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Induction of stomatal opening following a night-chilling event alleviates physiological damage in mango trees

Md Intesaful Haque [a], Or Shapira [a], Ziv Attia [a], Yuval Cohen [b], Dana Charuvi [b], Tamar Azoulay-Shemer [a,*]

[a] Fruit Tree Sciences, Volcani Center, Agricultural Research Organization, Newe Ya'ar Research Center, Ramat Yishay, Israel
[b] Institute of Plant Sciences, Volcani Center, Agricultural Research Organization, Rishon LeZion, Israel

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ABSTRACT

Chilling events have become more frequent with climate change and are a significant abiotic factor causing physiological damage to plants and, consequently, reducing crop yield. Like other tropical and subtropical plants, mango (*Mangifera indica* L.) is particularly sensitive to chilling events, especially if they are followed by bright sunny days. It was previously shown that in mango leaves stomatal opening is restricted in the morning following a night-chilling event. This impairment results in restraint of carbon assimilation and subsequently, photoinhibition and reactive oxygen species production, which leads to chlorosis and in severe cases, cell death. Our detailed physiological analysis showed that foliar application of the guard cell H^+-ATPase activator, fusicoccin, in the morning after a cold night, mitigates the physiological damage from 'cold night–bright day' abiotic stress. This application restored stomatal opening, thereby enabling gas exchange, releasing the photosynthetic machinery from harmful excess photon energy, and improving the plant's overall physiological state. The mechanisms by which plants react to this abiotic stress are examined in this work. The foliar application of compounds that cause stomatal opening as a potential method of minimizing physiological damage due to night chilling is discussed.

1. Introduction

Mango (*Mangifera indica* L.) is a major fruit crop tree in tropical and subtropical regions; with an annual production of ~57 million tons (FAOstat, 2021). It is second only to banana among tropical and subtropical fruit. Israel is one of the northernmost regions of the world where mangoes are commercially grown. Today, it is planted on over 3000 ha around Israel, producing ~70,000 tons annually, with one-third allocated for export.

Much research has been done on climate change and its effects on agriculture. Various models forecast an increase in the frequency of weather extremes and cold events worldwide, particularly around the Mediterranean region (Alpert et al., 2008). Tropical and subtropical plants are particularly sensitive to cold (Allen and Ort, 2001; Feng and Cao, 2005), with mango being the most sensitive tree among the common subtropical fruit trees grown in Israel (citrus, avocado, lychee). Most of the mango plantations in Israel are located around the Sea of Galilee, Jordan Valley, and the Valley of Springs, which are susceptible to night-chilling events.

In temperate regions, such as the Mediterranean, night-chilling events are often characterized by a temperature drop at night followed by a high-irradiance sunny day (Nir et al., 1997; Allen et al., 2000), termed 'cold night - bright day'. Mango trees exposed to this stress develop a chlorotic leaf phenotype (Gadallah et al., 2020). Under severe cold-stress conditions, the phenotype may become more severe, leading to leaf necrosis and dehydration, that necessitate pruning.

Various studies have suggested that the damage to tropical and subtropical trees following night-chilling events is not directly due to the drop in night temperature but depends on the high irradiance that follows the next day (Nir et al., 1997). In fact, it has been shown that after a cold night - bright day event, the outer sun-exposed sections of the tree exhibit an excessive damaged phenotype compared to the inner shaded

Abbreviations: A, net CO_2 assimilation; Ci, intercellular CO_2; FC, fusicoccin; Fv/Fm, the maximum quantum yield of PSII reaction centers; gs, stomatal conductance; NPQ, non-photochemical quenching; PSII, photosystem II; ΦPSII, PSII efficiency; PPFD, photosynthetic photon flux density; ROS, reactive oxygen species; TPU, triose phosphate-utilization rate.

* Corresponding author.
E-mail address: shemer.tamar@agri.gov.il (T. Azoulay-Shemer).

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ones, further supporting the link between radiation intensity and the severity of the physiological damage to the plant (Nir et al., 1997; Feng and Cao, 2005).

Night-chilling conditions affect various mechanisms, detected at the metabolic, transcriptomic, and proteomic levels (Janmohammadi et al., 2015; Kenchanmane Raju et al., 2018; Fürtauer and Nägele, 2020). One of the most significant physiological effects of cold night - bright day stress is a decrease in leaf photosynthesis. Many studies have found that this phenotype is caused by a delay in stomatal opening, which has been observed in several plant species, including tomato (Martin et al., 1981; Liu et al., 2012), alfalfa (Peoples and Koch, 1978), olive (Bongi and Long, 1987), coffee (Bauer et al., 1985; Guo and Cao, 2015), vines (Flexas et al., 1999), peanut (Bell et al., 1994), avocado (Joshi et al., 2020), soybean (Van Heerden et al., 2004) and mango (Nir et al., 1997; Allen et al., 2000).

According to various studies, the cold night–bright day event stimulates a series of processes within the plant, beginning with restriction of stomatal conductance and carbon fixation during the sunny day that follows the night chill (Allen et al., 2000). Because there are fewer electron acceptors, the accumulation of excess photon energy results in an increase in the production of reactive oxygen species (ROS) (Wise, 1995; Hajiboland, 2014), which cause damage to cellular systems (Chow, 1994; Michaeli et al., 2001), induce chlorosis (Weng et al., 2006; Liu et al., 2018) and at high levels, can cause cell death (Petrov et al., 2015) and necrosis.

Regulation of stomatal conductance is crucial for the plant's survival and growth. Stomata regulate gas exchange between the atmosphere and the interior of the leaf. Each stomate includes two specialized guard cells, which increase and decrease in size by changing their turgidity to control the magnitude of the gap between them, thereby controlling the rate of transpiration (diffusion of water vapor out of the leaf) and the diffusion of CO_2 into the leaf for photosynthesis (Schroeder et al., 2001; Matthews et al., 2017). Many signaling components tightly regulate stomatal opening and closure in response to different physiological and environmental stimuli (Kim et al., 2010; Murata et al., 2015; Assmann and Jegla, 2016).

Biotic and abiotic stresses have a profound effect on stomatal conductance regulation (Ku et al., 2018). Low temperatures have been found to cause a delay in stomatal opening in a few cold-sensitive species (Bauer et al., 1985; Nir et al., 1997; Van Heerden et al., 2004; Guo and Cao, 2015; Joshi et al., 2020). Furthermore, cold stress has been found to modulate a number of plant hormones that induce stomatal closure, such as salicylic acid, abscisic acid (ABA), brassinosteroids, and ethylene (Pallas and Kays, 1982; Guye et al., 1987; Lang et al., 1994; Miura and Tada, 2014; Agurla et al., 2018).

Fusicoccin (FC) is a fungal phytotoxin produced by *Fusicoccum amygdali* that is commonly used in basic studies on stomatal conductance regulation (Marra et al., 2021). Numerous *in-vitro* and *in-vivo* studies indicate that FC is a powerful activator of plasma membrane H^+-ATPase (Olivari et al., 1998; Kinoshita and Shimazaki, 2001). FC stabilizes protein–protein interactions between 14-3-3 adapter proteins and their phosphoprotein partners. This interaction initiates displacement of the H^+-ATPase autoinhibitory C-terminal domain, which leads to activation of the proton pump (Marra et al., 2021).

FC has been shown to activate H^+-ATPase in guard cells, induce stomatal opening (Kinoshita and Shimazaki, 2001), and to overcome different environmental factors that induce stomatal closure (impair stomatal opening), such as darkness (She et al., 2010), high CO_2 levels (Hashimoto et al., 2006) and ABA induced by drought/salinity (Huang et al., 2014).

We hypothesized that night chilling-induced stomatal dysfunction plays a primary role in the chain of events that lead to the physiological damage incurred by cold night - bright day stress. The effect of the stomatal opening elicitor FC was examined on cold-stressed mango trees to test whether induction of stomatal opening on the day following a night-chilling event can resolve the limitation in gas exchange and prevent the chilling damage. Here we bring solid evidence that FC can bypass the night chill-induced impairment in mango leaf stomatal opening. By resolving the limitation on gas exchange, the leaf's photosynthetic machinery is released from detrimental excess photon energy, mitigating the chilling damage.

2. Materials and methods

2.1. Plant material and growth conditions

Young (~1- to 2-year-old) mango trees (*Mangifera indica* L.) from the (cultivars 'Shelly' or 'Omer'), grafted on clonal polyembryonic rootstock 13-1 were obtained from the Zvieli Nursery (Moshava Kinneret, Israel). Based on the average calculation of 10 plants, the trees were ~110 cm high, posses plant leaf area of ~7.8 m^2 (avg leaf area 106.5 cm^2 × avg 73 leaves) and weight ~1.6 Kg of (Whole tree fresh weight, including all aerial parts and washed out roots).

Trees were grown in 4-L pots containing soil mixture (Klasmann-Deilmann) in a net-house/greenhouse at Newe Ya'ar Research Center, Israeli Ministry of Agriculture (GPS 32.70808, 3517937). From April to December, trees were grown in the net-house under a white net (50-mesh), exposed to a maximal photosynthetic photon flux density (PPFD) of ~1400 μmol photons m^{-2} s^{-1}. During the winter, from January–March, trees were grown in a temperature-controlled greenhouse to protect them from chilling. Conditions in the greenhouse were: maximal PPFD of 900–1100 μmol photons m^{-2} s^{-1} and temperatures of 20–24 °C during the day and 15–20 °C at night. Plants were irrigated daily using an automatic irrigation system and fertilized once a week (7:3:7 NPK Shefer, ICL). During the hot seasons, the trees were irrigated twice a day, and in the winter, only once a day, at 1 L/h for 60 min each time.

2.2. Night-chilling event: experimental design

Trees were moved at dusk from the greenhouse or net-house to a temperature-controlled dark room (18–22 °C) and randomly split into two treatments: (i) Control: trees were maintained during the night in the dark at ambient temperature (18–22 °C); (ii) Cold: trees were exposed to a moderate (7 °C), severe (4 °C), or extreme (0.5 °C) night-chilling event for different numbers of hours during the night (as described in each experiment). Exposure to night-chilling events was conducted in a temperature-controlled chamber (Inomak). The chamber was first set to room temperature (18–22 °C), and the plants were moved into the chamber at dusk, after sunset. For the 6 h cold-night treatment (7 °C, 4 °C, or 0.5 °C), the chamber started to cool at 11:00 p.m., reaching the desired temperature at ~11:15 p.m. At 5:30 a.m., the chamber stopped cooling, and the temperature gradually increased until it reached room temperature at 6:30 a.m. Similarly, for the 12 h cold-night treatment, the temperature was gradually reduced starting at 7:00 p.m. and maintained at the desired low temperature till 7:00 a.m. (as described in each experiment). To prevent sudden temperature drops in the soil and root system, we constructed a system in the cold chamber that maintains soil temperature between 13 and 15 °C. Two perspex containers (width 56 cm, length 61 cm, height 27.5 cm), wired with an electrical heating cable (Exo Terra), were installed in the temperature-controlled chamber. Mango tree pots were placed in the perspex containers and covered with a rockwool blanket (temperature insulator). A HOBO temperature-logger (Pendant MX2202, Onset, USA), which was installed in the pot, indicated that the soil temperature stayed between 13 and 15 °C during the night. At the end of the night (before exposure to direct sunlight), all trees were moved out of the chamber to the darkened room and acclimatized to room temperature together with the trees from the control treatment.

For post night-chilling-event treatment, two solutions were prepared: (i) Mock (0.02% Tween-20 surfactant in double distilled water (DDW)) and (ii) FC (0.02% Tween-20 + 10 μM FC in DDW). For each experiment, the Mock and FC working solutions were freshly prepared in

the morning. The 2 mM Fusiccocin (Santa Cruz Biotechnology, catalog number SC-200754) stock solution, which was made in 100% ethanol and kept at −20 °C, was diluted in 0.02% Tween-20 to the final concentration of 10 mM of FC. At the end of the night, after 1 h of acclimation to room temperature in the dark, the trees from the Control (18–22 °C) and Cold (7 °C, 4 °C or 0.5 °C) treatments were randomly divided into two subtreatments: (i) Mock and (ii) FC. Using a cotton swab, the indicated solution was applied on both the adaxial and abaxial sides of healthy and fully expanded mature leaves (~700 μl per each leaf). The solution was applied on more than 5 leaves of at least 4 trees, per each treatment. Trees were then placed outdoors, in the shade, for 30 min to acclimate under low light and so that the applied solution would evaporate from the leaf surface. Trees were then shifted to the net-house/greenhouse and exposed to a bright sunny day. More than 9 experiments were conducted under different cold-night temperatures (i.e., moderate (7 °C), severe (4 °C), or extreme (0.5 °C) night-chilling event). For each min temperature, a representative dataset was generated to demonstrate the various physiological effects of the varied night-chill temperatures.

2.3. Gas-exchange and chlorophyll-fluorescence measurements

Gas-exchange and chlorophyll-fluorescence measurements were conducted with an LI-6800 portable photosynthesis system (LI-COR Biosciences, USA) equipped with a Multiphase Flash™ Fluorometer (measured leaf area = 2 cm^2). All measurements were conducted on fully expanded mature leaves after at least 2 h of exposure to natural light. For gas-exchange measurements, reference CO_2 concentration was set to 400 ppm, and relative humidity and temperature were kept at ambient levels. Light, in the intensity of 1000 μmol m^{-2} s^{-1} PPFD, was provided by blue/red light-emitting diodes (10% blue and 90% red). The LI-6800 chamber airflow was set to 500 μmol s^{-1} with a boundary layer of ~3 mol m^{-2} s^{-1}. Measurements of gas exchange and light-adapted chlorophyll *a* fluorescence were recorded simultaneously. After leaf steady-state gas exchange was recorded for net CO_2 assimilation rate (*A*), stomatal conductance (g_s), and internal CO_2 (*Ci*), the steady-state chlorophyll fluorescence signal (*Fs*) was logged. A saturating light pulse of 8000 μmol m^{-2} s^{-1} was used to determine the maximum light-adapted fluorescence (*Fm'*). The overall efficiency of the photosystem II (PSII) reaction center in the light, calculated as $\Phi PSII = (Fm - Ft)/Fm'$, where *Fm* is the maximum fluorescence signal (when all PSII centers are in the closed state) measured from dark-adapted material, *Ft* is the steady-state yield of fluorescence in the light, and *Fm'* is the maximum fluorescence signal (when all PSII centers are in the closed state), measured from light-adapted material.

To determine the maximum PSII efficiency (*Fv/Fm*), by dividing the variable fluorescence (*Fv*) by the maximum fluorescence (*Fm*), chlorophyll-based fluorescence measurements were conducted in complete darkness, either predawn or 7 h after exposure to natural light (and 1 h of dark adaptation). For the evaluation of *Fv/Fm* at different time points, measurements were taken at each time point from the same leaves. For the calculation of non-photochemical quenching (NPQ), predawn *Fm* values were used as described in Ruban (2016).

2.4. Leaf chlorophyll concentration

Chlorophyll concentration (μmol Chl m^{-2}) was estimated non-destructively using a chlorophyll meter (MC-100, Apogee Instruments, USA). Measurements were conducted on 4 leaves from 4 trees (n = 4) for each treatment; for each leaf, 2 measurements were collected from both sides of the main vein.

2.5. Membrane damage evaluated by electrolyte-leakage assay

Leaf discs (1 cm diameter) were cut using a leaf puncher from fully developed leaves and rinsed with deionized water. To determine electrolyte leakage, electric conductivity (EC) was measured using a conductivity meter (CON 11/110, Oakton Instruments, USA). Samples were first immersed in 10 mL deionized water in a sealed 15-mL Falcon tube, incubated at room temperature on a shaker for 24 h, and then EC was measured (EC1). Next, the same samples were incubated at 100 °C for 1 h, cooled to room temperature, and EC was measured again (EC2). Electrolyte leakage (EL%) was calculated as (EC1/EC2) × 100 (Lutts, 1996).

2.6. A/Ci curves to study stomatal and biochemical photosynthesis limitations

Simultaneous measurement of leaf gas-exchange and modulated chlorophyll-fluorescence responses to light and CO_2 concentration enables to determine a wide range of key biochemical and biophysical limitations on photosynthesis. Leaf gas-exchange photosynthetic measurement can determine leaf CO_2 uptake (*A*) and the intercellular CO_2 concentration (*Ci*). Analysis of the ratio between them, using *A/Ci* curves, can quantitatively separate biochemical and stomatal limitations to photosynthesis and provide detailed information on the different photosynthetic components, including the maximal rate of carboxylation V_{cMax}, electron transport rate for regeneration of ribulose 1,5-bisphosphate (RuBP) (*J*), and triose phosphate-utilization rate (TPU) (Long and Bernacchi, 2003). To measure the leaf *A/Ci* ratio before and after the cold night - bright day stress, we obtained leaf *A/Ci* curves at different hours during the day. To calculate the *A/Ci* baseline levels of non-stressed leaves, 2 young mango trees were moved from the net-house to a temperature-controlled darkroom (18–22 °C). The following day, *A/Ci* response curves were obtained for 3 mature leaves per tree (see details below) using two LI-6800 portable photosynthesis systems equipped with the Multiphase Flash Fluorometer. At the end of the measurement, the plants were returned to the net-house for the rest of the day. At dusk on the second day, the same trees were moved back to the darkroom and randomly split into two treatments: (i) Control (18 °C–22 °C) and (ii) Cold (exposure to a moderate night-chilling event, 7 °C for 12 h). On the morning of day 3, the same 3 leaves were measured again, in the same order as on the first day (before treatments), to determine their *A/Ci* response curves. To prevent differential light exposure, the leaves were kept in the dark by covering them with aluminum foil until the measurements were performed. By using this protocol, we eliminated the effect of different cumulative light exposures and focused on the physiological effect produced by time of day. To test the impact of time on leaf photosynthetic performance, the first leaf from each tree was measured at 08:00 a.m., the second leaf at 10:00 a.m., and the third leaf at 12:00 p.m. The experiment was performed in five independent time blocks within 2 weeks. Each replication was measured over 2 consecutive days, generating a repeated-measures design for the analysis.

CO_2 response curves with the Dynamic Assimilation™ Technique (DAT) was recently developed by Saathoff and Welles (2021). Experiments were performed as recommended in the Li-Cor LTD user manual (https://www.licor.com/env/support/LI-6800/videos/dynamic-assimilation-technique.html). This technique represents an advanced and improved Rapid *A/Ci* Response technique, that features better accuracy of derived FcVB parameters and shorter replication time. In this study, the detailed rapid *A/Ci* curves were obtained after induction of photosynthesis for 1 h with linear PPFD ramping from 0 to 2000 μmol m^{-2} s^{-1}. Chamber conditions at the end of the light adaptation were as follows: CO_2 level – 415 μmol mol^{-1}, PPFD – 2000 μmol m^{-2} s^{-1}, leaf temperature – 29 °C, and relative humidity – 60%. Chamber CO_2 concentration was then reduced to 50 μmol mol^{-1} and then increased at a linear rate (200 μmol mol^{-1} min^{-1}) to 1800 μmol mol^{-1} min^{-1}. Data were recorded every 5 s for ~100 data points per curve (each curve took 9 min to complete). Data from *A/Ci* curves were fitted with the FvCB model at actual leaf temperature (Farquhar et al., 1980) using the msuRACiFit r script (https://github.com/poales/msuRACiFit).

2.7. Statistical analysis

The number of trees (biological replications) and of leaves measured within each replication was at least 3, as reported in the relevant figure legends. Results were analyzed using one-way ANOVA or two-way repeated-measures ANOVA followed by Tukey's or Sidak's multiple comparison tests. Data were checked for normality of the residuals and equal variance using the Shapiro–Wilk and Brown–Forsythe tests, respectively. In the few cases for which unequal variance was detected, Welch's ANOVA was used, followed by Dunnett's T3 pairwise comparison test. Results are reported as mean ± standard error of the mean (SEM) and *P*-values (adjusted for multiple comparisons) from Tukey, Sidak, or Dunnett's pairwise comparisons. When present, statistical interactions from ANOVA are reported as *P*-values. A/C_i curve data (Table 1) are presented as mean ± (SEM), mean difference, with 95% confidence intervals, and *P*-values for mean difference (P_{ES}). Analysis was conducted using GraphPad Prism version 9.5 (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

3. Results

3.1. Foliar treatment with FC after a moderate night-chilling event results in full recovery of leaf stomatal conductance and photosynthetic performance

To test whether FC can bypass the cold-induced impairment in stomatal opening of mango tree leaves and whether this treatment can reduce leaf physiological damage, young mango trees were exposed, during the night, to either ambient or moderate chilling (7 °C for 6 h) conditions, followed by application of FC in the morning. After ~2 h of natural light (~1000 μmol m^{-2} s^{-1}), leaves were measured for their gas-exchange and chlorophyll-fluorescence parameters; 5 h later (total of 7 h exposure to light), the same leaves were dark-adapted for 1 h and *Fv/Fm* was measured. The moderate night-chilling event induced an over 50% decrease in stomatal conductance (g_s) the following morning compared to control plants (Fig. 1A; Cold 26 ± 4 vs. Control 53 ± 8.0 mmol H_2O m^{-2} s^{-1}, $P_{ES} = 0.142$). FC treatment following the night chilling resulted in stomatal conductance that was similar to the control plants (Fig. 1A; Cold + FC 48 ± 13 mmol H_2O m^{-2} s^{-1}, $P_{ES\ Cold+FC-Control} = 0.986$). Furthermore, untreated mango plant leaves showed a reduction in the rate of net CO_2 assimilation (*A*) after exposure to the moderate night-chilling event compared to the control plants (Fig. 1B; Cold 3.3 ± 0.4 vs. Control 5.9 ± 0.4 μmol CO_2 m^{-2} s^{-1}, $P_{ES} = 0.028$). The effect size of the cold-night stress reached a reduction of ~50%, which was in agreement with the detected decrease in stomatal conductance. When leaves of cold-night stressed trees were treated with FC, net CO_2 assimilation levels were unaffected and remained stable and similar to those detected in the control trees (Fig. 1B; Cold + FC 5.7 ± 0.3 vs. Control 5.9 ± 0.4 μmol CO_2 m^{-2} s^{-1}, $P_{ES} = 0.992$). A comparison of leaf intercellular CO_2 (*Ci*) levels showed no statistically significant differences among treatments (Fig. 1C; Control 186 ± 32, Cold 175 ± 40, and Cold + FC 173 ± 29 μmol CO_2 mol air^{-1}). In addition, chlorophyll fluorescence parameters (*ΦPSII* and *Fv/Fm*) showed no statistically significant differences among treatments (*ΦPSII*: 0.11 ± 0.01, 0.11 ± 0.02 and 0.15 ± 0.02; *Fv/Fm*: 0.79 ± 0.01, 0.79 ± 0.01 and 0.79 ± 0.01, for Control, Cold and Cold + FC, respectively, Fig. 1D and E).

3.2. Foliar treatment with FC after a severe night-chilling event offers partial protection with respect to leaf CO_2 assimilation (A) and stomatal function (g_s), and complete protection of the light-harvesting and electron-transport systems (ΦPSII and Fv/Fm)

To evaluate the efficiency of FC protection from leaf physiological damage following a stronger night-chilling event, we conducted a similar experiment with a severe night-chilling event of 4 °C for 12 h. Severe cold-night stress resulted in a large decrease in stomatal conductance to ~40% of the control (Fig. 2A; Cold 20 ± 4 vs. Control 50 ± 8 mmol H_2O m^{-2} s^{-1}, $P_{ES} = 0.008$). Following the severe night-chilling event, FC-treated leaves showed higher (although not statistically significant) g_s than the untreated trees exposed to the cold night (Fig. 2A; Cold 20 ± 4 vs. Cold + FC 36 ± 4 mmol H_2O m^{-2} s^{-1}, $P_{ES} = 0.165$). Nevertheless, their g_s was somewhat (although not statistically significant) lower than in control non-stressed plant leaves (Fig. 2A; Control 50 ± 8 mmol H_2O m^{-2} s^{-1}, $P_{ES\ Cold\ +\ FC-Control} = 0.223$). A significant reduction in leaf CO_2 assimilation, by ~50%, was observed following the severe night-chilling event (Fig. 2B; Cold 2.59 ± 0.23 vs. Control 5.1 ± 0.35 μmol CO_2 m^{-2} s^{-1}, $P_{ES} = 0.006$). However, when FC treatment was applied after the cold night, leaf CO_2 assimilation rate

Table 1
A/Ci curve analyses reveal a temporary delay in mango leaf photosynthetic efficiency following night-chilling event. *A/Ci* curves data shown in Fig. 7 was fitted on the FvCB model using an R script (msuRACiFit). Table shows the mean ± SE, mean difference, 95% confidence intervals and P_{value} of the effect size (P_{ES}) of the maximal rate of carboxylation (V_{cMax}) electron transport rate for regeneration of RuBP (*J*), and triose phosphate utilization rate (*TPU*) for each treatment and time of measurement (at 08:00 a.m./10:00 a.m./12:00 p.m., respectively). The main parameters of the model were analyzed as repeated measurements using two-way ANOVA followed by Sidak's multiple comparison test (GraphPad Prism).

Time of measurement	Treatment	V_{cMax} (μmol m^{-2} s^{-1}) Mean ± SE		Mean difference & 95% CI	P_{ES}	*J* (μmol m^{-2} s^{-1}) Mean ± SE		Mean difference & 95% CI	P_{ES}	*TPU* (μmol m^{-2} s^{-1}) Mean ± SE		Mean difference & 95% CI	P_{ES}
		Before	After			Before	After			Before	After		
8:00 a.m.	Control	±44.7 9	43.2 ± 6.1	−0.8 −32.5 to 31.0	0.9959	74.3 ± 8.2	70.2 ± 9.4	2 −6.1 to 10.1	0.8682	5.4 ± 0.7	5.0 ± 0.6	−0.1 −1.9 to 1.7	0.9849
	Cold	±50.8 8.7	38.1 ± 4.4	−13.2 −5.0 to 18.5	0.393	79.2 ± 3.1	67 ± 5.3	−15.5 −23.6 to −7.4	0.0052	5.6 ± 0.1	4.6 ± 0.6	−1.1 −2.9 to 0.7	0.1983
10:00 a.m.	Control	51.3 ± 4.3	49.4 ± 5.1	−1.8 −16.0 to 12.3	0.9145	78.1 ± 3.6	77.0 ± 8.3	−1.1 −22.6 to 20.3	0.9861	5.2 ± 0.3	5.6 ± 0.7	0.1 −1.3 to 1.4	0.9909
	Cold	50.4 ± 5.6	34.5 ± 4.3	−17.2 −31.0 to −3.4	0.0205	73.4 ± 6.2	52.6 ± 5.1	−22.2 −42.8 to −1.5	0.0382	5.0 ± 0.6	3.9 ± 0.3	−1.3 −2.6 to 0.1	0.0593
12:00 p.m.	Control	49.7 ± 3	50.3 ± 2.6	0.6 −31.6 to 32.7	0.9945	79.0 ± 4.7	84.2 ± 2	5.3 −14.9 to 25.4	0.6581	5.4 ± 0.3	5.5 ± 0.5	0.1 −2.1 to 2.1	0.9984
	Cold	52.6 ± 4.2	42.0 ± 3.9	−10.6 −42.8 to 21.6	0.3248	82.9 ± 7.7	71.6 ± 2.1	−11.3 −31.4 to 8.9	0.2304	5.6 ± 0.5	4.9 ± 0.2	−0.7 −2.8 to 1.4	0.5144

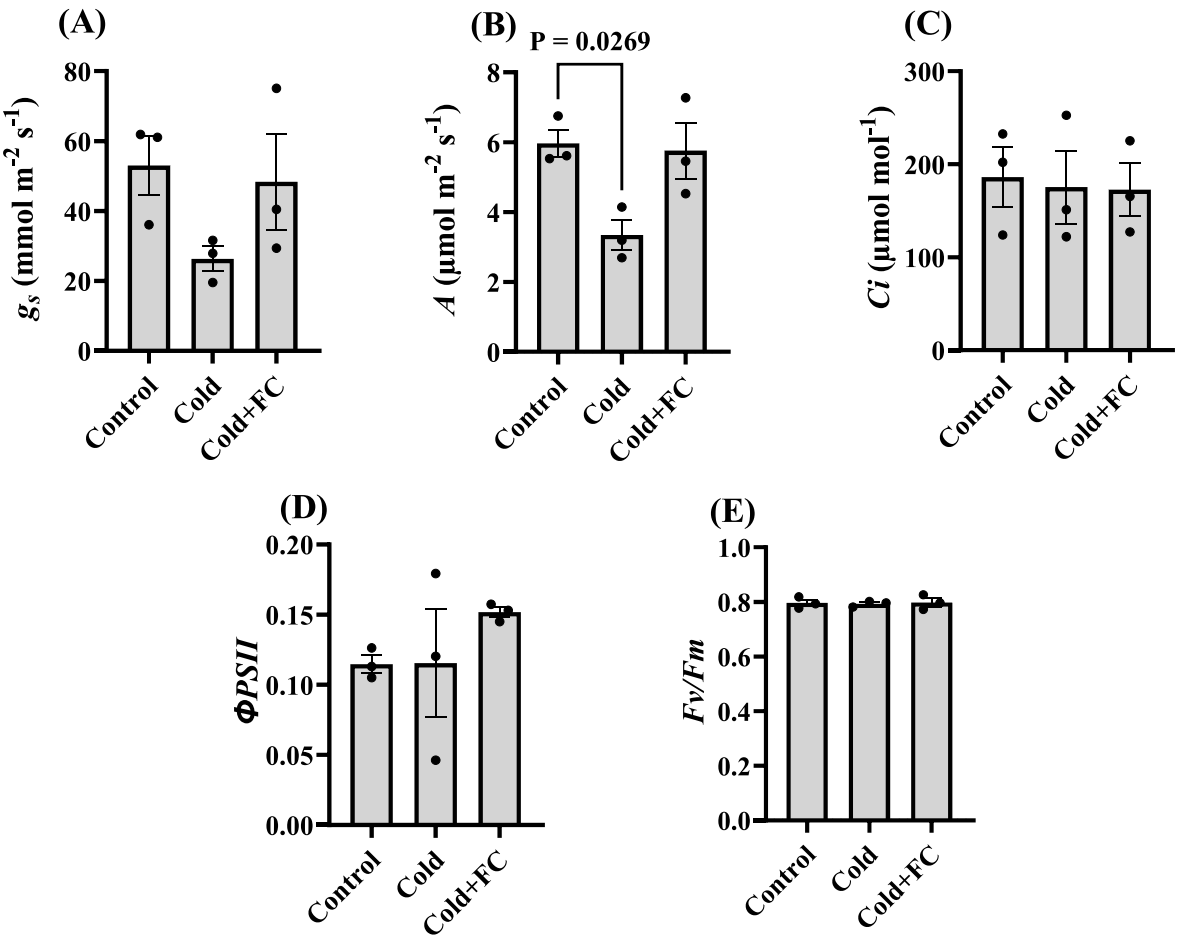

Fig. 1. Foliar treatment of fusicoccin after a moderate night-chilling event results in greater stomatal conductance and photosynthetic performances in mango leaves. Young mango trees were exposed to a moderate night-chilling event (Cold; 7 °C for 6 h) or kept under ambient growth conditions (Control). On the next day, just before sunrise, the cold stressed trees were treated with either 10 µM Fusiccocin in 0.02% Tween-20 (Cold + FC) or just with 0.02% TWEEN-20 solution (Cold). Control trees were treated with 0.02% TWEEN-20 solution (Control). All trees were then moved back to the greenhouse under natural light (~1000 µmol m^{-2} s^{-1}). After ~2 h, gas exchange and light-adapted chlorophyll fluorescence parameters were measured. **(A)** Stomatal conductance (g_s; mmol H_2O m^{-2} s^{-1}) **(B)** net CO_2 assimilation rates (A; µmol CO_2 m^{-2} s^{-1}), **(C)** intercellular CO_2 (Ci; µmol CO_2 mol air^{-1}), **(D)** actual efficiency of PSII ($\Phi PSII$), and **(E)** maximal efficiency of PSII (Fv/Fm) 7 h after exposure to natural light followed by 1 h of dark. Statistical analysis was conducted using one-way ANOVA followed by Tukey's multiple comparison test. The bars shown are means ± SEM; n = 3 trees for each treatment, and five leaves were measured from each tree. When a statistically meaningful Effect Size was found, the P_{value} is noted. Raw data is presented in Supplemental Table 1.

remained intact and was not substantially different from the control (Fig. 2B; Cold + FC 4.23 ± 0.60 µmol CO_2 m^{-2} s^{-1}, $P_{ES\ Cold+FC-Control}$ = 0.394). Similar *Ci* levels were identified in leaves of both control and cold-stressed mango tree (Fig. 2C; Cold 203 ± 28 vs. Control 207 ± 17 µmol CO_2 mol air^{-1}, P_{ES} = 0.991) and although not statistically significant, *Ci* levels in leaves of FC-treated cold-stressed plants were markedly lower (Fig. 2C; Cold + FC 146 ± 10 µmol CO_2 mol air^{-1}, $P_{ES\ Cold+FC-Control}$ = 0.153).

Chlorophyll *a* fluorescence measurements of plant leaves from the different treatments revealed that after a severe night-chilling event and exposure to a bright sunny day, both the effective quantum yield of PSII (*ΦPSII*) and the maximum quantum yield of PSII reaction centers (*Fv/Fm* measured after 1 h of recovery in the dark) were considerably reduced compared to non-stressed control leaves. The cold-night stress resulted in a large decrease (by ~54%) in leaf *ΦPSII* (Fig. 2D; Cold 0.057 ± 0.007 vs. Control 0.106 ± 0.010, P_{ES} = 0.008). Interestingly, here we found that using FC after the night-chilling event protected mango leaves from the subsequent alterations in the efficiency of linear flow through PSII (Fig. 2D; Cold + FC 0.090 ± 0.010, $P_{ES\ Cold+FC-Control}$ = 0.467). Leaf *Fv/Fm* measurements, conducted following exposure of the tree leaves to sunlight for 7 h (including 1 h of dark adaptation), revealed no noticeable difference between the Control and Cold + FC-treated plants, both showing higher values compared to the cold-stressed plants (Fig. 2E; Control 0.699 ± 0.024, Cold 0.571 ± 0.002, and Cold + FC 0.731 ± 0.039, $P_{ES\ Cold-Control}$ = 0.019, $P_{ES\ Cold-Cold+FC}$ = 0.005, $P_{ES\ Cold+FC-Control}$ = 0.69).

3.3. Foliar application of FC has a mild positive effect on leaf physiology after an extreme night-chilling event

It has been well-documented that physiological damage following a night-chilling event is typically time- and temperature-dependent, meaning that the damage increases as the temperature decreases and as the exposure time to the low-temperature increases (Nir et al., 1997; Ying et al., 2002). To further explore the protective effect of FC from the physiological outcome imposed by radical cold night–bright day stress, we repeated the experiment described in sections 3.1 and 3.2 under an extreme night-chilling event of 0.5 °C for 12 h. This event induced full stomatal closure. FC treatment resulted in a smaller (yet not statistically significant) reduction in stomatal conductance (Fig. 3A; Control 37 ± 8.0, Cold 1.1 ± 0.4, Cold + FC 11.1 ± 2.2 mmol H_2O m^{-2} s^{-1}, $P_{ES\ Cold-Control}$ = 0.001, $P_{ES\ Cold-Cold+FC}$ = 0.345, $P_{ES\ Cold+FC-Control}$ = 0.009). In correlation with g_s, a complete reduction in *A* was observed in leaves following the night-chilling event, reaching negative values (Fig. 3B;

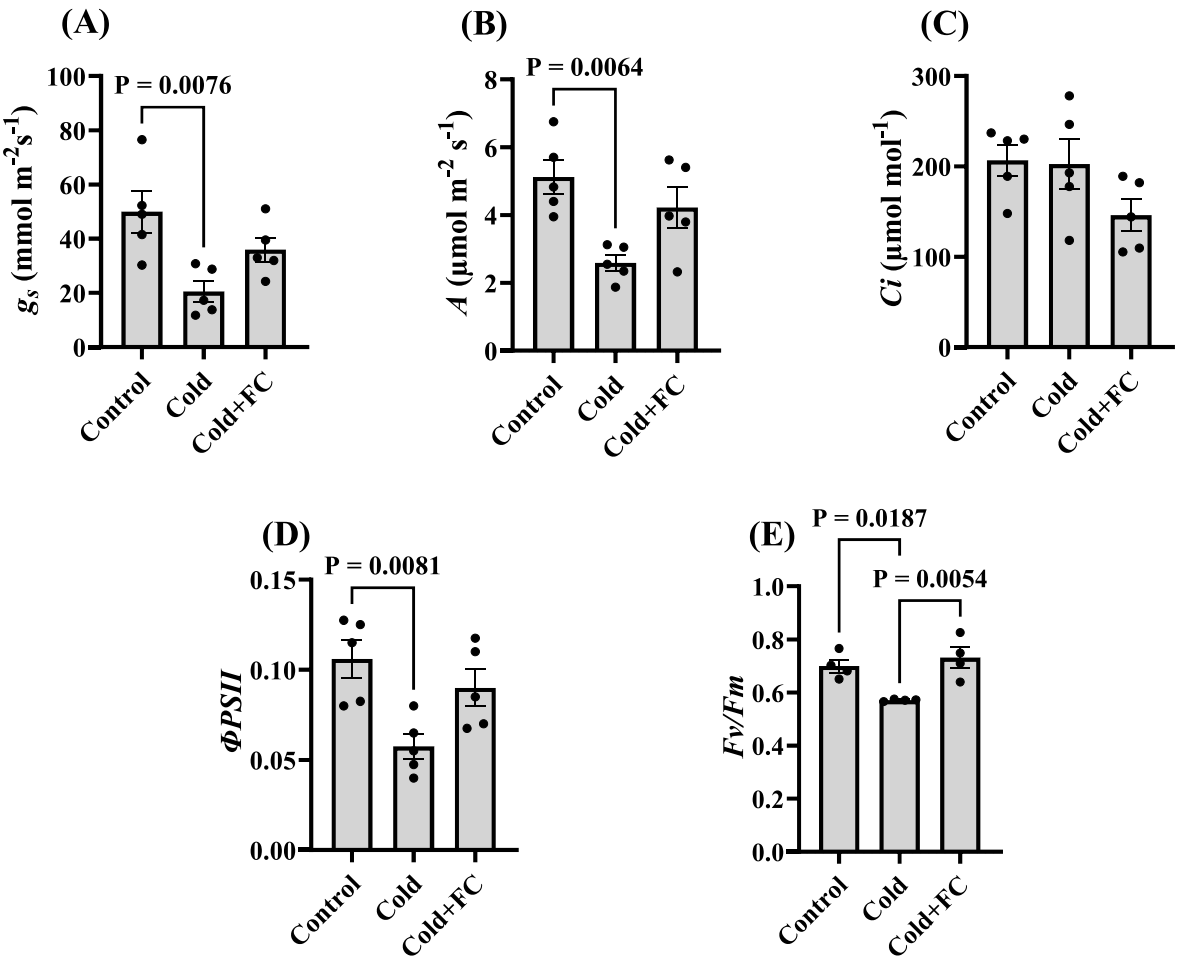

Fig. 2. Foliar treatment of fusicoccin after a severe night-chilling event results in a greater gas exchange and CO_2 assimilation rates and rescue from an impairment in mango leaf photosynthetic performances. The experiment was carried out as described in Fig. 1 where young mango trees were tested under severe cold night stress (4 °C for 12 h). Gas exchange and light-adapted chlorophyll fluorescence parameters were measured. Data shown is the average of: **(A)** stomatal conductance (g_s; mmol H_2O m^{-2} s^{-1}) **(B)** net CO_2 assimilation rates (*A*; µmol CO_2 m^{-2} s^{-1}), **(C)** intercellular CO_2 (*Ci*; µmol CO_2 mol air^{-1}), **(D)** actual efficiency of PSII (*ΦPSII*), and **(E)** maximal efficiency of PSII (*Fv/Fm*) 7 h after exposure to natural light followed by 1 h of dark. Statistical analysis was conducted using one-way ANOVA followed by Tukey's multiple comparison test. The bars shown are means ± SEM; n = 5 trees for each treatment, and five leaves were measured from each tree. When a statistically meaningful Effect Size was found, the P_{value} is noted. Raw data is presented in Supplemental Table 1.

Cold −0.7 ± 0.1 µmol m^{-2} s^{-1}). However, treatment with FC induced minimal stomatal opening, and increased *A* levels to positive values (Fig. 3B; Cold + FC 1.2 ± 0.4 µmol m^{-2} s^{-1}). Both treatments, Cold and Cold + FC, differed from the control (Fig. 3B; Control 5.0 ± 0.6 µmol m^{-2} s^{-1}, $P_{ES\ Cold\ -\ Control} < 0.0001$, $P_{ES\ Cold+FC-Control} = 0.001$, $P_{ES\ Cold-Cold+FC} = 0.06$).

Unlike moderate and severe night chilling (Figs. 1C and 2C), extreme night-chilling stress resulted in a massive increase in *Ci* compared to the control non-stressed plants (Fig. 3C; Cold 1074 ± 274 vs. Control 151 ± 9 µmol CO_2 mol air^{-1}, $P_{ES} = 0.067$). On the other hand, *Ci* of Cold + FC leaves was found to be lower than that of the untreated nigh-chill-induced plants, which further supports the positive effect of FC on the CO_2 assimilation process after the night-chilling event (Fig. 3C; Cold + FC 410 ± 75 µmol CO_2 mol air^{-1}, $P_{ES\ Cold+FC-Control} = 0.968$).

Chlorophyll *a* fluorescence parameters measured following the extreme night-chilling event revealed a reduction of ~40% in *ΦPSII* in cold-stressed plants (Fig. 3D; Cold 0.110 ± 0.015 vs. Control 0.182 ± 0.008, $P_{ES} = 0.002$). FC treatment of the cold-stressed plants could partially preserve the effective quantum yield of PSII, which was reduced by only ~13% compared to control plants (Fig. 3D; Cold + FC 0.157 ± 0.007, $P_{ES\ Cold+FC-Control} = 0.258$, $P_{ES\ Cold-Cold+FC} = 0.021$). Since cold stress might negatively affect the plant's photoprotection mechanism(s), nonphotochemical quenching of excessive excitation energy (NPQ) was calculated. Data showed slightly higher NPQ (yet not statistically significant) in leaves of both FC-treated/untreated cold-stressed plants (Fig. 3E; Control 3.1 ± 0.2, Cold 3.3 ± 0.4, Cold + FC 3.7 ± 0.2).

3.4. Foliar application of FC after an extreme night-chilling event protects from chronic photoinhibition

The *Fv/Fm* ratio is a chlorophyll fluorescence-based parameter representing the maximum potential quantum efficiency of PSII (Maxwell and Johnson, 2000). This parameter is a sensitive measurement tool that has been used as an indicator of environmental and temperature stress (Schulze and Caldwell, 1995). To assess the effect of FC foliar application on leaf photosynthetic performance after an extreme night-chilling event (0.5 °C for 12 h), and whether FC also has an effect on its recovery, leaf *Fv/Fm* measurements were recorded from the Control, Cold, and Cold + FC treatments (as described in section 3.3) at three different time points: (i) pre-dawn: after the cold stress and before any exposure to light; (ii) natural light 7 h: after the cold stress and exposure to 7 h of light (followed by 1 h of dark adaptation); and (iii) 1 week recovery: pre-dawn measurement conducted 1 week after the cold stress on trees that were grown under control conditions.

At the end of the night, *Fv/Fm* ratios of the cold-stressed plants (i.e.,

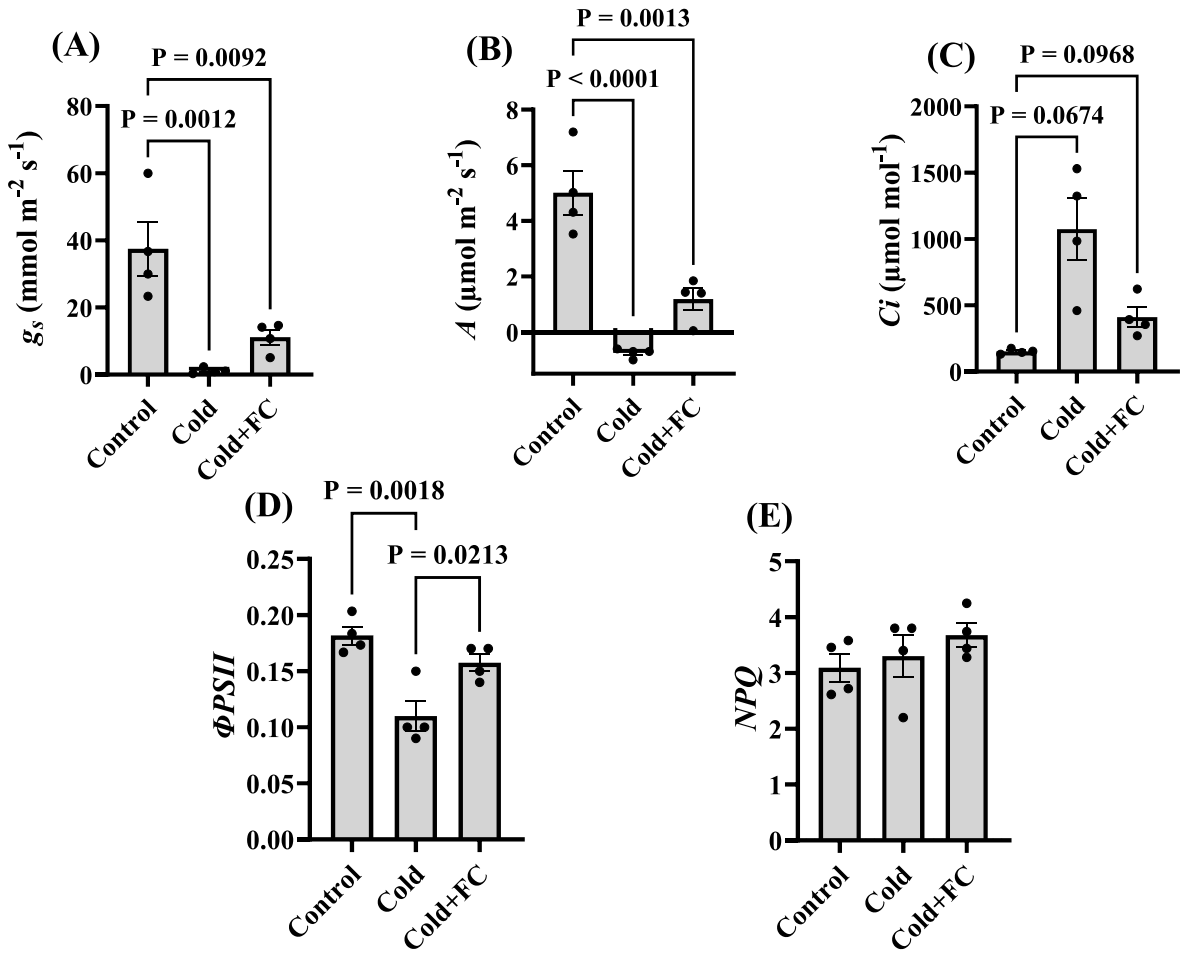

Fig. 3. Foliar treatment of fusicoccin after an extreme night-chilling event partially rescue from the physiological drop in mango leaf gas exchange and its PSII photochemical efficiency. The experiment was carried out as described in Fig. 1 where young mango trees were tested under extreme cold night stress (0.5 °C for 12 h). Gas exchange and light-adapted chlorophyll fluorescence parameters were measured. Data shown is the average of: **(A)** stomatal conductance (g_s; mmol H_2O m^{-2} s^{-1}) **(B)** net CO_2 assimilation rates (A; µmol CO_2 m^{-2} s^{-1}), **(C)** intercellular CO_2 (Ci; µmol CO_2 mol air^{-1}), **(D)** actual efficiency of PSII ($\Phi PSII$), and **(E)** non-photochemical quenching (NPQ). Statistical analysis was conducted using one-way ANOVA followed by Tukey's multiple comparison test. The bars shown are means ± SEM; n = 4 trees for each treatment, and five leaves were measured from each tree. When a statistically meaningful Effect Size was found, the P_{value} is noted. Raw data is presented in Supplemental Table 1.

Cold and Cold + FC treatments) were similar to that in the control plants (Fig. 4; Control 0.837 ± 0.007, Cold 0.826 ± 0.014, Cold + FC 0.852 ± 0.001), suggesting that the cold stress by itself did not affect the potential light capture and electron transport. We then measured *Fv/Fm* ratios after plants were exposed to natural sunlight for 7 h (plus 1 h of leaf dark adaptation). There was some reduction in the control non-stressed plants compared to pre-dawn values (Control 0.737 ± 0.001). In contrast, *Fv/Fm* ratio dramatically declined in the cold-stressed plant leaves, by ~27%, compared to the control. *Fv/Fm* ratio in the FC-treated cold-stressed plants was only reduced by ~16% of the control plants (Fig. 4; Cold 0.524 ± 0.027, Cold + FC 0.617 ± 0.021, $P_{ES\ Cold\ -\ Control}$ = 0.0063, $P_{ES\ Cold-Cold+FC}$ = 0.0791, $P_{ES\ Cold\ +\ FC-Control}$ = 0.0171), further supporting the positive protective effect of FC against extreme night-chilling damage. To assess the physiological status of the leaves after 1 week of recovery, trees were maintained under ambient growth conditions in the net-house, and the leaves that were previously measured were analyzed again for their pre-dawn *Fv/Fm* ratios. Data showed that foliar application of FC results in full recovery, similar to the control plant leaves (Fig. 4; Cold + FC 0.833 ± 0.022 vs. Control 0.835 ± 0.011, P_{ES} = 0.994). In comparison, untreated cold-stressed plants showed some sustained photoinhibition, with lower *Fv/Fm* ratios, and did not fully recover after 1 week (Cold 0.786 ± 0.033, $P_{ES\ Cold-Control}$ = 0.436).

3.5. Foliar application of FC after an extreme night-chilling event minimizes cellular damage and improves membrane stability in the leaves

Cold stress has been found to disturb membrane integrity (Campos et al., 2003). Electrolyte leakage (EL%) is an indicator of membrane stability, where increasing values of EL% indicate a decline in membrane stability and cellular damage. To test whether foliar application of FC after an extreme night-chilling event can protect from cellular damage, a similar experiment as in section 3.3 was conducted. After mango trees were exposed to night-chilling events and then to natural light conditions for 7 h, leaf discs were excised from the three different treatments (i.e., Control, Cold, and Cold + FC) and measured for electrolyte leakage. Results showed an ~37% increase in electrolyte leakage from mango leaf tissue following an extreme night-chilling event (Cold) compared to the control leaves (Fig. 5; Cold 21.21 ± 1.08 vs. Control 16.07 ± 0.30, P_{ES} = 0.002). However, the EL% of FC-treated cold-stressed mango trees was similar to that of the control plants (Fig. 5; Cold + FC 17.78 ± 0.66, $P_{ES\ Cold+FC-Control}$ = 0.287, $P_{ES\ Cold-Cold+FC}$ = 0.025). These findings suggest that foliar application of FC on mango tree leaves after a night chill can increase leaf tolerance to cold night–bright day stress and prevent membrane damage.

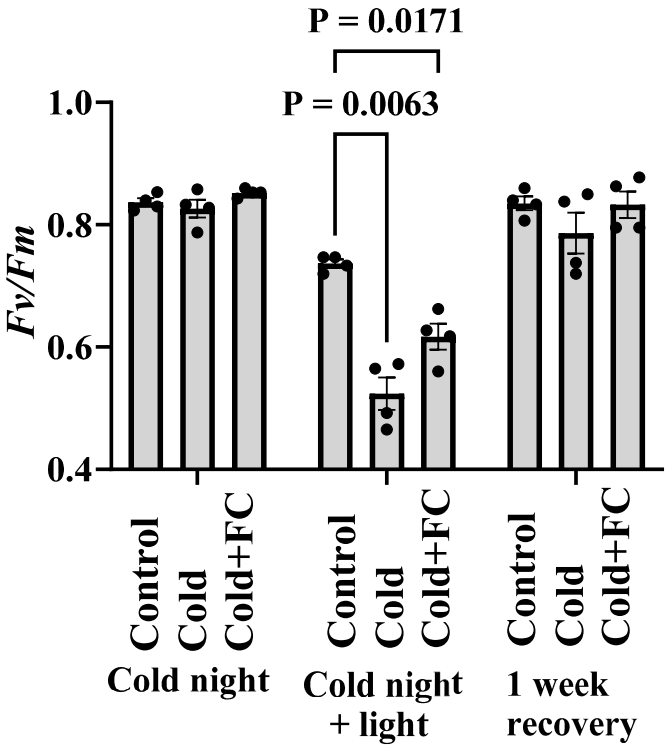

Fig. 4. Foliar treatment of fusicoccin after an extreme night-chilling event reduces the mango leaf photosynthetic damage and enhances its recovery. The experiment was carried out as described in Fig. 1 where young mango trees were tested under extreme cold night stress (0.5 °C for 12 h). Measurements of the maximal quantum efficiency (FV/FM) were conducted at: 1) "Cold-night" at the end of the night, before exposure to light, 2) "Cold-night + light" 8 h after exposure to natural light (dark adapted for 1 h) and 3) "1 week of recovery" after plants were exposed to the natural sunlight for 7 h (plus 1 h of leaf dark adaptation). Statistical analysis was conducted using two-way ANOVA, with treatment type and time point (as repeated measurement) as factors. Statistical interaction was evident ($P = 0.0024$). The bars shown are means (±SEM); n = 4 trees for each treatment; in each tree, 5 leaves were measured. When a statistically meaningful Effect Size was found within the same time point, the P_{value} is noted. Raw data is presented in Supplemental Table 1.

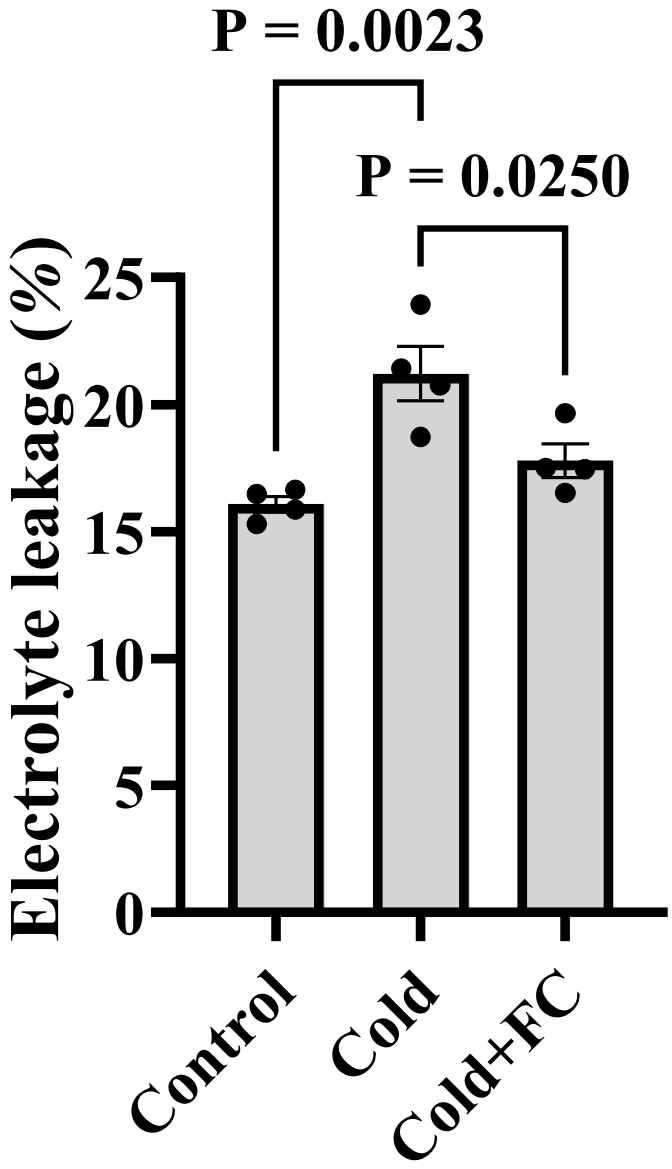

Fig. 5. Foliar treatment of fusicoccin after an extreme night-chilling event protects from bright-day induced membrane damage in mango leaves. The experiment was carried out as described in Fig. 1 where young mango trees were tested under extreme cold night stress (0.5 °C for 12 h). After 8 h under natural light conditions, leaf discs were excised from Control, Cold, and Cold + FC treated plants and measured for electrolyte leakage. Statistical analysis was conducted using 1-way ANOVA followed by Tukey's multiple comparison test. The bars shown are means ± SEM; n = 4 trees for each treatment, and five leaves were measured from each tree. When a statistically meaningful Effect Size was found, the P_{value} is noted. Raw data is presented in Supplemental Table 1.

3.6. FC induction of stomatal opening can rescue mango leaves from chlorosis following an extreme night-chilling event

Chlorophyll concentration after an extreme night-chilling event (as described in section 3.3) was determined 1, 2, 4, and 7 days after the chilling event. The chlorophyll concentration of the cold-stressed plants, measured after 7 h of light, was lower than that of the controls. Interestingly, leaves of the Cold + FC-treated plants showed only a negligible reduction (Fig. 6; Cold 469 ± 13, Control 528 ± 9, Cold + FC 517 ± 21 μmol m^{-2}, $P_{ES\ Cold\ -\ Control} = 0.0263$, $P_{ES\ Cold-Cold+FC} = 0.222$, $P_{ES\ Cold+FC-Control} = 0.877$). On day 2, the difference (ES) between the cold-treated and control leaves significantly increased (Fig. 6; Cold 437 vs. Control 530 μmol m^{-2}, $P_{ES} = 0.007$), suggesting that chlorophyll catabolism was still higher than its anabolism. On the other hand, although FC-treated cold-stressed plants (Cold + FC) showed some reduction in chlorophyll level on day 2 (compared to that on day 1), only a small, non-significant difference was found when compared to the control (Fig. 6; Cold + FC 490 μmol m^{-2}, $P_{ES\ Cold+FC-Control} = 0.239$). Similarly, analysis on day 4 revealed a further decrease in chlorophyll concentration in the cold-stressed plant leaves. On the other hand, leaves of FC-treated cold-stressed plants (Cold + FC) maintained the same chlorophyll concentration as on day 2, which was not statistically different from the control plants (Fig. 6; Cold 414, Control 513, Cold + FC 488 μmol m^{-2}, $P_{ES\ Cold\ -\ Control} = 0.038$, $P_{ES\ Cold-Cold+FC} = 0.107$, $P_{ES\ Cold+FC-Control} = 0.663$). After 7 days of recovery, the average leaf chlorophyll concentration increased in all treatments and reached similar levels (Fig. 6; Cold 490 ± 9, Control 541 ± 26, Cold + FC 525 ± 30 μmol m^{-2}, $P_{ES\ Cold\ -\ Control} = 0.284$, $P_{ES\ Cold-Cold+FC} = 0.552$, $P_{ES\ Cold+FC-Control} = 0.923$), showing recovery from the night-chilling event.

3.7. Photosynthetic leaf performance of night-chilled mango trees is affected by diurnal rhythm

To further study the mechanism involved in the inhibition of gas exchange and the subsequent leaf damage induced by cold-night stress, we tracked leaf photosynthetic performance, measuring *A/Ci* curves at

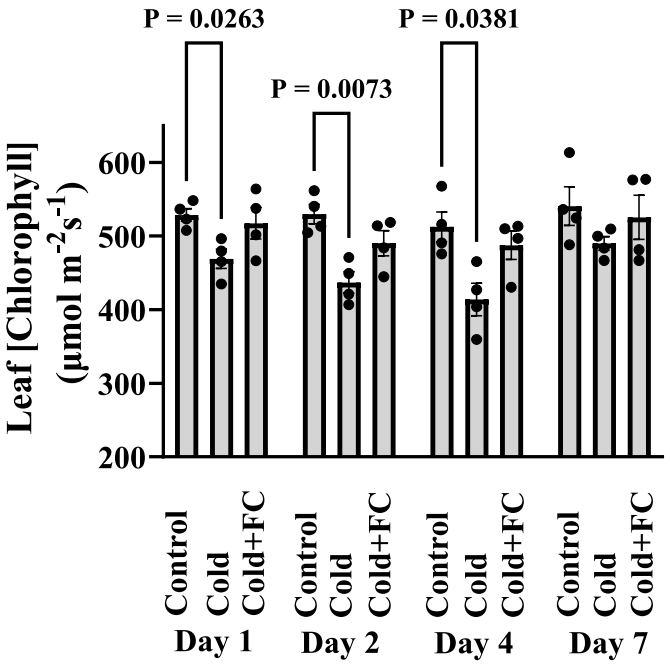

Fig. 6. Foliar treatment of fusicoccin after an extreme night-chilling event protects from bright-day induced chlorosis and necrosis in mango leaves. The experiment was carried out as described in Fig. 1 where young mango trees were tested under extreme cold night stress (0.5 °C for 12 h). Chlorophyll concentration was measured at 02:00 pm on days 1, 2, 4, and 7. Data shown is the chlorophyll concentration means ± SEM (µmol Chl m^{-2}); n = 4 trees for each treatment, 5 leaves were measured from each tree. Statistical analysis was conducted using two-way ANOVA, with 2 factors: Treatment and Time point (repeated measurement). No statistical interaction effect was found (P = 0.3508). When a statistically meaningful Effect Size was found within the same time point, the P_{value} is noted. Raw data is presented in Supplemental Table 1.

different times after the night-chilling event. We measured 3 leaves from 2 different trees sequentially at different times (leaf 1 at 8:00 a.m., leaf 2 at 10:00 a.m., and leaf 3 at 12:00 p.m.) as a control on the first day, before exposure to chilling. During the following night, one plant was exposed to a moderate night-chilling event (7 °C for 12 h), and the other was maintained under ambient temperature (Control). The same leaves that were measured on the previous day (non-stressed, steady-state levels) were measured again in the same sequence and at the same times after 1 h of photosynthetic induction (ramping of PPFD from 0 to 2000 µmol m^{-2} s^{-1}). As no variations were found in control non-stressed trees between the first and second day of measurements, Fig. 7A–C presents only the before-cold and after-cold *A/Ci* curves. The representative data show that the night-chilling event did not affect the rate of leaf photosynthetic CO_2 assimilation as a function of CO_2 concentration inside the leaf in the early morning (8:00 a.m.) (Fig. 7A). Two hours later (10:00 a.m.), the night chilling substantially decreased the *A/Ci* relationships (Fig. 7B), whereas after an additional 2 h (12:00 p.m.), partial recovery was observed (Fig. 7C). To quantify the effect of a moderate night-chilling event on photosynthesis, the FvCB model parameters were fitted to the whole dataset, and average values (n = 5) are summarized in Table 1. Solving the model for the control non-stressed plants revealed that all three parameters—V_{cMax}, J, and TPU—were very similar on the two consecutive measurement days, in all three leaves (measured at 08:00 a.m., 10:00 a.m., and 12:00 p.m.). In contrast, mango plants exposed to a moderate night-chilling event showed an interesting response. The effect of night chilling on leaf photosynthetic performance depended on the time during the diurnal cycle at which it was assessed. When cold-stressed leaves were measured at 08:00 a.m., J decreased considerably (79.2 ± 3.1 before vs. 66.9 ± 5.3 µmol m^{-2} s^{-1} after the night chilling, P_{ES} = 0.0052), whereas V_{cMax} and TPU were slightly lower (V_{cMax} 50.8 ± 8.7 before vs. 38.1 ± 4.4 µmol m^{-2} s^{-1} after night chilling, P_{ES} = 0.3930; TPU 5.6 ± 0.1 before vs. 4.6 ± 0.6 µmol m^{-2} s^{-1} after night chilling, P_{ES} = 0.1983). On the other hand, when cold-stressed leaves were measured at 10:00 a.m., all three parameters were substantially reduced, with V_{cMax} decreasing from 50.4 ± 5.6 to 34.5 ± 4.3 µmol m^{-2} s^{-1} (P_{ES} = 0.0205), J from 73.4 ± 6.2 to 52.6 ± 5.1 µmol m^{-2} s^{-1} (P_{ES} = 0.0382) and TPU from 5.0 ± 0.6 to 3.9 ± 0.3 µmol m^{-2} s^{-1} (P_{ES} = 0.0593) before and after the night-chilling event, respectively. When cold-stressed leaves were measured at 12:00 p.m., recovery was observed in all three parameters with no statistically significant differences compared to their previous levels (V_{cMax} was 52.6 ± 4.2 vs. 42.0 ± 3.9 µmol m^{-2} s^{-1}, $P_{ES=}$0.3248; J was 82.9 ± 7.7 vs. 71.6 ± 2.1 µmol m^{-2} s^{-1}, P_{ES} = 0.2304, and TPU was 5.6 ± 0.5 vs. 4.9 ± 0.2 µmol m^{-2} s^{-1}, P_{ES} = 0.5144, before and after the night-chilling event, respectively).

4. Discussion

Most warm-climate plant species are sensitive to non-freezing

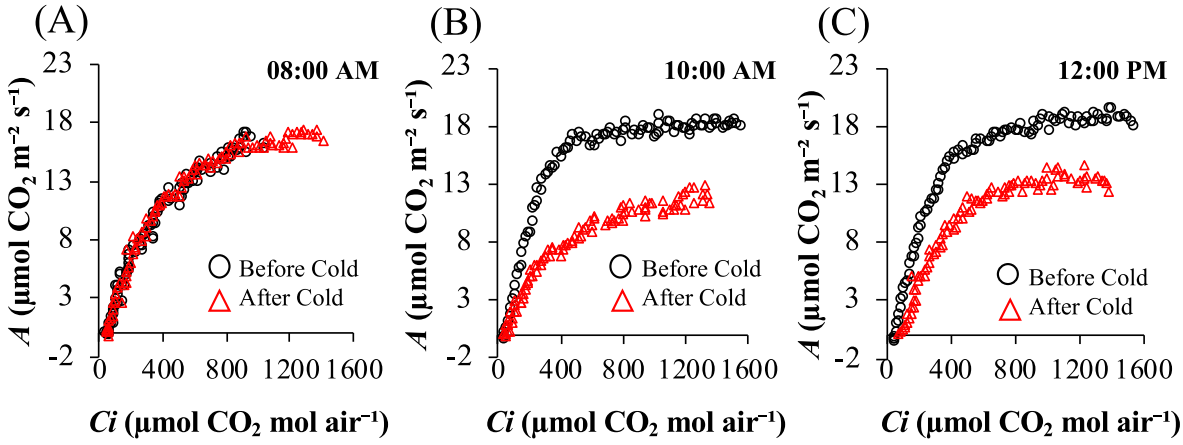

Fig. 7. The physiological reduction in mango leaf gas exchange was revealed by analyzing the CO_2 response curve (A/Ci) parameter after a moderate night-chilling event. Leaves of young mango trees were measured for their CO_2 response curves at three-time intervals, **(A)** 08:00 a.m., **(B)** 10:00 a.m., and **(C)** 12:00 p.m., before or after cold night stress. On the first day, leaf CO_2 response curve measurement were taken and then the analyzed plants were split into two treatment groups, I) cold-night stress, where plants were subjected to moderate night-chilling event (Cold; 7 °C for 6 h) or II) Control, where plants were kept under ambient growth conditions. On the proceeding day, the same plants were measured again for their CO_2 response curve at the same time of the day previously measured (08:00 a.m./10:00 a.m./12:00 p.m.).

chilling temperatures. Exposure to low temperatures and high radiation causes rapid and sometimes very severe inhibition of photosynthesis. Mango is characterized as an extremely chilling-sensitive species. Chilling disrupts various essential photosynthesis components, including both stomatal and non-stomatal components —it has been found to impair regulation of stomatal conductance (Martin et al., 1981; Bauer et al., 1985; Bongi and Long, 1987; Bell et al., 1994; Nir et al., 1997; Flexas et al., 1999; Allen et al., 2000; Van Heerden et al., 2004; Liu et al., 2012; Guo and Cao, 2015; Joshi et al., 2020), and the thylakoid electron-transport chain and carbon-reduction cycle (Kee et al., 1986; Baker, 1994; Leegood and Edwards, 1996; Fryer et al., 1998). In mango, Allen et al. (2000) showed that reduction in leaf net CO_2 assimilation rates at midday following a night-chilling event does not result primarily from PSII inhibition but from increased stomatal limitation and decreased Rubisco activity. Under these limitations, with exposure to light, part of the excess photon energy is target to a photoprotection path (reversible down regulation of PSII centers), where the rest of the energy results in leaf photodamage (Demmig-Adams and Adams, 1992; Long et al., 1994; Nir et al., 1997). One of the important tasks in this field of research is to identify the primary effects within this tightly regulated and complex plant system. The objective of this study was to evaluate the contribution of stomatal components (versus non-stomatal components) and their order within the sequence of events that ultimately result in the observed physiological damage following night-chilling events; in other words, to evaluate whether bypassing (i.e. eliminating) stomatal limitation on the day that follows the night chilling can mitigate leaf physiological damage. We showed that removing the stomatal limitation bottleneck using a foliar application of the guard cell H^+-ATPase activator FC (Yamauchi et al., 2016; Marra et al., 2021) decreases the physiological damage of the cold night - bright day stress, and improves the overall physiological state of the plant.

It has been previously shown that the severity of the night-chill effect on leaf physiological damage is time and temperature dependent, with the damage increasing as the temperature decreases and as the exposure time to the low temperature increases (Nir et al., 1997; Ying et al., 2002). In this study, we evaluated the effect of a foliar application of FC on leaf physiological parameters following exposure to moderate (7 °C), severe (4 °C), or extreme (0.5 °C) night-chilling intensities. Exposure to a prolonged and lower temperature during the night resulted in stronger physiological leaf damage, which could be rescued by FC treatment to varying extents.

We found that a moderate night-chilling event (7 °C for 6 h) induces a decrease in both stomatal conductance (g_s) and net CO_2 assimilation rates (A) (reduction of ~47% and ~40% compared to control non-stressed trees, respectively, Fig. 1A and B). Allen et al. (2000) suggested that the decline in A following a night-chilling event results from stomatal limitation and a decrease in Rubisco activity (V_{cMax}), supporting the involvement of both stomatal and non-stomatal components. Nonetheless, under these moderate cold-stress conditions, FC was able to fully recover g_s and A, suggesting that the effect of a moderate night-chilling event on A is mainly stomatal. However, when C_i levels were evaluated, no significant difference was observed between the treatments (i.e., Control, Cold, Cold + FC). If the observed decline in leaf net photosynthesis level of the cold-stressed plants was a consequence of stomatal limitation, C_i and g_s should have shown a correlated decrease. Together, the results presented in Fig. 1 suggest that a moderate chilling event induces inhibition of leaf A by a combination of both stomatal and non-stomatal components. Furthermore, FC treatment overcame the effects on both the stomatal and non-stomatal components inflicted by night chilling.

When the effect of FC treatment was tested on mango trees after a severe night-chilling event (4 °C for 12 h), g_s and A were higher in FC-treated cold-stressed plant leaves than in their untreated cold-stressed counterparts (Fig. 2A and B). Evaluation of their internal CO_2 levels revealed reduced *Ci,* which was negatively correlated to leaf g_s (Fig. 2C). This negative correlation in FC-treated cold-stressed plants further supports the role played by stomatal limitation in chilling-induced inhibition of photosynthesis. It has been previously shown that cold temperatures suppress leaf photochemical efficiency (Greer, 1990; Nir et al., 1997; Allen et al., 2000; Liu et al., 2012; Guo and Cao, 2015; Joshi et al., 2020). In this study, the photochemical efficiency of PSII, which was found intact following moderate night chilling (Fig. 1C and D), was substantially reduced following a severe night-chilling event. However, when FC treatment was applied, the photochemical efficiency of PSII fully recovered (Fig. 2D, *ΦPSII*; Fig. 2E, *Fv/Fm*). The results show that treatment with FC following a severe night-chilling event induces partial stomatal opening, albeit not as wide as in the non-stressed control plants, implying that FC treatment could only partially restore stomatal function and that additional components/pathways may be involved.

The physiological consequences of an extreme night-chilling event (0.5 °C for 12 h) were even more intense (Fig. 3). The cold-stressed plants' stomata were completely closed (Fig. 3A), a phenotype which was associated with negative A values (respiration losses greater than assimilation), whereas FC treatment had only a minor positive effect on gas exchange (Fig. 3A and B). On the other hand, FC provided impressive protection of the light-harvesting photosynthetic system (Fig. 3D, *ΦPSII*; Fig. 4, *Fv/Fm*) by preventing damage to cell membranes (Fig. 5, electrolyte leakage) and leaf chlorophyll (Fig. 6).

One of the essential protective mechanisms for the dissipation of excess photon energy is NPQ, a process in which excess absorbed light energy dissipates as heat before inducing electron transport (Ruban, 2016). It has been previously shown that the xanthophyll cycle plays a substantial role in regulating the NPQ mechanism. Weng et al. (2006) proposed that during the early morning (i.e., under low light) after a night-chilling event, mango plants downregulate the efficiency of PSII and enhance the xanthophyll de-epoxidation system, reducing potential oxidative damage to the photosynthetic system (Latowski et al., 2011).

Plants use regulatory processes to better control the dissipation of excess light energy following changes in plant environmental conditions. These include (i) dynamic photoinhibition, for flexible adjustment to actual environmental conditions, and (ii) chronic photoinhibition, the integrated response to long-term environmental stress, such as to a night-chilling event (Werner et al., 2002). In this study, we found that severe (4 °C for 6 h), and to a greater extent, extreme (0.5 °C for 12 h) night-chilling events affect leaf photochemical efficiency and induce chronic photoinhibition/protection in mango plant leaves (Figs. 2D and E, 3D and E and 4). NPQ values were similar across all treatments (Fig. 3E), indicating that the NPQ machinery was unaffected by the cold-stress conditions and/or the FC application.

Several studies have called into question the effect of cold stress on plant stomatal function (Agurla et al., 2018). The results reported here demonstrate that foliar application of FC partially prevents physiological night-chill damage to mango leaves by driving stomatal opening the following day. FC is a strong activator of the proton pump of plasma membrane H^+-ATPase (De Michelis et al., 1991), which is well known to be a key mechanism in guard cells during stomatal opening (Lohse and Hedrich, 1992; Kinoshita and Shimazaki, 2001; Ueno et al., 2005; Ando et al., 2022). In addition, FC participates in other plant signals that regulate stomatal conductance (Marra et al., 2021). It has been shown that: (i) FC stimulates the expression of various genes (Frick and Schaller, 2002), including at least one of the Ca^{2+}-ATPase genes (Liβ et al., 1991). Wilkinson et al. (2001) proposed that the cold-induced stomatal closure observed in intact *Commelina communis* leaves was caused by an increase in guard cell apoplastic calcium uptake; (ii) FC activates the enzyme nitrate reductase by disturbing its interaction with the regulatory 14-3-3 protein family (Moorhead et al., 1996). Both nitrate reductase and 14-3-3 proteins play a role in regulating stomatal conductance (Zhang et al., 2007; Wilson et al., 2008; Cotelle and Leonhardt, 2016), as well as in cold acclimation and tolerance to cold stress (Zhao et al., 2009; Visconti et al., 2019). It is interesting to note that nitrate reductase, which regulates the production of nitric oxide, is an essential component of the signaling network inducing stomatal

closure in response to the phytohormone ABA (Desikan et al., 2002; Bright et al., 2006); (iii) cold stress induces ABA production (Lang et al., 1994; Shinozaki and Yamaguchi-Shinozaki, 1996), a strong signal for stomatal closure, whereas it is inhibited by FC (Huang et al., 2014); (iv) ethylene, another phytohormone that is involved in various biotic and abiotic stress responses, increases following cold stress (Guye et al., 1987) and negatively regulates freezing tolerance in *Arabidopsis* (Shi et al., 2012). It has been previously shown that ethylene induces stomatal closure (Desikan et al., 2006; Zhang et al., 2021) and that its products may be inhibited by FC (Branca and Ricci, 1983); (v) the outward-rectifying K^+ channels in the guard cell constitute another mechanism that controls stomatal conductance (Hosy et al., 2003). Related to this, it was discovered that under cold stress, the guard cells' *GORK* gene—which encodes the K^+ release channel—is upregulated (Becker et al., 2003). The outward-rectifying K^+ channels are blocked by FC, similar to the other signaling elements detailed above (Blatt and Clint, 1989). The findings provided here suggest that one or more of the aforementioned signals are responsible for the defective stomatal opening phenotype, and that foliar application with FC can overcome the impaired stomatal opening in leaves after a night-chilling event in mango plants.

Previous research and the findings of this study indicate that chilling events inhibit leaf *A* via a combination of stomatal and non-stomatal components. According to the findings, FC treatment can overcome the non-stomatal components, which have a direct impact on leaf CO_2 uptake, in addition to its effect on the stomatal restriction induced by night chilling.

The circadian clock is an endogenous timer of plant metabolism and, among other processes, is involved in controlling stomatal conductance (g_s) (Hassidim et al., 2017) and photosynthesis (*A*) (Dodd et al., 2005) responses. However, it has been found that circadian regulation mediates *A* and *gs* in an independent matter (Dodd et al., 2004). In mango, the circadian rhythm of the guard cells is not affected by chilling, as the endogenous oscillation of the stomatal conductance is not affected, whereas photosynthesis is substantially compromised (Allen et al., 2000). On the other hand, cold stress severely disrupts the circadian regulation of key photosynthetic enzymes, as shown in chilling-sensitive herbaceous plants such as tomato. Dark chilling has been shown to disrupt the enzymatic activity of the enzyme sucrose-phosphate synthase, a key regulator of cellular carbon metabolism (Jones et al., 1998), and suspend the expression of the chlorophyll a/b binding protein and Rubisco activase genes (Martino-Catt and Ort, 1992). Disruption of circadian rhythm is likely to have a negative effect on photosynthetic performance. In this study, based on leaf *A/Ci* curves that were measured at different times of the day following a moderate night-chilling event, we found that the effect of night chilling on leaf photosynthetic performance depends on the time of day during the diurnal cycle. In the early morning (08:00 a.m.), *J* was significantly reduced. V_{cMax} and TPU rates were only significantly impaired 2 h later (10:00 a.m.). At noon (12:00 p.m.), all of the parameters showed recovery and were not considerably different from the control samples (Fig. 7 and Table 1). TPU-limited photosynthesis is very sensitive to temperature (Yang et al., 2016), even more than Rubisco- or RuBP regeneration-limited photosynthesis (Cen and Sage, 2005; Sharkey and Bernacchi, 2012). In this respect, our result implies that dark chilling induces a disruption in the circadian rhythm, and as a consequence, incompatibility between sucrose-phosphate synthase activity (Jones et al., 1998) and carbon-reduction cycle activities (Martino-Catt and Ort, 1992), resulting in transient V_{cMax} and TPU limitation of photosynthesis (Sun et al., 2011).

It has been well established that physiological damage following a night-chilling event is dependent on the severity of the night-chill (Nir et al., 1997; Ying et al., 2002), which affect both membrane-dependent photosynthetic processes and metabolic enzymatic reactions (Shen et al., 1990; Banerjee and Roychoudhury, 2019; Wei et al., 2022). Based on our results a moderate chilling event had no significant effect on the photosynthetic membrane dependent processes of mango leaves (*Fv/Fm* Fig. 1E), yet induced transient reduction of the enzymatic CO_2 assimilation reactions, as been observed in the *A/Ci* curve analyses (V_{cMax}, TPU, J, Table 1). On the other hand, an extreme night-chilling event resulted in damage and significant impairment of both the membrane-dependent photosynthetic processes (*Fv/Fm* Fig. 4, electrolyte leakage Fig. 5) and metabolic enzymatic reactions (negative correlation between the net CO_2 assimilation rates, Fig. 3b, and the intercellular CO_2 concentrations Fig. 3c).

Furthermore, photosynthesis is a complex and highly regulated process and is one of the most temperature-sensitive processes in the plant. It is well documented that cold temperatures affect photosynthetic related processes, which result in an imbalance between the energy source and the metabolic sink (Ensminger et al., 2006). Different studies revealed alternation in various photosynthetic components and photosynthetic carbon metabolism enzymes, which include the Chl a/b-binding proteins, photosynthesis antenna proteins, different electron transport chain components, and Calvin Benson cycle enzymes (Kawamura and Uemura, 2003; Van Heerden et al., 2004; Yu et al., 2021). Indeed, the leaf mesophyll is the primary photosynthetic and carbon-metabolizing tissue. Nevertheless, guard cells of most species also contain photosynthetically active chloroplasts (Outlaw et al., 1981; Zeiger et al., 1981; Shimazaki et al., 1982; Zemel and Gepstein, 1985; Gotow et al., 1988; Rother et al., 1988). In this respect, as photosynthesis plays a critical role in both the mesophyll and guard cells and since photosynthesis in mesophyll/guard cell affect stomatal conductance (Olsen et al., 2002; Lawson et al., 2008; Suetsugu et al., 2014; Azoulay-Shemer et al., 2015; Kromdijk et al., 2019), the impaired stomatal opening following night-chill may result from alternation in signals within the guard cells and/or long-distance signals from the mesophyll. Previous transcriptomic analysis of maize (whole leaf) response to cold stress revealed alternation in various pathways, which include photosynthesis-related genes as well a few stomata guard cell functioning-related DEGs (Yu et al., 2021). It is important to note that both the work by Allen and Ort (2001) and the results of our study (*A/Ci* Fig. 7, Table 1) imply that the impaired stomatal opening on the morning that follows the night-chill is the primary process which produces the inhibition of the photosynthetic system and result in plant physiological damage. Yet, further investigation, at the physiological and molecular levels, is still needed to reveal which pathway/s that regulate stomatal conductance in guard cells of mango leaves are impaired by the night chill. Guard cell transcriptome analysis from cold-stress mango leaves is currently being studied to shed light on this subject.

5. Conclusion

In the current study, we demonstrate that the exogenous application of FC to mango leaves shortly after a night-chilling event significantly reduces the chilling damage by causing stomatal opening and reducing non-stomatal limitations, thereby ameliorating the accompanying adverse physiological effects (i.e., *gs*, *A*, *Fv/Fm* and *ΦPSII*, cell membrane damage, leaf chlorophyll, chlorosis). We show that treatment with a specific compound that promotes stomatal opening can bypass the restricted gas exchange caused by night-chilling-induced stomatal closure in mango leaves, free the photosynthetic apparatus from harmful excess photon energy, and enhance the physiological state of the plant. Chemical induction of stomatal opening may be a promising tool for mitigating cold night - bright day physiological damage. Further research is needed to determine which pathways regulating stomatal conductance in the guard cells are impaired by night chilling, and whether other compounds that induce stomatal opening will be beneficial and practical for protecting mango orchards from night-chill damage.

Author statement

Conceptualization and design: T.A.-S.; Data acquisition: M.I.H., O.S., and Z.A.; Analysis and interpretation: M.I.H. and O.S.; Drafting of the article: M.I.H. and O.S.; Scientific feedback and discussions: T.A.-S., M.I.H., O.S., Y.C., and D.C.; Final approval: T.A.-S.

Statement of informed consent

No conflicts; informed consent, human or animal rights are not applicable.

Authors' agreement/declaration

All authors have seen and approved the final version of the manuscript. Furthermore, all authors have agreed to the authorship and submission of the manuscript to this journal.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2023.108221.

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