REGULAR ARTICLE

The possible role of extra magnesium and nitrogen supply to alleviate stress caused by high irradiation and temperature in lemon trees

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Abstract

Aims Citrus trees are subjected to variation of environmental conditions throughout the annual cycle, especially during the flowering and fruit set. Heat waves have affected the environmental conditions required for optimum crop growth and have reduced fruit yield and quality worldwide. This study aimed to evaluate an extra supply of magnesium (Mg) and/or nitrogen (N) to increase citrus tolerance to excessive increased irradiation and consequent air temperature.

Methods Young lemon trees were grown in a greenhouse with extra Mg, N and Mg + N supply for five months and then exposed to heat for 12 days. After imposing stress condition, photosynthetic and enzymatic antioxidant system parameters were assessed to evaluate the impact of the extra nutrient supply in alleviating stress induced by combination of elevated irradiance and air temperature.

Results Extra nutrient supply increased plant tolerance to the environmental stress, allowing maintenance of high photosynthetic and transpiration rates and low ratio of apparent electron transport rate per photosynthetic carbon assimilated. Enhanced Mg supply also increased activity of the antioxidant enzyme system and decreased the oxidative stress of plants. Trees treated with extra N exhibited increased chlorophyll concentrations and ability to harvest light energy.

Conclusions Citrus trees under stressed condition, such as elevated air temperature and radiation, associated to heat waves, exhibited less damage in the photosynthetic mechanism and high activity of the antioxidant enzymatic system when supplied with extra nutrient. It was concluded that extra Mg and N supply are important tools to prevent damages and enhance lemon trees biochemical and physiological responses to alleviate the harmful effects of unfavorable environmental condition.

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Abbreviations

A_N	photosynthetic rate
g_s	stomatal conductance
C_i	internal CO_2 concentration
E	transpiration
A_N/C_i	instantaneous carboxylation efficiency
WUE = A_N/E	water use efficiency
ETR	apparent electron transport rate
PSII	photosystem II

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qP	photochemical quenching
qNP	non-photochemical quenching
ROS	reactive oxygen species
SOD	superoxide dismutase
H_2O_2	hydrogen peroxide
MDA	malondialdehyde
CAT	catalase
APX	ascorbate peroxidase
GR	glutathione reductase

Introduction

Global climate change impacts life processes of plants and animals throughout the world (Lobell et al. 2011; Ray et al. 2015). Increased light radiation (mainly for UV and infra-red) and greenhouse gases concentration (e.g. CO_2) in the tropics has caused crop yield losses, mainly due to the increment of air temperature and water stress (drought) (Adam et al. 1998; Fares et al. 2017; Cassia et al. 2018; Krasensky-Wraczek and Kangajarvi 2018). Thereby higher daily air temperatures associated with extreme weather events have reduced the maximum yield potential of crops, such as sugarcane (Marin et al. 2013), cotton (Assad et al. 2013), and coffee (Camargo 2010) in the tropics. More recently, citrus production was significantly affected in the main Brazilian production region – the São Paulo state and Triângulo Mineiro (Brazilian Citrus Belt) – during three crop seasons (2014 to 2016) because of highest air temperatures during the flowering and fruit set stages, which caused losses in fruit yield (Fundecitrus 2016).

Under these stress conditions, there is an increase of reactive oxygen species (ROS) in plant tissues, leading to high photooxidative damage, reduction in stomatal conductance and photosynthetic capacity of trees, which will consequently impact nutritional status and growth of plants (Guo et al. 2006; Santini et al. 2012; Borges et al. 2019). Among the environmental stress conditions affecting crop production, unbalanced plant nutritional status represents a major barrier to highest yield of citrus fruits. Field observations in high yielding citrus orchards demonstrated increased symptoms of mineral nutrient deficiencies, especially magnesium (Mg) and nitrogen (N), when the trees are subjected to unfavorable environmental conditions as elevated irradiance and air temperature. This information suggests that current nutritional levels are not enough to meet plant demand under such limiting conditions and additional supply of Mg and/or N could alleviate stress in plants. However, there are few reports about the specific role of mineral nutrients in alleviating stress in plants (Waraich et al. 2012).

Decreased Mg concentration in cereal grains has been reported in recent decades, probably due to increased productivity of these crops, achieved after the Green Revolution, which consequently diluted the Mg concentration in the plant (Guo et al. 2016). Over the past 30 years, the average yield of citrus orchards varied threefold, from 13.5 t/ha to more than 40 t/ha (Fundecitrus 2019), which has increased nutrient removal and consequently Mg demand in these areas. Magnesium plays important roles in biochemical and physiological processes in plants (Verbruggen and Hermans 2013; Cakmak 2013). The nutrient is also responsible for synthesis of chlorophyll, phloem loading and photoassimilate transport into sink organs, formation and utilization of ATP, and activation of enzymes related to photosynthesis and the antioxidant system (Cakmak et al. 1994; Shaul 2002). Decrease in photosynthesis capacity is frequently associated with low Mg content in the leaves. Moreover, plants with limited Mg supply, when exposed to high light intensity, exhibit leaf chlorosis and necrosis, indicating that those plants will increase physiological requirement of Mg to avoid photooxidative damage caused by ROS generation (Cakmak and Kirkby 2008). Under low levels of Mg, wheat and maize plants are prone to heat stress-associated damages caused by elevated ROS levels (Mengutay et al. 2013). A correlation between Mg supply and antioxidant system in coffee seedlings was observed under controlled conditions, where the activity of such enzymes as catalase (CAT) and ascorbate peroxidase (APX) were greater in plants grown with low Mg supply, as well as those enzyme activities were dependent of the radiance level that the plants were exposed (Dias et al. 2018).

Nitrogen is crucial to plant growth and development, as well as to alleviation of abiotic stress conditions (Waraich et al. 2012; Lamaoui et al. 2018). Plants with extra N supply grown under high light intensity were more tolerant to photooxidative damage and exhibited greater photosynthetic capacity than those grown with low N supply (Kato et al. 2003). In this case, plants with extra N supply used absorbed light energy in the electron transport chain more efficiently than those with low N. Furthermore, N application also relates to reduced oxidative stress in plants under heat stress due to

increased antioxidant enzyme activities (Waraich et al. 2012; Lamaoui et al. 2018). Protein production increases with N availability and well-nourished plants exhibit greater quantities of mitochondrial and chloroplastic heat-shock proteins that influence plant tolerance to high temperatures (Heckathorn et al. 1996; Hippler et al. 2018a).

Elucidation of the interaction between physiological and biochemical processes with the nutritional status of plants would contribute to better understand potential effects of nutrient supply to alleviate stress observed in adverse environmental growing conditions. Therefore, we carried out this work to evaluate physiological traits and oxidative stress in young lemon trees grown outdoors, under elevated irradiance and air temperature, and supplied with additional Mg and N.

Materials and methods

One-year old Lisbon Limonera 8A lemon [*Citrus limon* (L.) Burm. f.] trees grafted onto Swingle citrumelo [*C. paradisi* Macfad. X *Poncitrus trifoliata* (L.) Raf.] were grown in 15 L plastic pots filled with substrate (80% pine bark, 5% carbonized materials, and 15% vermiculite) in a greenhouse. Initially, plants were grown for five months under a 50% shaded condition with an aluminized screen cover placed 2.5 m above the tree top, which reflected about 50% of the total radiation (shaded condition; Fig. 1). Plants were fertilized monthly with a complete macronutrient solution containing (per plant) 2.0 g N, 0.5 g P, 0.6 g K, 1.3 g Ca, 0.38 g Mg, and 0.5 g S (Hippler et al. 2015), whereas micronutrients were sprayed to the foliage twice during the experiment (Quaggio et al. 2010).

During the shade condition period, besides the control treatment, in which the plants were nourished with adequate levels for all nutrients, a set of plants were fertilized with extra Mg as $MgSO_4 \cdot 7H_2O$ (+ Mg), or N as NH_4NO_3 (+ N), or Mg and N as $Mg(NO_3)_2$ (+ Mg + N). The monthly additional nutrient level was 0.2 g Mg per plant and 0.25 g N per plant compared to control treatment. In this experiment, the extra application of sulfur (S; 0.26 g per plant) in the magnesium sulfate treatment was not expected to result in significant plant metabolism alteration since citrus responds only mildly to S doses (Mattos et al. 2007; Hippler et al. 2015). After this period (5 months under unstressed conditions) when

the second vegetative flush was physiologically mature the plants were placed to stress conditions for 12 days. The plants were exposed to direct sunlight (without aluminized screen cover), which promoted increased in the irradiance and a higher air temperature (Fig. 2). Also, a set of plants with no extra nutrient supply were maintained under unstressed conditions as the standard control. The total duration of the experiment was approximately of 5 1/2 months and throughout this period the plant pots were irrigated to maintain approximately 70% of the soil field capacity. Environmental conditions were automatically monitored and logged every 30 min throughout the experiment with two automatic weather stations (Vantage Pro2, Davies Instruments, California, USA), one in shaded condition and another in a stress condition, placed at the height of the plant canopy.

Chlorophyll content

After 12 days under the stress condition, total chlorophyll (*Chl*) and chlorophyll a/b ratio were quantified in physiologically mature leaves according to Barnes et al. (1992) with modifications (Hippler et al. 2015). Fresh leaf tissues were collected in glass vials, cut into small strips, and suspended in dimethyl sulfoxide. The vials were heated in a water bath at 68 °C for approximately 4 h (until tissue depigmentation). The absorbance (*A*) of extracts was read at 646 and 663 nm (Multiskan™ Go, Thermo Fisher Scientific, Vantaa, Finland) and *Chl* was determined as $Chl = 7.49 \times A_{663} + 20.34 \times A_{646}$ according to Barnes et al. (1992).

Gas exchange and chlorophyll a fluorescence measurement

The net photosynthetic rate (A_N), stomatal conductance to water vapor (g_s), internal CO_2 concentration (C_i) and transpiration (E) were determined in expanded leaves in the middle third of the most mature vegetative flush of growth. Water use efficiency (WUE; A_N/E) and the apparent carboxylation efficiency (A_N/C_i) were calculated. The evaluations were performed 12 days after the beginning of stress conditions on a clear day in the morning (between 9:00 h and 10:00 h) and in the afternoon (between 13:00 h and 14:00 h) with an open path infrared gas analyzer system (LI-6800, LI-COR, Lincoln, NE, USA) equipped with an integrated fluorescence chamber head. Ambient measurement conditions
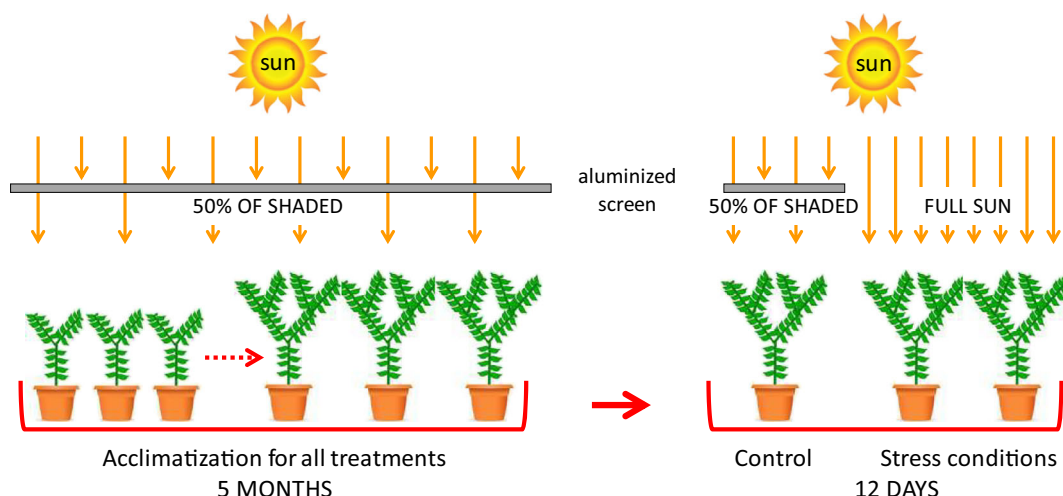
Fig. 1 Schematic representation of the experiment. Young citrus plants fertilized with different levels of magnesium and nitrogen were grown under unstressed conditions (aluminized screen blocking 50% of sunlight) for five months. After this period the plants were exposed to direct sunlight (without aluminized screen cover) for 12 days. A set of plants with adequate nutritional supply was maintained under unstressed conditions as the standard control
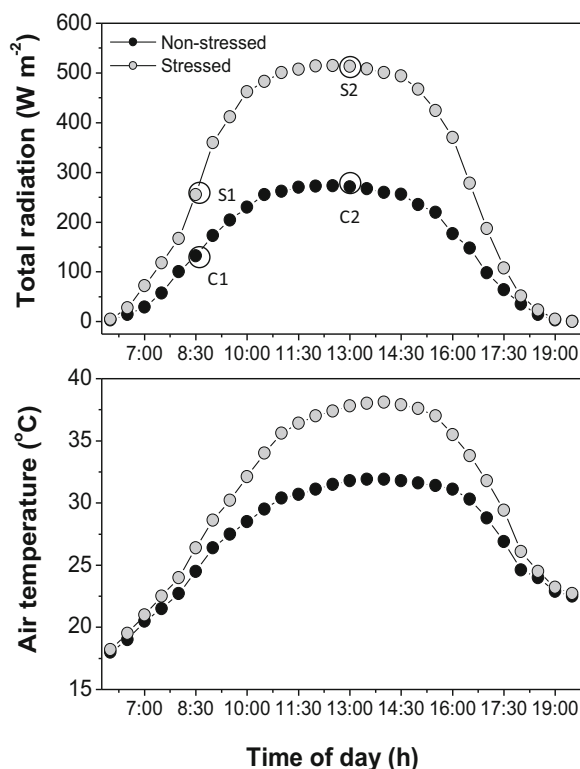

Fig. 2 Average of air temperature and total radiation in daylight period during the 12 days in the unstressed treatments (shaded) and stressed (sunlight). Legend: S1: Photosynthetically Active Radiation (PAR) = 950 μmol m^{-2} s^{-1}; S2: PAR = 1820 μmol m^{-2} s^{-1}; C1: PAR = 620 μmol m^{-2} s^{-1}; C2: PAR = 940 μmol m^{-2} s^{-1}

were controlled to maintain 40 Pa CO_2 partial pressure and an artificial photosynthetic photon flux density (PPFD) of 1,000 μmol photons m^{-2} s^{-1} at the leaf level, the duration for stabilization inside the chamber was quick, about 3 to 5 min per measurement.

Steady-state (F_o') and maximum (F_m') fluorescence yields were assessed in light adapted leaves between 13:00 h and 14:00 h and minimum (F_o) and maximum (F_m) fluorescence yield were assessed in dark-adapted (over-night) leaf tissues. F_m and F_m' were measured after a light saturation pulse ($\lambda < 710$ nm, PPFD ~ 10,000 μmol photon m^{-2} s^{-1}, 0.8 s). Chlorophyll fluorescence measurements were taken with infrared gas analyzer system (LI-6800, LI-COR, Lincoln, NE, USA) under the same ambient condition as described for gas exchange measurements. The variable fluorescence yield in both dark-adapted ($F_v = F_m - F_o$) and light-adapted ($\Delta F = F_m' - F_o'$) leaves was calculated. The maximum quantum yield of PSII [$F_v/F_m = (F_m - F_o)/F_m$], the effective quantum yield of PSII [$\Delta F/F_m' = (F_m' - F_s)/F_m'$], the photochemical quenching coefficient [qP = $(F_m' - F_S)/(F_m' - F_o')$], and the non-photochemical quenching coefficient [qNP = $(F_m - F_m')/(F_m - F_o')$] were calculated (Baker 2008). The apparent electron transport rate (ETR) was calculated according to Schreiber et al. (1998). The ratio ETR/A_N was calculated to estimate the use of electrons in processes not related to photosynthetic CO_2 assimilation.

Hydrogen peroxide and MDA levels

On the same day as photosynthetic measurements, physiologically mature leaves were collected and frozen at -80 °C for further measurements of hydrogen peroxide (H_2O_2) content and malondialdehyde (MDA), as well as the activities of the enzymes superoxide dismutase (SOD, EC 1.15.1.1), catalase (EC 1.11.1.6), ascorbate peroxidase (EC 1.11.1.11), and glutathione reductase (GR, E.C. 1.11.1.9).

Quantification of H_2O_2 and MDA were performed with the same extraction in which 500 mg of fresh leaf mass was homogenized in 0.1% (w/v) trichloroacetic acid (TCA) and 4% (w/v) polyvinylpolypirrolidone (PVPP), and centrifuged at $5590 \times g$ for 15 min at 4 °C (Alexieva et al. 2001). For H_2O_2 concentrations, the supernatant was mixed with 100 mM potassium phosphate buffer (pH 7.0) and 1.0 M potassium iodide (1:1:4), and incubated at 4 °C for 1 h in darkness and then for 20 min at 25 °C. Sample absorbance was measured at 390 nm and H_2O_2 content was calculated using a standard curve with known concentrations of H_2O_2.

Lipid peroxidation was determined by the presence of MDA according to Heath and Packer (1968). To the extracted supernatant, 1 mL of a solution containing 20% (w/v) TCA and 0.5% (w/v) thiobarbituric acid (TBA) was added and then incubated at 95 °C for 30 min. The mixture was then quickly cooled at 4 °C to stop the reaction. The samples were re-centrifuged for 5 min at $12,100 \times g$ and the supernatant were measured at 535 and 600 nm. The absorbance of the formed TBA reactive substances was determined at 535 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. Using an extinction coefficient of 155 mM^{-1} cm^{-1}, the amount of MDA was calculated.

Enzymatic antioxidant system

For protein quantification and enzyme activities, one gram of fine leaf powder was homogenized in 5.0 mL of 100 mM potassium phosphate buffer (pH 7.5) with 3 mM dithiothreitol, 1 mM EDTA, and 4% (w/v) PVPP (Gratão et al. 2015). The suspension was centrifuged at $12,100 \times g$ at 4 °C for 35 min, and the supernatant was stored at -80 °C until further analysis. Total protein content was determined using bovine serum albumin as a standard (Bradford 1976).

Superoxide dismutase activity (SOD) staining was carried out according to Beauchamp and Fridovich (1971) with some modifications (Hippler et al. 2015). Electrophoresis was carried out under non-denaturing condition in 12% polyacrylamide gel (PAGE) with 75 µg protein for leaves, using a BioRad (PowerPac Universal Power Supply, Bio-Rad Laboratories, Inc. CA, USA) voltage of 100 V and amperage of 15 mA. After non-denaturing PAGE separation, the gel was incubated in the dark in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium, and 0.3% N,N,N9,N9-tetramethylethylene-diamine. One unit of bovine liver SOD (Sigma, St. Louis, USA) was used as a positive control for activity. SOD isoenzyme characterization was performed as described by Azevedo et al. (1998); briefly, they were distinguished by their sensitivity to inhibition with 2.0 mM potassium cyanide and 5.0 mM H_2O_2. Densitometry of SOD was performed using the software ImageJ®.

The activity of CAT was determined according to Kraus et al. (1995) with modifications (Azevedo et al. 1998). The reaction was initiated with the addition of 20 µL of plant extract to a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.5) and H_2O_2 (30% solution) at 25 °C. The enzyme activity was determined by following the decrease in absorbance at 240 nm, which represents the decomposition of H_2O_2, for 1 min against a plant extract-free blank. Catalase activity was calculated using an extinction coefficient of 39.4 M^{-1} cm^{-1}.

The activity of APX was determined with the method of Nakano and Asada (1981) where the rate of ascorbate oxidation at 290 nm and 30 °C was monitored. The reaction medium contained 50 mM phosphate buffer (pH 7.0) with 0.5 mM ascorbate, 0.1 mM EDTA, and 0.1 mM H_2O_2. The reaction began with the addition of ascorbate and a molar extinction coefficient of 2.8 mM^{-1} cm^{-1} was used in calculations.

Glutathione reductase activity was determined according to Smith et al. (1988) in a mixture consisting of 1 mL 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM 5,5″- dithiobis (2-nitrobenzoic acid), 1 mM oxidized glutathione, and 1 mM NADPH. The reaction was performed at 30 °C and initiated with the addition of 50 µL of plant extract. The enzyme activity was determined by monitoring the reduction of oxidized glutathione at 412 nm over a 1 min period.

Plant growth and nutritional status

After the physiological evaluations, plants were destructively collected for the determination of dry mass and leaf area (LI-3100C, LI-COR, Lincoln, USA. Specific leaf area was evaluated by measuring of dry weight of 30 leaf discs (diameter = 0.52 cm), randomly sampled on leaf blade from recent mature leaves, fully exposed to the light. All the plant material (roots, shoots and leaves) was washed and then dried at 58–60 °C to constant weight to determine dry mass; the leaves were ground and digested in nitric-perchloric acid for the determination of N, P, K, Ca, Mg, S, B, Cu, Fe, Mn and Zn as described by Bataglia et al. (1983).

Statistical analysis

Analysis of variance (ANOVA) at $p < 0.05$ was used to compare measured variables among treatment groups, and when significant, means were compared using Tukey test ($\alpha = 0.05$). Control treatments (stressed and unstressed) were compared using an F test ($\alpha = 0.05$).

Results

Plant nutritional status and chlorophyll concentration

Nutrient concentrations in the leaves of young lemon plants changed according to the nutritional treatment (Table 1). Plants with + N exhibited higher N (25 mg kg^{-1}) concentrations than + Mg and control plants (17 mg kg^{-1}), whereas plants with + Mg + N exhibited intermediate concentration (22 mg kg^{-1}). Conversely, plants with + Mg and + Mg + N presented greater Mg concentrations in their leaves than control and + N plants. Plants in treatments with extra N supply (+ N and + Mg + N) had lower phosphorus (P) and potassium (K) concentrations compared to control and + Mg plants. An extra N supply reduced the concentration of copper (Cu) in leaves compared to the other stressed treatments. Regarding stress conditions, the control plants under stress presented lower iron (Fe) and Cu concentrations in leaves than unstressed control plants (Table 1).

In the afternoon period (13:00 h to 14:00 h) the air temperature differed up to 8.0 °C between the stressed and unstressed conditions, while in the morning period (9:00 h to 10:00 h) this difference was nearly to 2.0–3.0 °C (Fig. 2). Total radiation was twice higher in the stressed condition than unstressed one, being over 500 W m^{-2} in the afternoon period (Fig. 1). A greater chlorophyll concentration was verified in the plants supplied with extra N (+ N and + Mg + N), although, no differences were observed in the chlorophyll a/b ratio among the treatments (Fig. 3).

Leaf gas exchange and chlorophyll a fluorescence

After 12 days of imposing the stress condition, plants supplied only with extra N exhibited lower g_s, C_i, and E and a higher A_N/C_i and WUE when compared to the other treatments under stress in the morning period. However, no differences in A_N were observed among the stressed plants, regardless of the nutritional treatment (Fig. 4).

In the afternoon period plants with + N or + Mg exhibited greater A_N/C_i and plants supplied with + Mg + N exhibited higher WUE when compared to control plants (Fig. 4). In addition, +Mg plants exhibited greater A_N, g_s, E, and A_N/C_i when compared to control plants under the stress condition.

Plants in the + N treatment exhibited greater maximum quantum yield of PSII (F_v/F_m), effective quantum yield of PSII ($\Delta F/F_m'$) and qP than control treatment, while the plants of the treatment + Mg + N showed intermediate values. No differences were observed for non-photochemical quenching (q_NP) (Fig. 5).

In the morning period the extra N supply increased the ETR as well as the ETR/A_N ratio when compared to control and + Mg plants. Although, in the afternoon, all the treatments with extra nutrients supplies (+ N, +Mg and + Mg + N) and the control plants unstressed exhibited a higher ETR when compared with control plants exposed to the stress condition. The ETR/A_N in stressed plants with + Mg was lower than in control plants, whereas stressed plants with + N or + Mg + N exhibited intermediate ETR/A_N (Fig. 6).

Oxidative stress and antioxidant enzymatic system

Control stressed plants exhibited higher leaf concentrations of H_2O_2 and MDA compared to unstressed plants. Likewise, plants in the + Mg and + Mg + N treatments

Table 1 Nutrient concentrations in leaves of young lemon plants at the end of the experiment (five months of acclimatization + 12 days of stress)

Treatments	N	P	K	Ca	Mg	S	Fe	Mn	Cu	Zn	B
						g kg^1	kg^1				mg
Control	17 b	4.4 a	27 a	17	3.0 b	2.3	124	18	24 a	43	89
+Mg	17 b	4.2 a	28 a	15	4.0 a	2.5	117	19	27 a	53	91
+N	25 a	3.2 b	21 b	19	3.1 b	2.5	113	20	16 b	44	77
+Mg + N	22 ab	3.6 b	23 b	18	4.3 a	2.5	138	20	26 a	50	91
Unstressed control	18	4.2	24	18	2.8	2.4	161*	22	33*	55	84

Legend: means followed by different letters are significantly different according to Tukey test ($p < 0.05$);

Asterisk (*) represents significant difference between stressed and unstressed controls

had reduced levels of H_2O_2 in their leaves under the stress condition than the control plants. Furthermore, extra nutrient supplies reduced MDA levels compared to control plants (Fig. 7). Superoxide dismutase activity increased in leaves of plants when grown with extra + Mg, +N, or + Mg + N. The primary SOD activities that increased were for the isoforms Mn-SOD, Fe-SOD II, Cu/Zn-SOD I, and Cu/Zn-SOD III (Fig. 8).
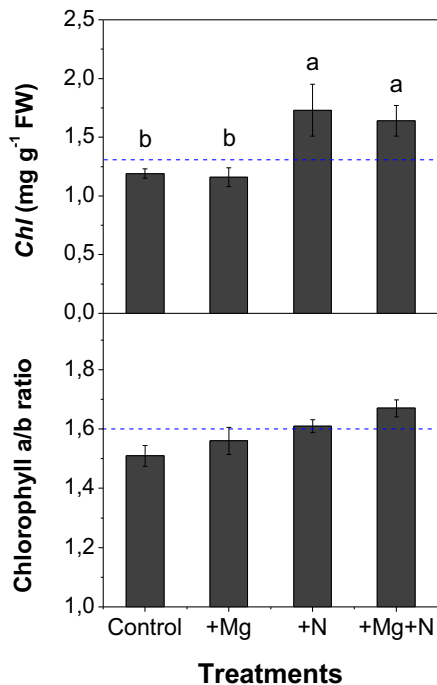
Besides SOD, the activities of the enzymes CAT, APX and GR were higher in plants supplied with extra N (+ N and + Mg + N) compared to the control plants, and the + Mg treatment plants had intermediated activities (Fig. 9). APX was the only enzyme to exhibit greater activity in control plants under the stress condition when compared to unstressed plants. In the control treatment no differences were observed between stressed and unstressed plants for the other enzyme activities (Fig. 9).

Fig. 3 Mean (± standard error, n = 4) total chlorophyll concentration (*Chl*) and chlorophyll a/b ratio in leaves of young lemon plants supplied with additional magnesium (+ Mg), nitrogen (+ N), or both (+ Mg + N) after 12 days of heat and radiation stress. Legend: Control - plants exposed to the stress condition with standard fertilization; +Mg - plants exposed to the stress condition with extra supply of Mg; +N - plants exposed to the stress condition with extra supply of N; + Mg + N - plants exposed to the stress condition with extra supply of Mg and N. The dashed line is the mean of control plants under unstressed condition. Statistics: Within a panel, bars marked with different letters are significantly different according to Tukey test ($p < 0.05$)

Discussion

The outcome of extra nutrients supplies to mitigate abiotic stress

Heat and drought stress are likely the most deleterious events to plants, especially when compared to flood or cold stress (Lesk et al. 2016). In citrus production, heat and light stress were also identified as potential deleterious factors impairing fruit yield and quality (Otero et al. 2011). Citrus trees under excessive radiation and elevated air temperature present scorching of leaves; as well peel burn in fruits that likely reduces external and internal quality. Further, severe flower and fruit drop is observed
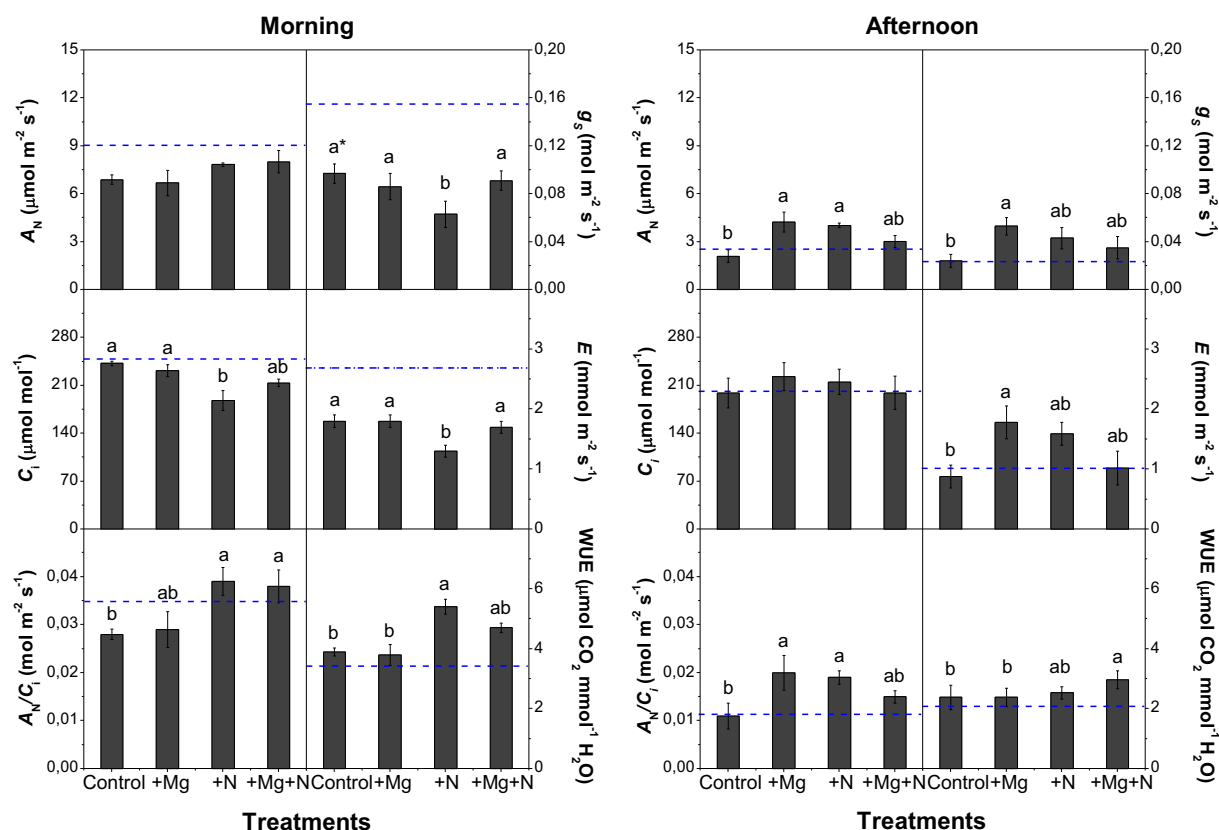
Fig. 4 Mean (± standard error, n = 4) photosynthetic rate (A_N), stomatal conductance (g_s), internal CO_2 concentration (C_i), transpiration (E), instantaneous carboxylation efficiency (A_N/C_i) and water use efficiency (WUE = A_N/E) in leaves of young lemon plants supplied with additional magnesium (+ Mg), nitrogen (+ N), or both (+ Mg + N) during the morning (9:00 h to 10:00 h) and the afternoon (13:00 h to 14:00 h) after 12 days of heat stress. Legend: Control - plants exposed to stress condition with standard fertilization; +Mg - plants exposed to the stress condition with extra supply of Mg; +N - plants exposed to the stress condition with extra supply of N; + Mg + N - plants exposed to the stress condition with extra supply of Mg and N. The dashed line is the mean of the control plants under unstressed conditions. Statistics: Within a panel, bars marked with different letters are significantly different according to Tukey test ($p < 0.05$). Asterisk (*) indicates significant difference between stressed and unstressed controls

in Brazilian citrus orchards after short periods under high temperatures, even when trees are irrigated. Such high temperature periods are especially limiting during the blooming and beginning of fruit set (Fundecitrus 2019).

Furthermore, nutrient deficiencies significantly limit maximum yield potential and product quality of agricultural crops, defining nutrient management as an important tool to minimize losses also associated to abiotic stress conditions, including aluminum toxicity (Riaz et al. 2018), water deficit (Salehi-Eskandari et al. 2018) and heat stress (Jiang and Huang 2001). Our results demonstrate that extra supply of Mg and/or N improve plant responses to heat and light stress for 12 days.

Extra supply of N reduced P, K, and Cu concentrations in leaves (Table 1). Decrease in the concentrations of these nutrients resulted likely by dilution effect

caused by greater plant growth when extra N was supplied (Supplementary Fig. 1). Excess vigor due to extra N fertilization is usually observed in non-bearing trees, which can cause Cu deficiency as revealed by visual symptoms and biochemistry (Hippler et al. 2018a). Differently from the treatment with extra N (+ N and + N + Mg), the additional supply of Mg (+ Mg) did not change the plant growth and only affected the Mg concentration in the leaves.

We should not expect differences in nutrient concentrations of leaves within the short period of exposure of plants to stress conditions. However, after twelve days, Fe and Cu concentrations in leaves of unstressed control plants were higher than the control plant under stress (Table 1). An interaction between these two nutrients is observed in the citrus trees, which exhibit a regulatory mechanism of metal
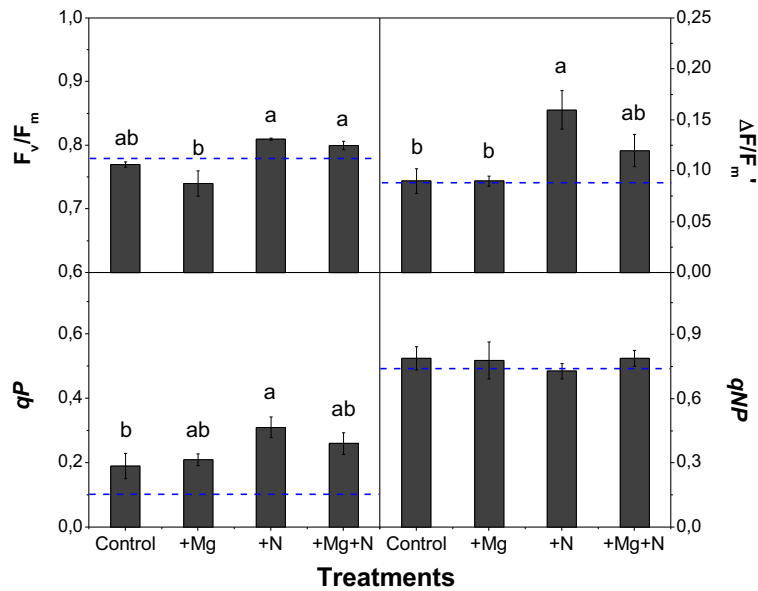
Fig. 5 Mean (± standard error, n = 4) maximum (F_v/F_m) and effective ($\Delta F/F_m'$) quantum yield of photosystem II, photochemical (qP) and non-photochemical quenching (qNP) in leaves of young lemon trees supplied with additional magnesium (+ Mg), nitrogen (+ N), or both (+ Mg + N) during the afternoon (13:00 h to 14:00 h) after 12 days of heat stress. Legend: Control - plants exposed to the stress condition with standard fertilization; +Mg - plants exposed to the stress condition with extra supply of Mg; +N - plants exposed to the stress condition with extra supply of N; + Mg + N - plants exposed to the stress condition with extra supply of Mg and N. The dashed line is the mean of control plants under unstressed conditions. Statistics: Within a panel, bars marked with different letters are significantly different according to Tukey tests ($p < 0.05$)
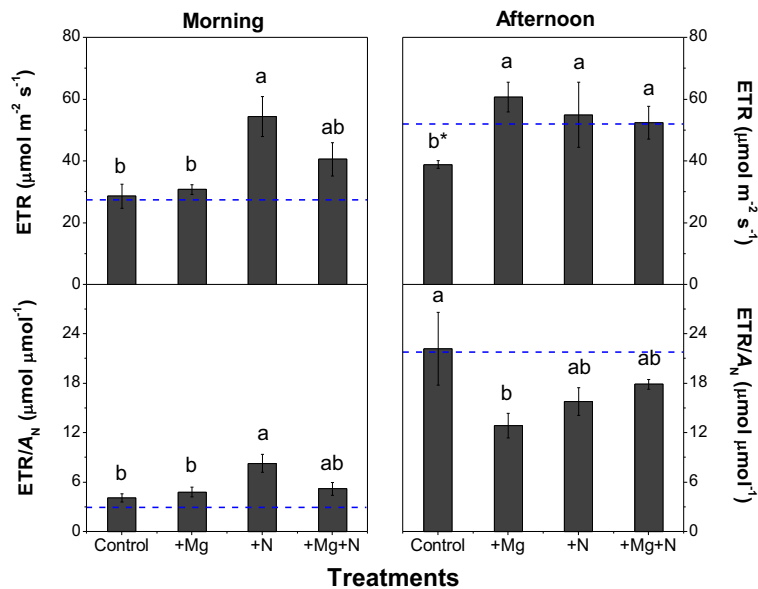

Fig. 6 Mean (± standard error, n = 4) apparent electron transport rate (ETR) and ratio between ETR and photosynthetic rate (ETR/A_N) in leaves of young lemon trees supplied with additional magnesium (+ Mg), nitrogen (+ N), or both (+ Mg + N) measured in the morning (9:00 h to 10:00 h) and afternoon (13:00 h to 14:00 h) after 12 days of heat stress. Legend: Control - plants exposed to the stress condition with standard fertilization; +Mg - plants exposed to the stress condition with extra supply of Mg; +N - plants exposed to the stress condition with extra supply of N; + Mg + N - plants exposed to the stress condition with extra supply of Mg and N. The dashed line is the mean of control plants under unstressed conditions. Statistics: Within a panel, bars marked with different letters are significantly different according to Tukey test ($p < 0.05$). Asterisk (*) indicates significant difference between stressed and unstressed controls
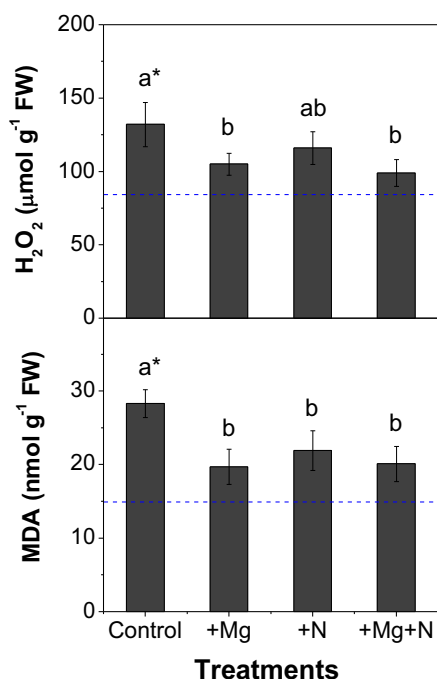
Fig. 7 Mean (± standard error, n = 4) hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in leaves of young lemon trees supplied with additional magnesium (+ Mg), nitrogen (+ N), or both (+ Mg + N) after 12 days of heat stress. Legend: Control - plants exposed to the stress condition with standard fertilization; +Mg - plants exposed to the stress condition with extra supply of Mg; +N - plants exposed to the stress condition with extra supply of N; + Mg + N - plants exposed to the stress condition with extra supply of Mg and N. Dashed line mean average of control plants under no-stress condition. Statistics: Within a panel, bars different letters are significantly different by the Tukey test ($p < 0.05$). Asterisks (*) indicate significant difference between stressed and unstressed controls

homeostasis (Hippler et al. 2018b). The transport of nutrients from roots to shoot is severely affected by drought as well the rate of transpiration (Tripathi et al. 2018). In this way, the g_s and E of the unstressed plants were higher than the stressed ones specially during the morning (Fig. 4) which could partly explain the difference in concentrations of Cu and Fe in the leaves.

Physiological responses to extra nutrient supplies under stress conditions

The higher chlorophyll concentration improved plant ability to harvest light energy (Fig. 6), which difference in chlorophyll concentration may be also linked to the plant acclimatization period. During this period, the extra N supply created a more favorable acclimation under shaded condition, indicating that higher

chlorophyll represents better acclimation to shade rather than better high irradiance tolerance *per se* (Syvertsen and Smith 1984).

During the morning, plants under stress condition and with + N exhibited lower g_S and C_i compared to Control or + Mg trees (Fig. 4). However, since there was no reduction in A_N in plants with + N, A_N/C_i and WUE were higher compared to Control or + Mg lemon trees (Fig. 4). Under water deficit condition, *Coffea canephora* exhibited similar responses in gas exchange parameters when plants were supplied with high N (DaMatta et al. 2002), in which the ratio between A_N and g_S increased compared to plants with limited supply of N. In this case, besides transpiration was reduced, efficiency to assimilate CO_2 was higher with less water loss. Furthermore, young plants of *Coffea arabica* with high N supply were less affected by photoinhibition under light stress compared with limited supply of N, in which those plants with high N supply exhibited better control of stomata opening-closure that was also associated with changes at the photochemical level (Nunes et al. 1993). The same results were observed in the present study in young lemon trees with + N, which exhibited higher values of $\Delta F/F_m'$ and qP (Fig. 5).

Photosynthetic activity (A_N) of the young lemon trees were lower in the afternoon compared to the morning, even for shaded trees (Fig. 4). Citrus species exhibit light saturation in a photosynthetically active radiation (PAR) of 700–800 $\mu mol\ m^{-2}\ s^{-1}$ (Syvertsen and Smith 1984). In the present study, PAR in the afternoon was 940 $\mu mol\ m^{-2}\ s^{-1}$ for shaded condition and 1820 $\mu mol\ m^{-2}\ s^{-1}$ for unshaded one (Fig. 2), which this latter was not enough to cause stomatal closure. However, the stomatal conductance in a same light radiation of citrus trees fluctuates over different season (Ribeiro and Machado 2002), in which temperature and water availability have been considered more relevant on stomatal aperture responses for citrus trees than irradiance.

During the afternoon, besides observed reduction of the photosynthetic parameters compared to the morning measurements, the stomatal conductance (g_s) of trees supplied with + Mg was less affected, which resulted in higher A_N, A_N/C_i and E than Control (Fig. 4). Moreover, extra supply of Mg did not increase chlorophyll content in the leaves when compared to plants with + N (Fig. 3). Magnesium has an important effect on photosynthesis and in many chloroplast enzymes (Hermans and Verbruggen 2005). Furthermore, trees with extra Mg exhibited higher ETR as well lower ETR/A_N than Control in the afternoon, which
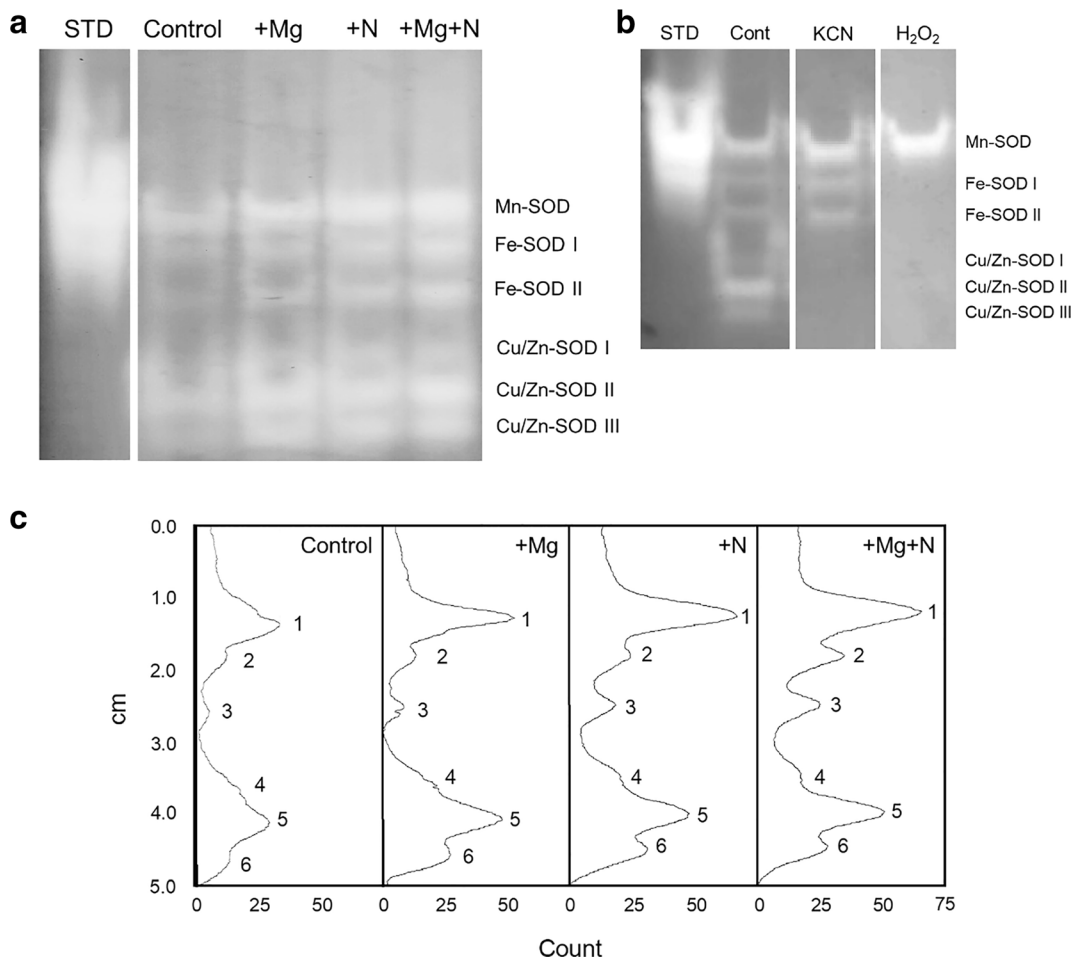
Fig. 8 Superoxide dismutase (SOD) protein abundance (A) a 12% polyacrylamide gel after electrophoresis, (B) SOD isoforms identification, and (C) densitogram of SOD activity in leaves of young lemon trees supplied with additional magnesium (+ Mg), nitrogen (+ N), or both (+ Mg + N) and after 12 days of heat stress. Legend: Control - plants exposed to the stress condition with standard fertilization; +Mg - plants exposed to the stress condition with extra supply of Mg; +N - plants exposed to the stress condition with extra supply of N; + Mg + N - plants exposed to the stress condition with extra supply of Mg and N; STD = Bovine SOD standard; Cont = without any inhibitor; KCN = 2 mM potassium cyanide (Cu/Zn-SOD inhibitor); H_2O_2 = 5 mM hydrogen peroxide (Cu/Zn-SOD and Fe-SOD inhibitor); 1 = Mn-SOD; 2 = Fe-SOD I; 3 = Fe-SOD II; 4 = Cu/Zn-SOD I; 5 = Cu/Zn-SOD II; 6 = Cu/Zn-SOD III

was related to lower H_2O_2 and MDA levels observed in plant leaves (Fig. 7). In general, under abiotic stress condition, the photosynthetic activity is repressed via destabilization of Rubisco and damage to PSII (Nishiyama and Murata 2014). When this damage occurs, the utilization rate of absorbed light energy in the electron transport is reduced and the electrons that accumulate in the transport chain generate oxidative stress (Guo et al. 2006). However, extra Mg supply enabled the maintenance of high A_N and ETR because the high irradiance stress environment did not increase H_2O_2 and MDA concentrations (Fig. 5).

Trees with + N also exhibited higher A_N and A_N/C_i than Control, but the higher supply of N provided intermediated g_s and E (Fig. 4). During the afternoon, high air temperature and radiation did not limit transpiration of trees with extra N supply, rather these trees did not exhibited decrease in A_N or A_N/C_i compared to Control, but intermediated values for g_s and E (Fig. 4). Similar results were observed for citrus under heat stress when compared with trees under shade or sprayed with sun protector to reduce leaf temperature (Otero et al. 2011).

Nutrient supply and the antioxidant enzymatic system

Accumulation of ROS and other reactive species under stress conditions are harmful to cell structure, unbalance
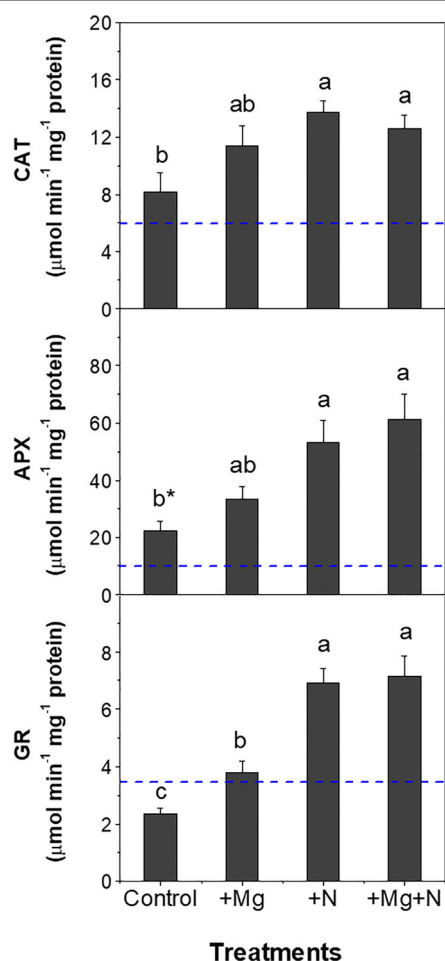
Fig. 9 Mean (± standard error, n = 4) catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) activities in leaves of young lemon trees supplied with additional magnesium (+ Mg), nitrogen (+ N), or both (+ Mg + N) after 12 days of heat stress. Legend: Control - plants exposed to the stress condition with standard fertilization; +Mg - plants exposed to the stress condition with extra supply of Mg; +N - plants exposed to the stress condition with extra supply of N; + Mg + N - plants exposed to the stress condition with extra supply of Mg and N. The dashed line is the mean of control plants under unstressed conditions. Statistics: Within a panel, bars marked with different letters are significantly different according to Tukey test ($p < 0.05$). Asterisk (*) indicates significant difference between stressed and unstressed controls

plant metabolisms, and reduce the integrity of chloroplasts (Foyer and Noctor 2005). In the presented work, when plant nutritional management was enhanced, the antioxidant enzymatic system was activated, which was important to reduce damages caused by heat stress. Highest temperature and radiation promoted ROS accumulation, as observed by higher H_2O_2 and MDA levels in leaves of the Control stressed treatment compared with unstressed trees

(Fig. 7). Accumulation of ROS, such as H_2O_2, in plant tissues increases MDA, which is a secondary product of lipid peroxidation, a marker of cell membrane injury (Hippler et al. 2018a). Under stress condition, trees with + Mg showed lower concentrations of H_2O_2 and MDA than trees without extra nutrient supply (Fig. 7). This demonstrated the role of Mg supply in reducing ROS, which could ameliorate tree horticultural responses under unfavorable environmental conditions.

Superoxide dismutase is the first enzyme in the ROS scavenging chain, and it is responsible for reducing superoxide (O_2^-) into H_2O_2 (Alscher et al. 2002; Borges et al. 2019). In lemon trees, six isoforms were identified in the leaves, including one Mn-SOD, two Fe-SOD, and three Cu/Zn-SOD. Specific SOD activity was probably important in alleviating the damage caused by the elevated air temperature and radiation in leaves. Six isoforms of SOD were more intense when plants were supplied with extra Mg, N, or both, with main differences observed in Mn-SOD, Fe-SOD II and Cu/Zn-SOD II activities (Fig. 8).

Activities of CAT, APX and GR were responsive to N supply (+ N and + Mg + N), whereas trees with only extra Mg supply exhibited intermediate activities of these enzymes (Fig. 9). Highest activities of CAT, APX and GR were measured in trees with extra nutrient supply, leading to lower H_2O_2 and MDA levels when compared to control tress under stress conditions (Fig. 7), similarly, citrus grown in nutrient solution with high Cu concentration, when supplied with high N accumulated less H_2O_2 and MDA and exhibited higher antioxidant enzyme activities than those plants grown with adequate N (Hippler et al. 2018a).

Under stress, there is a compensatory mechanism that regulate the equilibrium of protective enzymes. Then, balance of the activities of SOD, CAT, APX, and GR in plants is essential for determining the levels of ROS (Prasch and Sonnewald 2015; Choudury et al. 2016). In the same way, the activities of antioxidative enzymes, such as APX and GR, were higher with the increase of Mg deficiency, especially under high light intensity (Cakmak 2013). Our results indicate that trees supplied with extra N and Mg activated a defense mechanism that avoided ROS production and accumulation under heat stress, which could be demonstrated by a greater detoxification ability of ROS, that was catalyzed by SOD to H_2O_2 and then the H_2O_2 was eliminated by CAT, APX, and GR enzymes (Figs. 7, 8 and 9). Our study also demonstrated that improved Mg and N management contribute to alleviate the harmful effects of heat waves,

in citrus production, caused by climate changes. None-theless it is important to point out that environmental conditions other than irradiation and temperature can affect physiological responses under field condition (Otero et al. 2011), therefore future studies are required in order to elucidate such further question.

Conclusions

Lemon trees with extra Mg and N supplies subject to elevated air temperature and radiation stress exhibited low damage to photosynthetic functioning. Also, those trees maintained higher transpiration and utilization of absorbed light, as well exhibited a greater antioxidant enzyme activity that reduced oxidative stress. Overall, our results highlight the potential for improved nutrient management in citrus orchards to minimize crop losses resulting from unfavorable environmental conditions. Since stress caused by heat waves have occurred frequently in the Brazilian citrus producing region, actions to support alleviating these injuries with a more rationale nutrient management, can greatly support the sustainability of the citrus industry. Therefore, supply of soluble Mg well-balanced with N fertilization appears as an effective way to increase fruit quality and yield of citrus orchards.

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