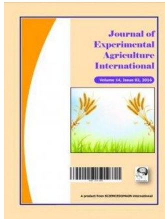

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Antioxidant Defense in Sunflower against Drought

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Authors' contributions

This work was carried out in collaboration between all authors. Author MMLCC designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MEF, CFP, SBT, GEÁ, JB and CD managed the analyses of the study. Author SD managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The sunflower has a fundamental role in the global economy, being one of the most important crops for oil production. The plants have a short growing season and it adapts well to different soil conditions and unfavorable weather. The present study aimed to evaluate the impact of drought on leaf water potential, the content of photosynthetic pigments and antioxidant responses in two cultivars of sunflower. Seeds of the M735 cv. and MG2 cv. were sown in pots and four plants were maintained per pot that was arranged randomly. Plants were grown in a greenhouse, and two months after sowing, subjected to water stress: Irrigated and not irrigated. Evaluations were performed after 1, 5 and 12 days of induction the stress treatment. Significant reduction in leaf water potential at 5 and 12 days in M735 cv. and 12 days for MG2 cv., were observed. The pigment content did not differ between treatments. There was no change in antioxidant enzyme activity for the M735 cv., though the levels of H_2O_2 increased in non-irrigated plants after 12 days. The SOD

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and CAT activities increased in the MG2 cv. as a function of water deficit at five and 12 days of water stress. In MG2 cv. was also observed increased levels of H_2O_2 and lipid peroxidation after 12 days of drought. These results suggest that the major effect of water stress can vary with sunflower cultivars.

Keywords: Helianthus annuus L.; water stress; pigments; antioxidant enzymes.

1. INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the oilseed crop cultivated in different parts of the world. Moreover, its cultivation has intensified with the use of this species as raw material for biofuel production, driven by the creation of the National Biodiesel Program that has as goals the inclusion of family farming in the production of agrofuels [1]. In addition, sunflower use, due to the increase in domestic demand for edible vegetable oils of good quality and the possibility of its cultivation as the succeeding crop after corn or soybeans, allows greater use of land, machinery, and labour [2].

Despite the favorable outlook for the sunflower crop in Brazil, the occurrence of adverse weather is still a risk factor and failure, especially when grown in the off-season, more subject to variations period at edaphoclimatic conditions, in particular the soil water availability [3] and may lead to loss of production and quality grains/sunflower seeds [4]. According to Reddy et al. [5], the drought during the growing season can lead to a reduction of achenes production in the range of 15 to 25% and water stress during flowering can increase this decline up to 50%.

In addition, water stress leads to changes in both morphological and physiological characteristics, such as reduction in cell growth and leaf area, increase in leaf abscission, a reduced relationship between the biomass of the root to shoot, stomatal closure and reduction in net photosynthesis [6]. Depending on the intensity and duration, water deficit can induce metabolic changes, such as increased production of reactive oxygen species (ROS). Under normal conditions ROS such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) can be produced without causing any cellular damage to the plant as intermediates in a series of metabolic reactions (signaling) and then, specific agents of the antioxidant system eliminate them. However, its accumulation is potentially harmful to the plant cells, causing damage to biomolecules such as DNA, RNA, proteins and cell membranes [7]. The intensity of cell damage is determined by the ability of plants to remove ROS or minimize its effects through an antioxidant defense system, including non-enzyme compounds such as ascorbate, glutathione, flavonoids as well as enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidases (POX), ascorbate peroxidase (APX) and glutathione reductase (GR) [8,9].

One of the key points of the plants in drought conditions is the knowledge of their dependence severity of stress and tolerance of each genotype. Thus, this study was aimed to evaluate the effect of drought on leaf water potential, the content of photosynthetic pigments, and the activity of enzymes involved in the removal of ROS and cell damage in two sunflower cultivars in early stages of development for identifying possible physio-logical and biochemical mechanisms in response to drought.

2. MATERIALS AND METHODS

2.1 Plant Material and Growth Conditions

Sunflower cultivars (M735 and MG2), provided by Embrapa (Brazilian Agricultural Research Corporation) and widely grown in several regions of Brazil were used in the experiment. Seeds were sown in plastic pots with a capacity of 10 L containing soil (Planosol Haplic) as a substrate. The physicochemical characteristics of the soil were: water pH: 5.0; O.M.: 1.7%; K: 55.5 mg dm^{-3}; P: 1.8 mg dm^{-3}; Al: 0.9 cmolc dm^{-3}, Ca: 1.3 cmolc dm^{-3}; Mg: 1.3 cmolc dm^{-3}; and clay content: 18%.

After germination, the seedlings were thinned to four plants per pot, with a total of 18 pots for each cultivar. Plants were watered daily and supplied with a nutrient solution containing nitrogen once per week [10] at half strength in the first two weeks, followed by the full strength at the remaining period until the beginning of the treatments.

The plants were subjected to two water regimes at 60 days after sowing (DAS): Irrigated (I); the plants were kept irrigated daily with soil near to field capacity (10 kPa) and Not Irrigated (NI), with a total suspension of irrigation. The plant cultivars were grown in a greenhouse with a mean temperature of 28.6ºC and a relative humidity of 82.3%. At harvest with 1, 5 and 12 days under water stress, three biological replicates for each cultivar in each drought treatment were taken randomly for evaluation and kept frozen (-80ºC) until analysis.

2.2 Leaf Water Potential

The leaf water potential (Ψ_W maximum) was evaluated before dawn using a pressure pump type Scholander (Soilmoisture 3000 model) in a fully expanded 4th leaf from the top of each plant (three pots per treatment and cultivars).

2.3 Photosynthetic Pigments

Chlorophyll content (a, b, and total) and carotenoids were quantified from approximately 200 mg of leaf tissue after extraction with 80% acetone, as methods described by Lichtenthaler [11].

2.4 Antioxidative Enzyme Activity

The antioxidative enzyme activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POX) were determined in leaf and root tissues. Plant material (~200 mg) was ground to a powder in liquid nitrogen using a pestle and mortar with 20% polyvinylpolypyrrolidone (PVPP). The powder was homogenized in 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA and 10 mM ascorbic acid. The homogenate was centrifuged at 12 000 g for 15 min at 4 ºC. The supernatant was collected and used for both antioxidative enzyme activity and total protein quantification [12].

The SOD activity was estimated by enzyme's ability to inhibit the photoreduction of nitroblue tetrazolium (NBT) [13] in a reaction medium containing 100 mM potassium phosphate buffer (pH.7,8), 14 mM methionine, 0.1 uM EDTA, 75 µM NBT and 2 µM riboflavin. The absorbance was read at 560 nm in a spectrophotometer, and one unit (U) of SOD was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction. The CAT activity was monitored by the decrease in absorbance at 240 nm for 2 min at 10 s intervals in a reaction medium containing 100 mM potassium phosphate buffer (pH 7.0) and 12.5 mM H_2O_2, incubated at 28 °C [14]. The APX activity was monitored by ascorbate oxidation rate at 290 nm for 2 min in reaction medium composed of 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid and 0.1 mM H_2O_2 incubated at 28°C [15]. The POX activity was determined according to Chance and Maehly [16], in reaction medium composed of 100 mM potassium phosphate buffer (pH 6.8), 30 mM H_2O_2, 30 mM guaiacol and the extract enzymatic. The increase in absorbance was recorded at 420 nm for 2 min.

2.5 Cellular Damage and Oxidative Metabolites

Cellular damage and oxidative metabolites were estimated by measuring the levels of lipid peroxidation and hydrogen peroxide (H_2O_2), respectively. Extracts were obtained from 200 mg of leaf and root tissues in liquid N_2 and homogenized in 0.1% trichloroacetic acid (TCA) (w/v). The homogenate was centrifuged at 12 000 g for 15 min at 4ºC.

The lipid peroxidation is an indicator of oxidative stress, determined by measuring the concentration of thiobarbituric acid reactive species (TBARS) [17]. The crude extract obtained above was added to 0.5% (w/v) thiobarbituric acid (TBA) and 10% (w/v) TCA. The TBA form red-colored complexes with low molecular weight aldehydes, such as malondialdehyde (MDA), a secondary product of the peroxidation process. The reaction medium was incubated at 95ºC for 30 min followed by a rapid cooling in an ice bath and water to stop the reaction. The absorbance of TBARS formed was determined by a spectrophotometer at 535nm and 600nm and the level of peroxidation of lipids expressed in nmoles using the molar extinction coefficient of MDA (1,55 mM^{-1} cm^{-1}).

The H_2O_2 content was estimated using the methods described by Sinha et al. [18]. The crude extract was added to 10 mM potassium phosphate buffer (pH 7.0) and 1M potassium iodide. Readings were taken spectrophotometry at 390 nm and H_2O_2 content calculated by comparing the readings with a standard curve obtained using different known concentrations of H_2O_2.

2.6 Statistical Analysis

The experimental design was completely randomized with three biological replicates and

the experimental unit consisted of four plants per pot, the data were interpreted independently for each cultivar comparing the water regimes in the three periods. The data were analyzed by one-way analysis of variance (ANOVA). When *F* was significant, the treatment means for each cultivar were compared by Tukey test ($p \leq 0.05$).

3. RESULTS AND DISCUSSION

The suspension of irrigation induced a significant reduction in leaf water potential (Ψ_W) of sunflower plants (Fig. 1). For M735 cv. there was a difference between irrigated treatments (I) and non-irrigated (NI) at 5 and 12 days after submission to stress (Fig. 1A), and only 12 days for MG2 cv. (Fig. 1B). In the last analysis period (12 days), the Ψ_W for non-irrigated plants of M735 cv. Reduced to -1.20 MPa, approximately 54% lower than that observed in the irrigated plants. For MG2 cv., this reduction was even greater, with Ψ_W of -1.72 MPa for non-irrigated plants, close to 133% lower in comparison to their control, showing greater stress on this cultivar.

Evaluating drought stress in sunflower plants [19] observed a similar behavior, with a proportional reduction in Ψ_W with increasing water stress after six days. According to Silva et al. [6], one of the first strategies to minimize water loss due to transpiration under low water potential is the stomatal closure. However, this process causes a reduction in the photosynthetic assimilation of CO_2 and growth, and plant adaptation to different environmental conditions are related to photosynthetic efficiency which, in turn, is associated with the pigment content in the leaves [20].

Chlorophylls and carotenoids can be used as indicative of the level of damage to plants when grown under adverse conditions, varying with the species and cultivar studied and exposure time, resulting in the loss of these pigments during stress [21]. In this study, the chlorophyll *a*, *b* and total contents did not differ significantly between treatments for M735 cv. in the periods studied. For MG2 cv., differences were observed only in the first evaluation. The carotenoid content, in turn, showed no significant variation in both sunflower cultivars (Table 1).

Corroborating these results, previous studies [21,22] found that the chlorophyll content did not differ significantly in *Allium schoenoprosum* and sugarcane when exposed to drought, which according to the authors, indicates the absence of oxidative stress. However, in severe drought conditions, plants can present destruction of photosynthetic pigments due to oxidative damage. On the other hand, plants can protect themselves by synthesizing antioxidants compounds such as ascorbate, α-tocopherol, glutathione, and flavonoid, or by increasing antioxidant enzymes activities [22]. If the antioxidant defense mechanisms do not operate efficiently in severe stress conditions, ROS accumulation is intensified and can promote the oxidation of photosynthetic pigments, membrane lipids, proteins and nucleic acids [23].

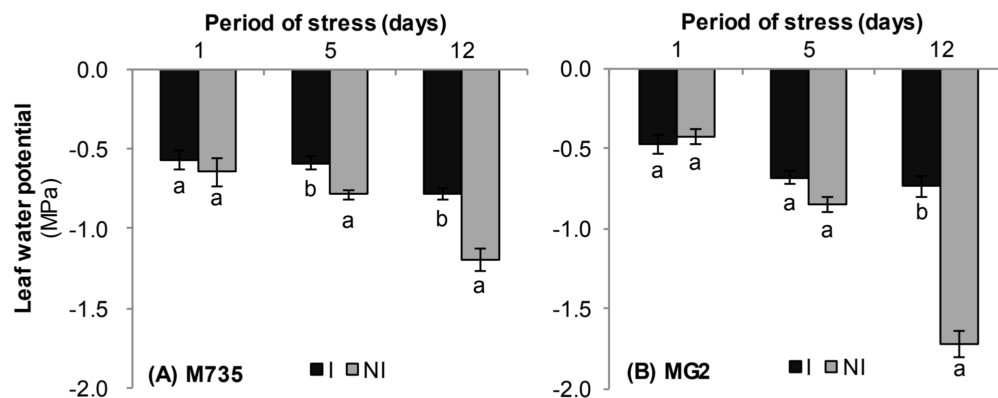

Fig. 1. Leaf water potential (Ψ_W) in sunflower plants, M735 cv. (A) and MG2 cv. (B), assessed after 1, 5 and 12 days of water stress (Irrigated (I) and Non-Irrigated (NI)). Bars bearing the same letter comparing the water regimes (irrigated and non-irrigated) in each period of stress do not differ by Tukey's test (*p* ≤ 0.05). Values represent the mean ± SE (*n*=3)

Table 1. Levels of chlorophyll and carotenoids in sunflower plants, M735, and MG2 cultivars, subjected to two water regimes: Irrigated (I) and Non-Irrigated (NI)

Cultivar	Period of stress (days)	Chlorophyll *a*		Chlorophyll *b*		Total chlorophyll		Carotenoids	
		...............μg mg^{-1} MF...............							
		I	NI	I	NI	I	NI	I	NI
M735	1	1.05a*	1.15a	0.35a	0.36a	1.39a	1.50a	76.89a	82.89a
	5	0.97a	0.92a	0.36a	0.33a	1.47a	1.33a	85.72a	78.17a
	12	1.11a	1.00a	0.30a	0.35a	1.26a	1.20a	74.49a	69.81a
MG2	1	0.91b	1.30a	0.30a	0.17b	1.21b	1.47a	68.76a	69.35a
	5	0.97a	0.92a	0.31a	0.29a	1.28a	1.21a	71.90a	70.80a
	12	0.84a	0.86a	0.29a	0.30a	1.13a	1.16a	66.99a	67.46a

** Means followed by the same letter comparing the water regimes (irrigated and non-irrigated) in each period of stress do not differ by Tukey's test ($p \leq 0.05$). Values represent the mean ± SE (n=3)*

In sunflower plants of the present study, the activity of antioxidant enzymes in leaves, for the M735 cv., did not differ significantly between irrigated and non-irrigated treatments (Figs. 2A, C, E, and G). Although Ψ_W showed significant variation, reaching about 54% of reduction at 12 days in plants under water deficit in relation to irrigated (Fig. 1A), it did not reflect in an increase in antioxidant enzymes activities. Plants from MG2 cv., showed a significant difference in SOD (Fig. 2B) and CAT (Fig. 2D) activity when subjected to water stress in 5 and 12 days, with the highest activity observed in non-irrigated plants. The APX (Fig. 2F) and POX (Fig. 2H) activities showed no difference. The increased SOD and CAT enzymes activities can be justified by the significant reduction in Ψ_W suffered by these plants to 12 days of stress (Fig. 1B).

Manivannan et al. [24] while evaluating the water stress in sunflower plants at intervals of three, six and nine days observed an increase in the SOD, CAT and APX activities in leaves and roots. We also performed antioxidative enzymes activity measurements in roots of both M375 and MG2 sunflower cultivars. However, no statistical differences were found in these tissues under water stress (data not shown).

Among the enzymes involved in the removal of ROS, SOD is considered a key enzyme and generally the first line in the defense mechanism against oxidative stress [25], which catalyzes the dismutation of $O_2^{\bullet-}$ into H_2O_2 which is the central mechanism of defense needed to prevent the formation of $OH^{\bullet}$ radicals [26], known to confer tolerance to oxidative stress. The CAT is abundant in peroxisomes of C_3 plants and breaks down H_2O_2 into H_2O and O_2 produced mainly during photorespiration via glycolate oxidase (GO) [26]. GO may increase its activity in leaves of stressed plants to adapt themselves to water stress [27]. Thus, for sunflower plants MG2 cv. submitted to drought, the highest CAT activity can be justified for the removal the H_2O_2 produced by SOD activity and also a possible induction of photorespiration rate occurred in these plants.

Other studies have also reported an increase in the activity of antioxidant enzymes, especially SOD, CAT and APX, due to the drought, as *Olea europaea* [27], *Carapa guianensis* [28] and *Coffea arabica* plants [29], featuring an efficient defense system against ROS produced in these conditions.

Another important factor in antioxidant capacity is the affinity of the enzyme for the substrate. CAT has a low affinity for H_2O_2, being activated only when it is present at high concentrations and APX and peroxidases have a high affinity, removing it when in low concentrations in the tissues. In addition, CAT acts in peroxisomes, while APX and POX basically act in chloroplasts and cell walls, respectively [8].

The higher CAT activity can be related to a high concentration of H_2O_2 in the plant cells. When analyzing the content of this compound in the leaves of sunflower plants, we observed an increase in the production along with the period of water stress, however, a significant difference between treatments was obtained only at 12 days in the two cultivars (Fig. 3). It is noteworthy that the difference was more significant in MG2 cv. (Fig. 3B), where H_2O_2 content in the non-irrigated plants was approximately 67% higher than irrigated plants, compared to approximately 51% between treatments in M735 cv. (Fig. 3A). These results suggest that the sunflower plants, and in particular MG2 cv. might show a high rate of photorespiration besides the H_2O_2 produced by the activity of SOD, leading to increased H_2O_2 levels, since the APX and POX enzymes did not differ between treatments.

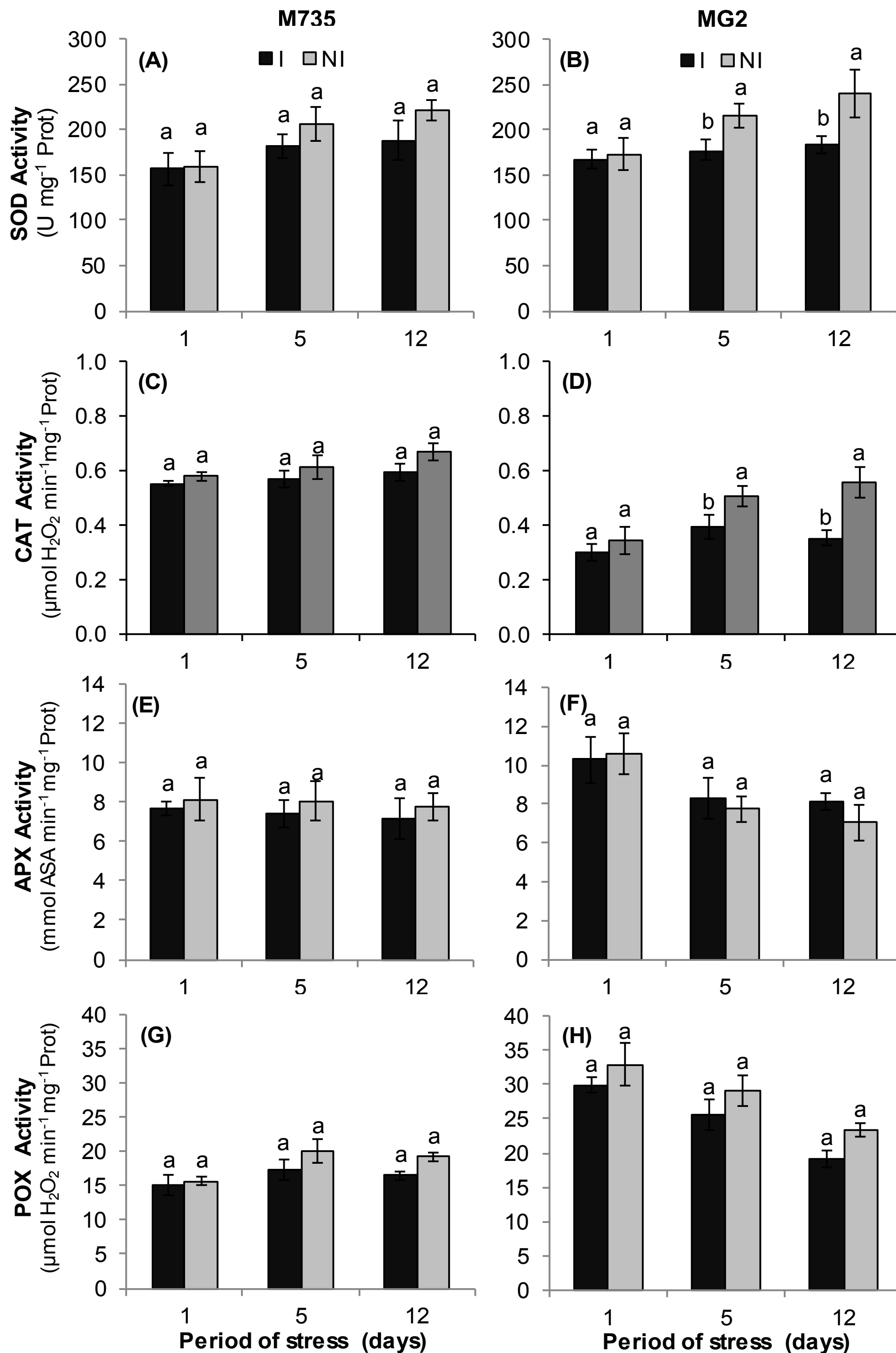

Fig. 2. Antioxidant enzyme activity in leaves of sunflower cultivars M735 (A, C, E, and G) and MG2 (B, D, F and H), assessed after 1, 5 and 12 days of water stress (Irrigated (I) and Non-Irrigated (NI)). SOD – superoxide dismutase; CAT – catalase; APX – ascorbate peroxidase; POX – peroxidases. Bars bearing the same letter comparing the water regimes (irrigated and non-irrigated) in each period of stress do not differ by Tukey's test ($p \leq 0.05$). Values represent the mean ± SE (*n*=3)

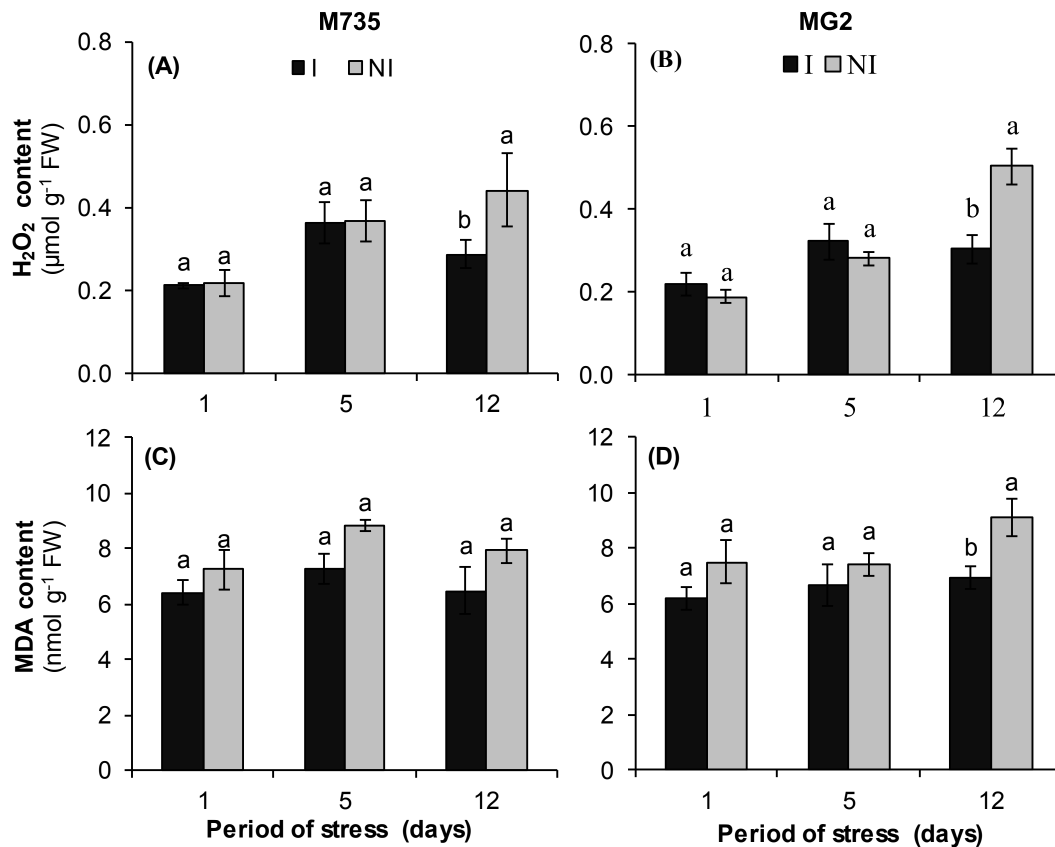

Fig. 3. Hydrogen peroxide content (H_2O_2) and lipid peroxidation (MDA - malondialdehyde) in leaves of sunflower cultivars M735 (A, C) and MG2 (B, D), assessed after 1, 5 and 12 days of water stress (Irrigated (I) and Non-Irrigated (NI)). Bars bearing the same letter comparing the water regimes (irrigated and non-irrigated) in each period of stress do not differ by Tukey's test ($p \leq 0.05$). Values represent the mean ± SE (*n*=3)

The ability to maintain the activity of SOD, CAT, and APX at high levels in environmental stress conditions is essential to keep the balance between the formation and removal of H_2O_2 intracellular to avoid oxidative stress [30]. If the ROS levels are not kept low, they can cause damage to cell membranes due to lipid peroxidation. The malondialdehyde (MDA) is a by-product of the peroxidation process and its accumulation is often used as an indicator of oxidative stress [31]. Although in the leaves of the M735 cv., the H_2O_2 content has shown significant change after 12 days, this compound did not induce increases in lipid peroxidation in the cells during water stress (Fig. 3C). On the other hand, in MG2 cv. (Fig. 3D) was a significant increase in the MDA levels with 12 days in non-irrigated plants, which may be related to increased H_2O_2 content. In roots, the H_2O_2 levels and lipid peroxidation did not differ under water stress (data not shown).

The MDA content was efficiently controlled by maintaining the SOD, CAT and APX activities under water deficit in *Poa pratensis* plants [32] and Cechin et al. [19] observed an increase in MDA content in young and adult plants of leaves of sunflower, also under water deficit. These authors reported that the increase in MDA levels can be correlated with an inadequate antioxidant enzymes activity of SOD and CAT, which may explain the high levels of H_2O_2 and lipid peroxidation observed in MG2 cv.

4. CONCLUSION

Water stress alters the metabolism of sunflower cultivars (M735 and MG2). However, the effect is more severe on the MG2 cv., showing to be more sensitive to 12 days of water restriction, which, even with the increase of antioxidant enzymes activities, stress leads to H_2O_2 accumulation and to cell membrane damage.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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