammahi and Gururani, 2020). The lower φE_0 value indicates that heat stress is limited by electron transport on the acceptor side of PSII (Jahan *et al.*, 2021).

Performance indices (PIs) are the sum of three (PI_{ABS}) or four (PI_{total}) efficiency indicators that describe distinct energy conversion processes (Swoczyna *et al.*, 2020). Stressed plants had considerably lower PI levels as compared to melatonin-treated plants, suggesting that the latter plants were in better health under stress (Alyammahi and Gururani, 2020; Shomali *et al.*, 2021). Because PI_{ABS} is a function of ABS/RC and other chlorophyll fluorescence characteristics, changes in PI_{ABS} correlate to changes in ABS/RC (Shomali *et al.*, 2021). These findings are in line with our findings for cold-stressed plants. For all degrees of cold stress, a rise in the ABS/RC and DI_0/RC ratios, together with a drop in F_v/F_m, resulted in a decrease in PI_{total}. Our findings revealed that cold stress may damage active RCs and decrease PI_{ABS}, implying that PSII activities and the rate of electron transfer from PQ to PSI may be inhibited (Umar *et al.*, 2019). Furthermore, plants with higher PI_{ABS} dissipate extra energy more efficiently than plants

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with lower photosynthetic efficiency, as seen by enhanced F_v/F_m in treated plants (Shomali *et al.*, 2021). In this study, the performance parameters (PI_{ABS} and PI_{total}) compared to other parameters had a moderate share in changes among treatments. Research has shown that the use of melatonin increases the efficiency of photosynthesis and upregulates the expression of the genes of the antioxidant enzymes in response to cold (Li *et al.*, 2016; Sun *et al.*, 2018).

One of the most important indicators of damage due to cold stress is electrolyte leakage measurement (Saleem *et al.*, 2021). Free radicals, by acting on unsaturated fatty acids, cause lipid peroxidation and reduced cell membrane fluidity. In this study melatonin reduced electrolyte leakage compared to the control. Our findings are consistent with previous studies that have shown that electrolyte leakage markedly decreased when the pistachio seedlings sprayed with melatonin under freezing stress (Barand *et al.*, 2020). Lower levels of electrolyte leakage have also been reported in tea (Li *et al.*, 2019) sprayed with melatonin under cold stress. It has been suggested that melatonin is located between two layers of the cell membrane to maintain fluidity and thus reduce electrolyte leakage (Garcia *et al.*, 2014). In addition, melatonin decreases electrolyte leakage by increasing antioxidant capacity in response to various stresses (Ren *et al.*, 2020).

Acetic acid has been reported to maintain membrane permeability and fluidity through regulation of lipid metabolism and contribution to make higher unsaturated levels (Hu *et al.*, 2021). Acetic acid reduced electrolyte leakage under cold stress compared to control. Our findings are consistent with previous studies that have shown that exogenous application of acetic acid could decrease electrolyte leakage in soybean under drought stress and mung bean under salt stress. Acetic acid suppress ROS accumulation and protect membrane integrity (Rahman *et al.*, 2021; Rahman *et al.*, 2019).

In this study, melatonin and acetic acid treatments had no significant effect on MDA content, while it was reported that the application of melatonin in pistachio seedlings (Barand *et al.*, 2020) and tea plants (Li *et al.*, 2019) under cold stress reduced MDA content. Contrary to our results it has been reported that the use of acetic acid in soybean under drought stress (Rahman *et al.*, 2021) and mung bean under salt stress (Rahman *et al.*, 2019) reduced MDA content. These differences may be due to different plant species or concentrations of compounds used.

Conclusions

The present study showed that the exogenous use of natural compounds (melatonin) and cost-effective agent (acetic acid) can increase the tolerance of seedlings to cold stress by improving the photosynthetic parameters. In general, melatonin and acetic acid, or their interaction, increased the efficiency of energy trapping and helped maintain the photosystem II activity. Nowadays, more attention should be paid to the exogenous use of bioactive compounds that in addition to increasing plant yield, also increase tolerance to environmental stresses. In this study, the most appropriate parameters to identify the best treatments included Area, F_v/F_m, F_0/F_m, and F_v/F_0. However, more research is needed to investigate the mechanism of the mentioned bioactive compounds to reduce damage caused by cold stress in plants.

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