

Antioxidant defense in sunflower crops against drought

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, content of photosynthetic pigments and antioxidant responses in two cultivars of sunflower. Seeds of the M735 cv. and MG2 cv. were sown in pots (10 l) and kept four plants were maintained per pot that was arranged randomly arranged. 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 H₂O₂ increased in non-irrigated plants after 12 days. The SOD 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 H₂O₂ and lipid peroxidation after 12 days of drought. These results show suggest that the major effect of water stress can vary with sunflower cultivars in MG2 cv.

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

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28 1. INTRODUCTION

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

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

43 In addition, water stress leads to changes in both morphological and physiological
44 characteristics, such as reduction in cell growth and leaf area, increase in leaf abscission,
45 reduced relationship between the biomass of the root to shoot, stomatal closure and reduction
46 in net photosynthesis [6]. Depending on the intensity and duration, water deficit can induce
47 metabolic changes, such as increased production of reactive oxygen species (ROS). Under
48 normal conditions ROS such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), hydrogen
49 peroxide (H_2O_2) and singlet oxygen ($^1O_2$) can be produced without causing any cellular

50 damage to the plant as intermediates in a series of metabolic reactions (signaling) and then,
51 they are eliminated by specific agents of the antioxidant system. However, its accumulation is
52 potentially harmful to the plant cells, causing damage to biomolecules such as DNA, RNA,
53 proteins and cell membranes [7]. The intensity of cell damage is determined by the ability of
54 plants to remove ROS or minimize its effects through an antioxidant defense system,
55 including non-enzyme compounds such as ascorbate, glutathione, flavonoids as well as
56 enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidases (POX), ascorbate
57 peroxidase (APX) and glutathione reductase (GR) [8,9].

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

65 2. MATERIALS AND METHODS

66 2.1. Plant material and growth conditions

67 Sunflower cultivars (M735 and MG2), widely grown in several regions of Brazil were
68 used in the experiment. Seeds were sown in pots (10 l), containing soil (Planosol Haplic) as a
69 substrate. After germination, the seedlings were thinned to thinning was performed while
70 keeping four plants per pot, with a total of 18 pots for each cultivar. Plants were watered
71 daily and supplied with a nutrient solution containing nitrogen once per week [10] at half
72 strength in the first two weeks, followed by the full force strength at the remaining period
73 until the beginning of the treatments.

Comment [R1]: Mention here the seed source.

Comment [R2]: What is this? Is this the size of the pots used? Or the ratio (10:1) of seeds sown per pot? What was the size of the pots?

Comment [R3]: You should provide the basic physicochemical characteristics of the soil.

74 | The plants were subjected to two water regimes **when reached at** 60 days after sowing
75 (DAS): Irrigated (I) the plants were kept irrigated daily with soil near to **field capacity** and
76 Not Irrigated (NI), with total suspension of irrigation. The plant cultivars were grown in a
77 greenhouse under **natural light and temperature**, arranged randomly on the benches. At
78 harvest with 1, 5 and 12 days under water stress, three biological replicates for each cultivar
79 | in each **water system drought treatment** were taken randomly for evaluation and kept frozen
80 (-80 °C) until analysis.

Comment [R4]: Mention the soil moisture at field capacity.

Comment [R5]: Mention the values for the greenhouse conditions. What about relative humidity during the study period??

81

82 **2.2. Leaf water potential**

83 The leaf water potential (Ψ_w maximum) was evaluated before dawn using a pressure
84 pump type Scholander (Soilmoisture 3000 model) **in a fully expanded leaf** of each plant
85 (three pots per treatment and cultivars).

Comment [R6]: Was any uniformity followed for measurements? For example, fully expanded 3rd or 4th or 5th leaf from the top.

86

87 **2.3. Photosynthetic pigments**

88 Chlorophyll content (a, b, and total) and carotenoids were quantified from approximately
89 200 mg of leaf tissue after extraction with 80% acetone, as methods described by
90 | [Lichtenthaler](#) [11].

91

92 **2.4. Antioxidative enzyme activity**

93 The antioxidative enzyme activities of superoxide dismutase (SOD), catalase (CAT),
94 ascorbate peroxidase (APX) and peroxidase (POX) were determined in leaf and root tissues.
95 Plant material (~200 mg) was ground to a powder in liquid nitrogen using a pestle and mortar
96 with 20% polyvinylpyrrolidone (PVPP). The powder was homogenized in 100 mM
97 potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA and 10 mM ascorbic acid.
98 The homogenate was centrifuged at 12 000 g for 15 min at 4 °C. The supernatant was

99 collected and used for both antioxidative enzyme activity and total protein quantification
100 [12].

101 The SOD activity was estimated by enzyme's ability to inhibit the photoreduction of
102 nitroblue tetrazolium (NBT) [13] in a reaction medium containing 100 mM potassium
103 phosphate buffer (pH.7,8), 14 mM methionine, 0.1 uM EDTA, 75 µM NBT and 2 µM
104 riboflavin. The absorbance was [taken read](#) at 560 nm in a spectrophotometer, and one unit
105 (U) of SOD was defined as the amount of enzyme required to inhibit 50% of the NBT
106 photoreduction. The CAT activity was monitored by the decrease in absorbance at 240 nm for
107 2 min at 10 s intervals in a reaction medium containing 100 mM potassium phosphate buffer
108 (pH 7.0) and 12.5 mM H₂O₂, incubated at 28 °C [14]. The APX activity was monitored by
109 ascorbate oxidation rate at 290 nm for 2 min in reaction medium composed of 100 mM
110 potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid and 0.1 mM H₂O₂ incubated at
111 28 °C [15]. The POX activity was determined according to [Chance and Maehly](#) [16], in
112 reaction medium composed of 100 mM potassium phosphate buffer (pH 6.8), 30 mM H₂O₂,
113 30 mM guaiacol and the extract enzymatic. The increase in absorbance was recorded at 420
114 nm for 2 min.

115

116 **2.5. Cellular damage and oxidative metabolites**

117 Cellular damage and oxidative metabolites were estimated by measuring the levels of
118 lipid peroxidation and hydrogen peroxide (H₂O₂), respectively. Extracts were obtained from
119 200 mg of leaf and root tissues in liquid N₂ and homogenized in 0.1% trichloroacetic acid
120 (TCA) (w/v). The homogenate was centrifuged at 12 000 g for 15 min at 4 °C.

121 The lipid peroxidation is an indicator of oxidative stress, determined by measuring the
122 concentration of thiobarbituric acid reactive species (TBARS) [17]. The crude extract
123 obtained above was added to 0.5% (w/v) thiobarbituric acid (TBA) and 10% (w/v) TCA. The

124 TBA form red-colored complexes with low molecular weight aldehydes, such as
125 malondialdehyde (MDA), a secondary product of the peroxidation process. The reaction
126 medium was incubated at 95°C for 30 min followed by a rapid cooling in an ice bath and
127 water to stop the reaction. The absorbance of TBARS formed was determined by a
128 spectrophotometer at 535nm and 600nm and the level of peroxidation of lipids expressed in
129 nmoles using the molar extinction coefficient of MDA ($1,55 \text{ mM}^{-1} \text{ cm}^{-1}$).

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

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135 2.6. Statistical analysis

136 The experimental design was completely randomized with three biological replicates and
137 the experimental unit consisted of four plants per pot, the data were interpreted independently
138 for each cultivar comparing the water regimes in the three periods. The data were analyzed by
139 one-way analysis of variance (ANOVA). When F was significant, the treatment means for
140 each cultivar were compared by Tukey test ($p \leq 0.05$).

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142 3. RESULTS AND DISCUSSION

143 The suspension of irrigation induced a significant reduction in leaf water potential (Ψ_w)
144 of sunflower plants (Fig. 1). For M735 cv. there was difference between irrigated treatments
145 (I) and non-irrigated (NI) at 5 and 12 days after submission to stress (Fig. 1A), and only 12
146 days for MG2 cv. (Fig. 1B). In the last analysis period (12 days), the Ψ_w for non-irrigated
147 plants of M735 cv. reduced to -1.20 MPa, approximately 54% lower than that observed in the
148 irrigated plants. For MG2 cv., this reduction was even greater, with Ψ_w of -1.72 MPa for non-

Comment [R7]: A two-way ANOVA is better suited for this experimental design as there were two factors (cultivars and drought conditions).

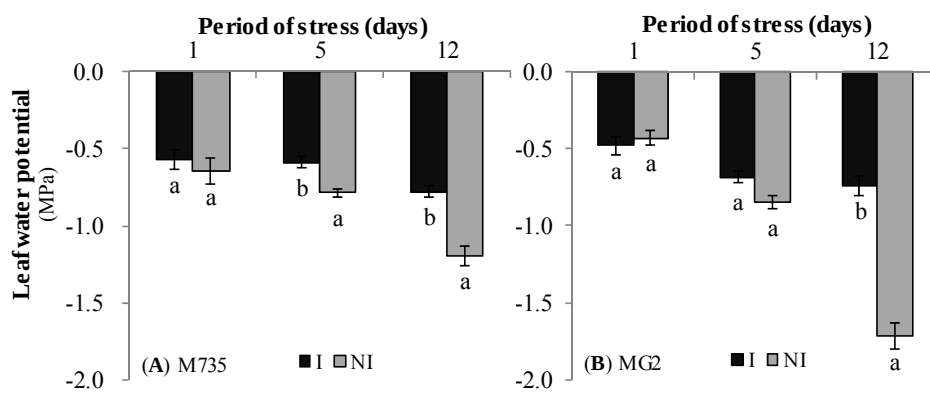


Figure 1. Leaf water potential (Ψ_w) in sunflower plants, M735 cv. (A) and MG2 cv. (B), assessed after 1, 5 and 12 days of water stress (Irrigated (I) and Non-Irrigated (NI)). Means followed by the Bars bearing same letter comparing the water regimes (irrigated and non-irrigated) in each period of stress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

Evaluating drought stress in sunflower plants [19] observed 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 in under low water potential conditions 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

178 exposure time, resulting in the loss of these pigments during stress [21]. In this study, the
 179 chlorophyll *a*, *b* and total contents did not differ significantly between treatments for M735
 180 cv. in the periods studied. For MG2 cv., differences were observed only in the first
 181 evaluation. The carotenoid content, in turn, showed no significant variation in both sunflower
 182 cultivars (Table 1).

183

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

Cultivar	Period of stress (days)	Chlorophyll <i>a</i>		Chlorophyll <i>b</i>		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

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

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190 Corroborating these results, [previous studies \[21, 22\]](#) and [21] found that the chlorophyll
 191 content did not differ significantly in plant *Allium schoenoprosom* and sugarcane when
 192 exposed to drought, which according to the authors, indicates the absence of oxidative stress.

193 However, in severe drought conditions, plants can present destruction of photosynthetic
 194 pigments due to oxidative damage. On the other hand, plants can protect themselves by
 195 synthesizing antioxidants compounds such as ascorbate, α -tocopherol, glutathione, and
 196 flavonoid, or by increasing antioxidant enzymes activities [22]. If the antioxidant defense
 197 mechanisms do not operate efficiently in severe stress conditions, ROS accumulation is

Comment [R8]: These species are monocots and the drought response of C3 and C4 plants can differ substantially.

intensified and can promote the oxidation of photosynthetic pigments, membrane lipids, proteins and nucleic acids [23].

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, they 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 this 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 $O_2^{\cdot -}$ into H_2O_2 which is the central mechanism of defense needed to prevent the formation of $OH^{\cdot}$ 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

223 removal the H₂O₂ produced by SOD activity and also a possible induction of photorespiration
224 rate occurred in these plants.

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

229 Another important factor in antioxidant capacity is the affinity of the enzyme for the
230 substrate. CAT has a low affinity for H₂O₂, being activated only when it is present at high
231 concentrations and APX and peroxidases have a high affinity, removing it when in low
232 concentrations in the tissues. In addition, CAT acts in peroxisomes, while APX and POX
233 basically act in chloroplasts and cell walls, respectively [8].

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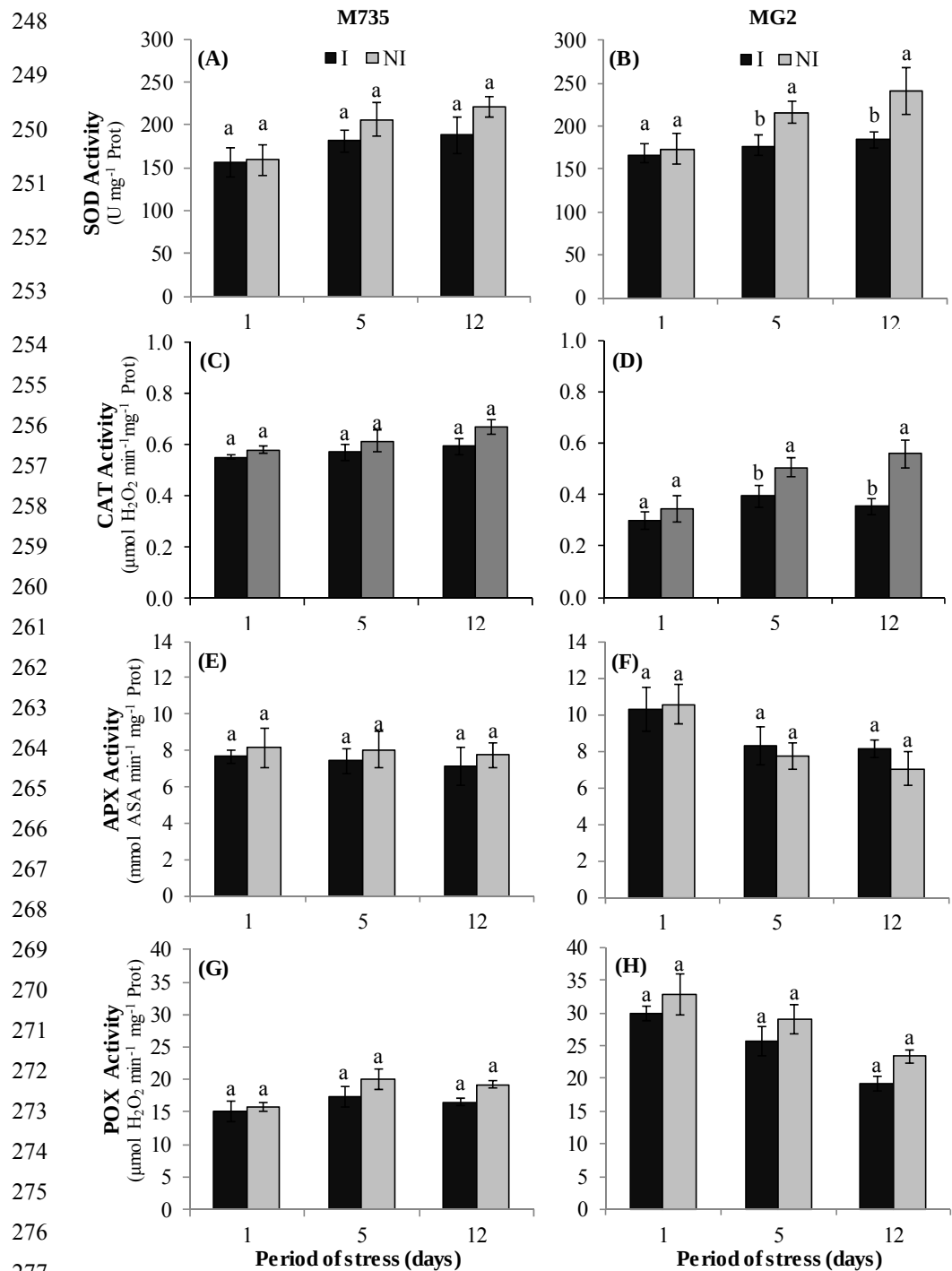


Figure 2. Antioxidant enzyme activity in leaves of sunflower cultivars M735 (A, C, E, and G) and MG2 (B, D, F and H), assessed after 1, 5 and 12 days of water stress (Irrigated (I) and Non-Irrigated (NI)). SOD – superoxide dismutase; CAT – catalase; APX – ascorbate peroxidase; POX – peroxidases. Means followed by 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 $\pm$ SE ($n=3$).

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.

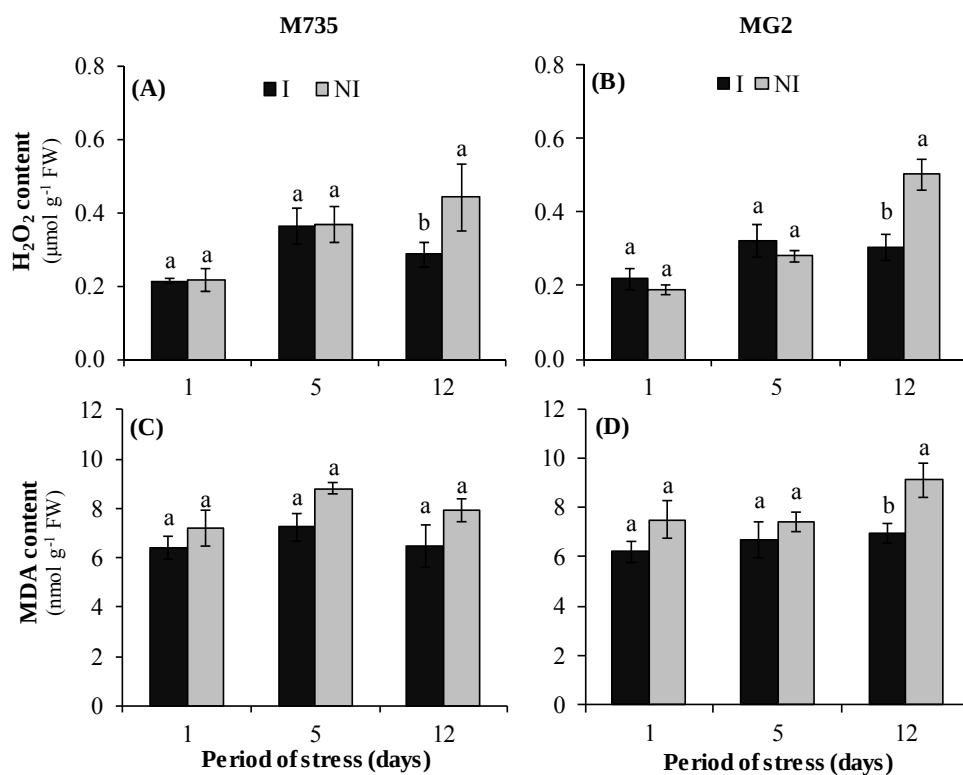


Figure 3. Hydrogen peroxide content (H₂O₂) and lipid peroxidation (MDA - malondialdehyde) in leaves of sunflower cultivars M735 (A, C) and MG2 (B, D), assessed after 1, 5 and 12 days of water stress (Irrigated (I) and Non-Irrigated (NI)). Means followed by Bars bearing the same letter comparing the water 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 $\pm$ 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 H₂O₂ 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 H₂O₂ content has shown significant change after 12 days, this compound did not induce

335 increases in lipid peroxidation in the cells during water stress (Fig. 3C). On the other hand, in
336 MG2 cv. (Fig. 3D) was a significant increase in the MDA levels with 12 days in non-irrigated
337 plants, which may be related to increased H₂O₂ content. In roots, the H₂O₂ levels and lipid
338 peroxidation did not differ under water stress (data not shown).

339 The MDA content was efficiently controlled by maintaining the SOD, CAT and APX
340 activities under water deficit in *Poa pratensis* plants [32] and [Cechin et al.](#) [19] observed an
341 increase in MDA content in young and adult plants of leaves of sunflower, also under water
342 deficit. These authors reported that the increase in MDA levels can be correlated with an
343 inadequate antioxidant enzymes activity of SOD and CAT, which may explain the high levels
344 of H₂O₂ and lipid peroxidation observed in MG2 cv.

345

346 4. CONCLUSION

347 Water stress alters the metabolism of sunflower cultivars (M735 and MG2). However,
348 the effect is more severe on the MG2 cv., showing to be more sensitive to 12 days of water
349 restriction, which, even with the increase of antioxidant enzymes activities, stress leads to
350 H₂O₂ accumulation and to cell membrane damage.

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