



## Mycorrhization and saline stress response in *Hyptis suaveolens*

Marília Cristina Gomes de Souza<sup>1</sup>  Marciana Bizerra de Moraes<sup>1\*</sup>  Marcelo da Silva Andrade<sup>1</sup>   
Mayron Alves de Vasconcelos<sup>1,2</sup>  Silvana Saker Sampaio<sup>3</sup>  Cynthia Cavalcanti de Albuquerque<sup>1</sup> 

<sup>1</sup>Programa de Pós-graduação em Ciências Naturais, Universidade do Estado do Rio Grande do Norte (UERN), 59600-000, Mossoró, RN, Brasil. E-mail: marciana.bio@gmail.com. \*Corresponding author.

<sup>2</sup>Departamento de Patologia e Medicina Legal, Laboratório Integrado de Biomoléculas, Universidade Federal do Ceará (UFC), Fortaleza, CE, Brasil.

<sup>3</sup>Departamento de Engenharia de Pesca, Laboratório de Bioquímica Marinha, Universidade Federal do Ceará (UFC), Fortaleza, CE, Brasil.

**ABSTRACT:** *The objective of this study was to evaluate the physiological and biochemical mechanisms of adaptation of Hyptis suaveolens under osmotic stress due to salinity inoculations with a mycorrhizal fungal species. H. suaveolens seeds were germinated in polyethylene pots containing a substrate associated with or without arbuscular mycorrhizal fungi (AMF). After plant formation, treatments were treated with different salt concentrations (0.0, 35, 70, and 105 mM) and fungi (control and two types of AMF), totaling 12 treatments with three replicates. The experimental design used randomized blocks in a 4 x 3 factorial scheme, totaling 12 treatments with three replicates each. Salinity affected all measured physiological and biochemical variables, and the stress reduced dry matter content. Plants associated with AMF had increased dry matter compared to non-associated plants, and there were increased biochemical and physiological responses of AMF-colonized plants in the 35 mM NaCl treatment. However, saline stress negatively affected the development of H. suaveolens; and therefore, no attenuation of fungi was observed.*

**Key words:** antioxidant activity, abiotic stress, salinity, arbuscular mycorrhizal fungi.

## Micorrização e resposta ao estresse salino em *Hyptis suaveolens*

**RESUMO:** *Este estudo teve como objetivo avaliar mecanismos fisiológicos e bioquímicos de adaptação de Hyptis suaveolens submetidas ao estresse osmótico decorrente da salinidade inoculadas com espécies de fungos micorrízicos. Sementes de H. suaveolens foram colocadas para germinar em vasos de polietileno contendo substrato associado, ou não, a FMA. Após o estabelecimento das plantas, foram iniciados os tratamentos com diferentes concentrações de sais (0,0; 35; 70 e 105 mM) e fungos (controle e dois tipos de FMA), perfazendo 12 tratamentos com três repetições. O delineamento experimental foi em blocos casualizados em esquema fatorial 4 x 3, perfazendo 12 tratamentos com três repetições cada. Observou-se, através de variáveis fisiológicas e bioquímicas, que a salinidade afetou todas as variáveis analisadas e o estresse promoveu redução no conteúdo de matéria seca. Verificou-se que plantas associadas com FMA apresentaram ganho de matéria seca em relação as não associadas e incremento de respostas bioquímicas e fisiológicas das plantas colonizadas no tratamento 35mM de NaCl. No entanto, o estresse salino afeta negativamente o desenvolvimento de H. suaveolens não sendo observado, portanto, atenuação dos fungos.*

**Palavras-chave:** atividade antioxidante, estresse abiótico, salinidade, fungos micorrízicos arbusculares.

## INTRODUCTION

*Hyptis suaveolens* (L.) Poit is a weed from the Labiatae family, a subshrub with a wide geographical distribution in Brazil, but occurring more frequently in the Cerrado regions. It occurs annually and inhabits agricultural soils, roadsides, lands, and soil drastically changed by anthropogenic action. This species is widely distributed in tropical and subtropical regions (SAKTHIVADIVEL et al., 2015).

The species is recorded in the literature as a producer of an essential oil whose chemical composition consists mainly of monoterpenes, sesquiterpenes, alkanes, benzothiazole, diterpenes, triterpenes, and steroids (MARTINS, 2006). There are many studies on the action of this essential oil, which has proven antifungal, antibacterial, anticarcinogenic, and antiseptic properties (CHATRI et al., 2014), antioxidant effects (TAFURT-GARCÍA, 2015), and other actions.

Plants are subject to various abiotic conditions in their natural habitat that change their metabolism. Due to the semiarid climate in the western region of Rio Grande Norte, the cultivation of important crops requires the use of fertigation, which over time salinizes and degrades the soil. This problem is associated with the abandonment of agricultural land, causing environmental damage and social problems (OLIVEIRA et al., 2019). To make use of these soils, it is necessary to investigate plant species that are adapted to semiarid conditions with the potential to generate income people living in the region. In this respect, the effect of saline stress has been extensively investigated.

Salinity causes major disturbances in most plant species, resulting in growth restriction and productivity loss, triggering an imbalance in the plant's redox system and causing physiological stress, which can impair growth and development (MORAIS et al., 2018; OLIVEIRA et al., 2019). A strategy that has been investigated to minimize the deleterious effects of excess salt in plants is the symbiotic association of plants with arbuscular mycorrhizal fungi (AMF). The symbiosis between plants and AMF is advantageous for both organisms, since plants provide fungi with energy products resulting from photosynthesis, allowing growth and maintenance; in turn, AMF provides the plant with water and nutrients such as phosphorus and nitrogen (HODGE; STORER, 2014). In this process, fungi associated with plant roots minimize damage and promote development through tolerance/resistance mechanisms (FOLLI-PEREIRA et al., 2012).

There are new possibilities to cultivate plants with economic potential that are adapted to high concentrations of salts and are able to maintain the production levels. The use of microorganisms may represent an option for expanding cultivation to saline areas. In view of this, the objective of the present study was to understand the physiological and biochemical mechanisms of adaptation of *H. suaveolens* to conditions of osmotic stress due to salinity when inoculated with mycorrhizal fungi species.

## MATERIALS AND METHODS

Research was conducted in a greenhouse located in a semiarid region in the city of Mossoró, RN, 05° 12'10" east and 37° 18' 57" west, with an average temperature of 35.9 °C ± 2.7 °C, natural photoperiod (approximately 12 h:12 h light: dark), and a relative humidity of 49% ± 5.5%. According to the Köppen classification, the climate of the region is

a BSW'h' type, characterized by being very hot and dry, with a rainy season in the summer that is delayed before the fall.

The arbuscular mycorrhizal fungi (AMF) *Gigaspora albida* and *Claroideoglossum etunicatum* were provided by the Federal Rural University of Pernambuco (UFRPE). They were multiplied in sand previously sterilized in an autoclave at 121 °C with pressure of 1 atm for one hour and placed in a forced circulation oven at 70 °C for two days, mixed with Vermiculite Expanded®. To germinate them in a greenhouse, this mixture was distributed in pots containing *Panicum miliaceum* (Millet), a plant used as an AMF host. The substrate used in the experiment was the result of a mixture of washed sand, natural soil, and organic fertilizer in a 2:1:1 ratio, which was subsequently autoclaved at 121 °C with pressure of 1atm for one hour to completely remove any microorganisms. After sterilization, the soil was dried for two days in a forced circulation oven at 70 °C. After preparation, the substrates were characterized according to their physical and chemical characteristics (Table 1).

### Experimental strategy

*H. suaveolens* seeds were collected, taken to the laboratory, selected and treated with chemical scarification with calcium oxide (CaO) for dormancy. Then, 1,080 seeds were sown in 36 pots, totaling 30 seeds per pot. Thinning took place 30 days after sowing, leaving one plant per pot. The nutritional conditions of the substrate were maintained with the use of a nutrient solution (HOAGLAND & ARNON, 1950) every five days throughout the cycle. After the acclimation period, the plants were treated with different sodium chloride (NaCl) concentrations: 0.0, 35, 70, and 105 mM, which corresponded to electrical conductivities of: 0.41, 2.41, 5.76, and 8.47 dS cm<sup>-1</sup> respectively. Plants were either inoculated with *Gigaspora albida* or *Claroideoglossum etunicatum* or not inoculated, and experienced systematic daily watering according to pot capacity (which was established using the capillarity method until the substrate was completely saturated). Pot capacity was considered as the water content retained in the soil after complete drainage.

The experimental statistical design was a 4x3 factorial arrangement in randomized blocks (four levels of NaCl and three treatments with fungi, including a control treatment and two types of AMF), totaling 12 treatments with three replicates. The Assisat® version 7.7 statistical software was used for the statistical analysis with ANOVA, and means were

Table 1 - Physical and chemical characteristics of substrates inoculated with either *Gigaspora albida* or *Claroideoglossum etunicatum*.

Characteristics	Natural soil	<i>G. albida</i>	<i>C. etunicatum</i>
Texture	Sand	Sand	Sand
Silt (%)	4.7	8.14	3.58
Clay (%)	0.78	2.47	0.32
Sand (%)	94.51	88.42	95.64
DA (g.cm <sup>3</sup> )	1.53	1.47	1.51
ST (ppt)	198.4	201	204
TSD (ppt)	194.8	197.3	212.41
N (g kg <sup>-1</sup> )	3.01	1.75	2.38
pH (H <sub>2</sub> O)	7.70	7.00	7.40
OM (g kg <sup>-1</sup> )	20.98	19.45	21.20
P (mg dm <sup>-3</sup> )	273.6	241.6	242.2
K <sup>+</sup> (mg dm <sup>-3</sup> )	157.8	286.4	821.3
Na <sup>+</sup> (mg dm <sup>-3</sup> )	209.0	404.1	1009.9
Ca <sup>2+</sup> (cmol <sup>c</sup> dm <sup>-3</sup> )	8.40	7.60	8.90
Mg <sup>2+</sup> (cmol <sup>c</sup> dm <sup>-3</sup> )	0.80	4.10	2.40
Al <sup>3+</sup> (cmol <sup>c</sup> dm <sup>-3</sup> )	0.00	0.00	0.00
Cu (mg dm <sup>-3</sup> )	0.31	1.27	0.75
Fe (mg dm <sup>-3</sup> )	13.8	45.3	48.7
Mn (mg dm <sup>-3</sup> )	62.7	29.3	30.7
Zn (mg dm <sup>-3</sup> )	25.13	15.47	18.17
H <sup>+</sup> Al (cmol <sup>c</sup> dm <sup>-3</sup> )	0.00	0.33	0.00
SB (cmol <sup>c</sup> dm <sup>-3</sup> )	10.51	14.19	17.79
t (cmol <sup>c</sup> dm <sup>-3</sup> )	10.51	14.19	17.79
CTC	10.51	14.52	17.79
V (%)	100	98	100
m (%)	0	0	0
PST (%)	9	12	25

compared using the Tukey test at 5% significance. After collection, plant material was immediately frozen in liquid nitrogen and kept at -20 °C for subsequent biochemical analysis.

#### *Evaluated variables*

Relative water content (RWC) was determined following the method by CAIRO (1995). In each replicate, six samples were taken from the central region of the penultimate leaf produced, packed in ice and immediately transferred to the laboratory. Fresh mass (FM) was determined using an analytical scale up to two hours after excision. These samples were then kept in deionized water for 24 h and, after removing excess water on filter paper, the turgid mass (TM) was obtained. After that, the samples were dried at 70 °C for 48 h to determine the dry mass (DM) of the tissue. RWC values were determined by the equation: (FM-DM)/(TM-DM) x 100.

Plant material was dried in an oven with forced air circulation at 70 °C for 48 h to determine the dry biomass of the aerial part and root. Based on biomass values of the dry aerial part and root, the root/aerial part (R/AP) ratio was calculated according to BENINCASA (1988).

Proline content was quantified according to the method proposed by BATES et al. (1973) and the reducing sugars were determined using the 3,5-dinitrosalicylic (DNS) colorimetric method described by MILLER (1959). The standard curve of the spectrophotometric determination of reducing sugars was prepared using the standard 1 g.L<sup>-1</sup> of glucose.

The analysis of chloroplast pigments (chlorophyll and carotenoids) was determined by the 80% acetone extraction method, according to LICHTENTHALER & WELLBURN (1983). Absorbance readings of the samples obtained in a spectrophotometer at wavelengths 470, 645 and 663

nm were used to calculate a, b and total chlorophyll levels (WITHAM et al., 1971).

Lipid peroxidation was determined according to HEATH & PACKER (1968), with modifications, where the reaction was determined through the production of malondialdehyde (MDA), a metabolite reactive to 2-thiobarbituric acid (TBA), and the percentage of membrane damage (MD) was estimated by the equation: %MD = (L1/L2) x 100, estimated by electrolyte leakage (%EL) and measured according to BLUM & EBERCON (1981). Antioxidant activity, which is measured by the ability to sequester the DPPH radical (2,2-diphenyl-1-picrylhydrazyl - Sigma D9132), was determined according to DUAN et al. (2006), with some modifications.

The PHILLIPS & HAYMAN (1970) methodology was modified and used to evaluate the percentage of mycorrhizal colonization, where thin roots were diaphanized in 2% KOH at 90 °C in a water bath for 20 minutes, acidified in 1% HCl in a water bath for four minutes and then colored with 0.05% trypan blue in lactoglycerol at 90 °C in water bath for ten minutes. Colonization rate was estimated using the slide method by GIOVANETTI & MOSSE (1980).

## RESULTS AND DISCUSSION

There was a significant interaction between dry biomass of the aerial part, dry biomass of the root, and the R/AP ratio (ANOVA;  $p < 0.01$ ) (Table 2). When subjected to different levels of salinity, *H. suaveolens* showed a marked reduction in biomass production, with the roots more affected than the aerial part (Figure 1A and 1B), probably

due to direct contact of the roots with the salt in the soil and corroborating the results by RAGAGNIN et al. (2014). This fact affected the R/AP ratio (Figure 1C). The reduced growth rate of the plants was attributed to photosynthetic rate restrictions due to lower stomatal conductance and the consequent limitation in CO<sub>2</sub> absorption capacity, as well as stress directly affecting cell division and expansion, which suppressed plant development (MORAIS et al., 2018). Visual symptoms characterized by chlorosis followed by necrosis were seen in basal and median *H. suaveolens* leaves as a result of saline stress toxicity. These symptoms in stressed leaves probably occurred due to the ionic stress caused by toxic levels of saline ions in leaf tissue (OLIVEIRA et al., 2019).

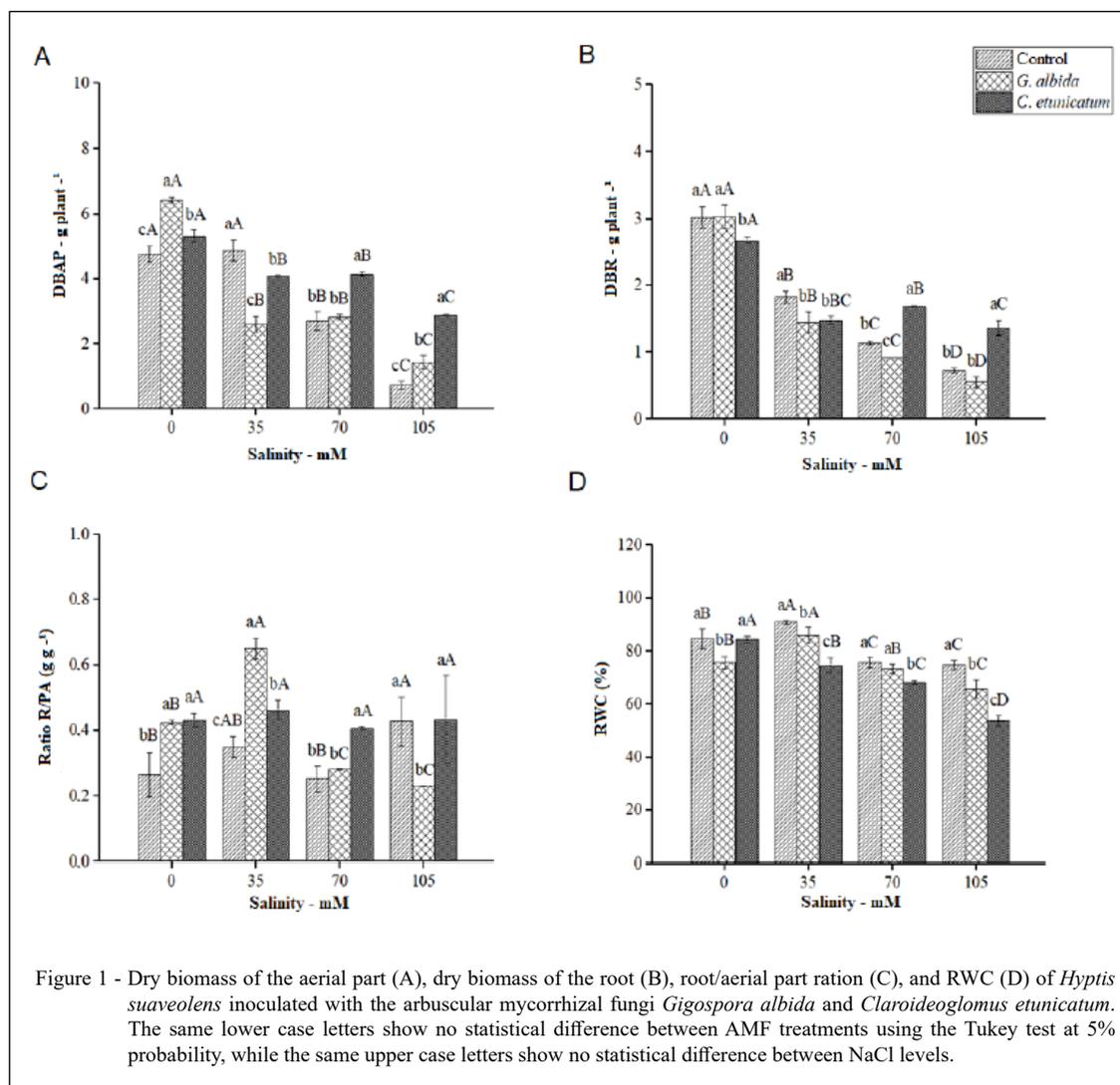
Although, the plants were negatively affected by salt, the association of plants with mycorrhizal fungi significantly increased dry matter in the aerial part compared with non-associated plants, especially at the highest salt concentrations (Figure 1A). The initial growth reduction caused by excess sodium is often associated with osmotic stress, which can cause a loss of RWC that results in decreased cell turgor (MUNNS, 2002), as observed in this study (Figure 1D). In a second step, growth reduction is attributed to Na<sup>+</sup> accumulation in tissues, which is toxic and results in chlorosis and necrosis, as seen in plants under severe stress (MORAIS et al., 2019; OLIVEIRA et al., 2019).

Salinity proportionally reduced biomass production in plants, with a reduction up to about 50% in the most severe treatment, regardless of whether or not they were inoculated with AMF; however, plants associated with *C. etunicatum* had greater biomass under the same stress conditions (Figure 1A and 1B). In this regard, some of the AMF benefits

Table 2 - Summary of the analysis of variance of the evaluated characteristics dry aerial part biomass (DAPB), dry root biomass (DRB) and root/aerial part ratio (R/AP) in *Hyptis suaveolens* subjected to different salt concentrations associated with mycorrhizal fungi.

Variation Factor	GL	DAPB (g)	DRB(g)	R/AP
Fungi (F1)	2	76.3959 **	29.4694 **	15.2949**
Salinity (F2)	3	623.2078 **	680.5532 **	19.4823**
F1xF2	6	83.7608 **	31.2306 **	15.6909**
Treatments	11	229.5436 **	207.9983 **	16.6529**
Blocks	2	0.6319 <sup>ns</sup>	0.5116 <sup>ns</sup>	2.0248 <sup>ns</sup>
		5.33	6.15	13.00

\*\*Tukey test at 1% probability was applied \*Tukey test at 5% probability was applied.



are a greater availability of nutrients to plants and a compartmentalization of  $\text{Na}^+$  in hyphae, which can reduce the damage caused by excess salts (GUTJAHR & PARNISKE, 2013). Studies with melon plants (*Cucumis melo*) under high salt concentrations showed decreased dry biomass production in aerial parts (LUCIO et al., 2013). However, the authors observed that when inoculated with AMF, plants had increased dry matter, despite the salt.

The results suggested that 75 mM NaCl is already a toxic level for *H. suaveolens*, as the plant stopped growing to deal with the level of stress. There was decreased RWC (Figure 1D), increased membrane damage (Figure 2A) and MDA accumulation (Figure 2B), with the accumulation of osmoregulators, such as

proline, in an attempt to minimize the effects of saline stress (Figure 2C). This scenario can be attributed to oxidative damage caused by stress. Ionic stress in the protoplasm results in high levels of toxicity and disturbances related to ionic balance, changing the structure and stability of cell membranes (SHABALA et al., 2012).

The RWC is completely associated with the water retention capacity of the plant, and the higher this value, the better the water status of plant tissue. Studies of plants inoculated with AMF showed a relatively higher RWC compared to plants that were not inoculated (COLLA et al., 2008). As for lipid peroxidation, MDA has been considered a common biomarker of NaCl toxicity in plants. *Lycopersicon esculentum* inoculated with *Glomus mosseae* under

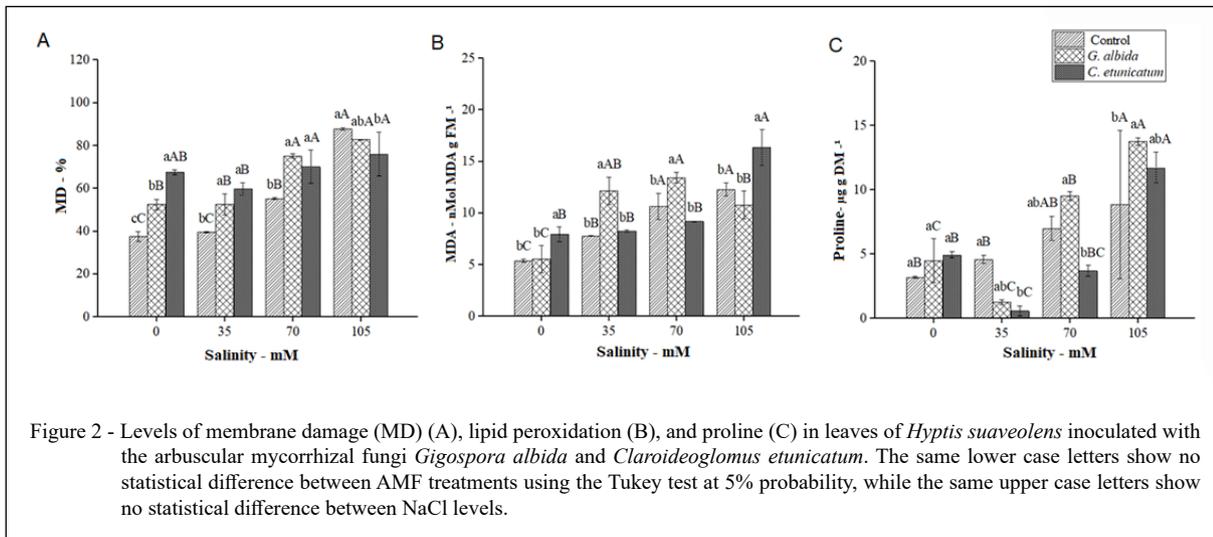


Figure 2 - Levels of membrane damage (MD) (A), lipid peroxidation (B), and proline (C) in leaves of *Hyptis suaveolens* inoculated with the arbuscular mycorrhizal fungi *Gigaspora albida* and *Claroideoglomus etunicatum*. The same lower case letters show no statistical difference between AMF treatments using the Tukey test at 5% probability, while the same upper case letters show no statistical difference between NaCl levels.

different salinity levels showed reduced MDA content (LATEF & CHAOXING, 2011). In this study, the significantly increased MDA content in more severe treatments showed metabolic damage occurred and reflected the effects of oxidative stress.

Increased proline when exposed to salinity was also reported in another study (SARWAT et al., 2016). When plants were subjected to excess salts, they triggered osmotic adjustment mechanisms to maintain cell turgidity. The accumulation of this solute is a sensitive physiological indicator of plant

responses to salt and other stresses (GUERZONI et al., 2014), as it also has other functions such as maintaining and protecting the integrity of the plasma membrane (MARIJUAN & BOSCH, 2013).

In plants, sugar is used in growth-related metabolism by providing energy through the biological oxidation process (MORAIS et al., 2019). The increase in carbohydrates observed in severe salinity stresses (70 mM and 105 mM) may reflect an accumulation of osmoregulators in order to minimize the effects of stress (Figure 3B). High concentrations

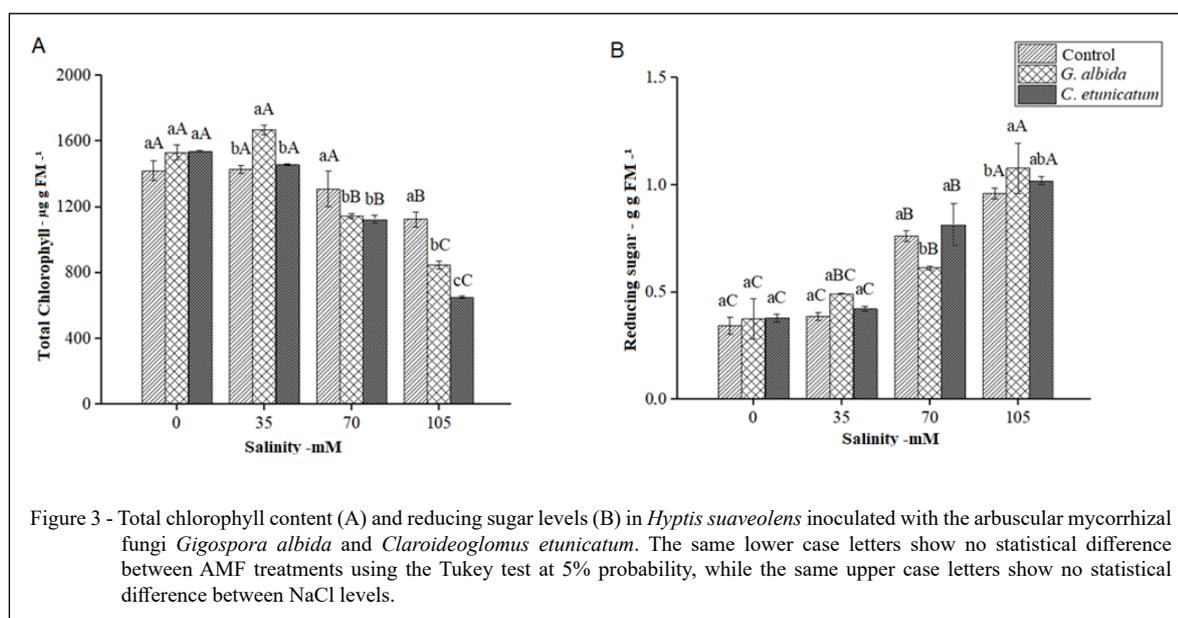


Figure 3 - Total chlorophyll content (A) and reducing sugar levels (B) in *Hyptis suaveolens* inoculated with the arbuscular mycorrhizal fungi *Gigaspora albida* and *Claroideoglomus etunicatum*. The same lower case letters show no statistical difference between AMF treatments using the Tukey test at 5% probability, while the same upper case letters show no statistical difference between NaCl levels.

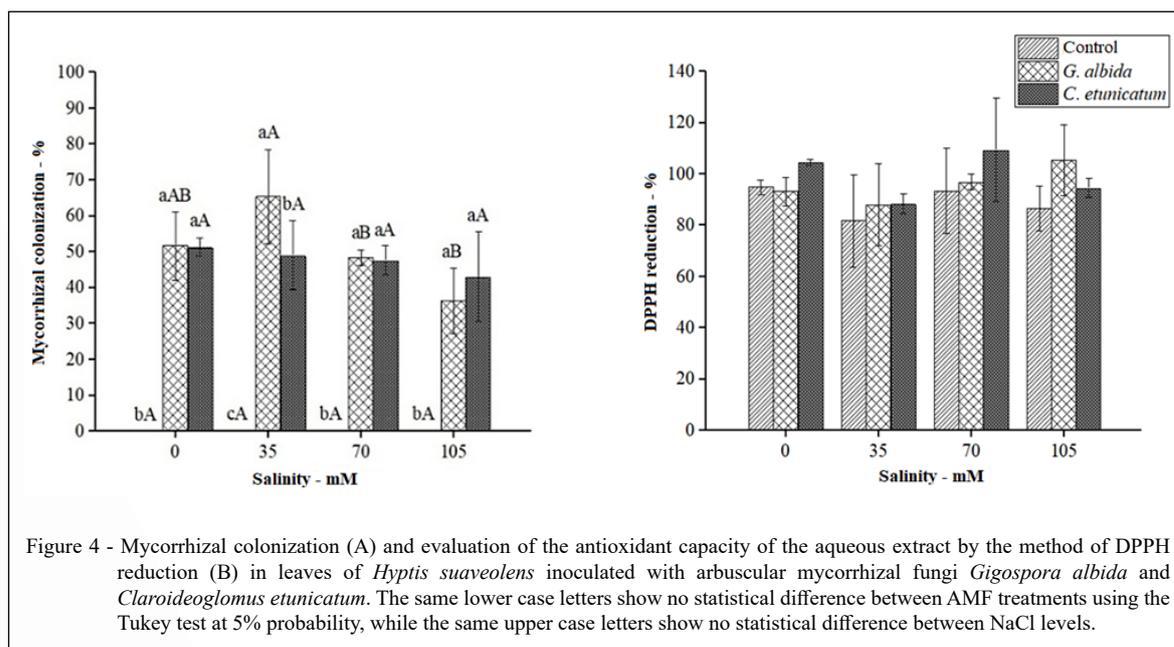
of reducing sugars stabilize some macromolecular structures of the cells, which helps to restore plasma membrane integrity. Of the various organic osmolytes, sugars provide about 50% or more of the total osmotic potential of glycophytes under abiotic stresses, in addition to preventing dehydration and being a source of energy for active cells under stress conditions (ELAVUMOOTIL et al., 2003).

The plants were able to remain photosynthetically active under moderate salinity conditions (35 mM) (Figure 3A), which is considered important for plant development under stress and is usually treated as an important variable when determining salinity tolerance (MORAIS et al., 2019). However, more severe conditions had adverse effects on chlorophyll content, with saline stress causing a reduction in pigment content. ARAGÃO et al. (2009) proved that increased salt levels in the soil negatively influenced chlorophyll production in melon plants (*C. melo* L.); according to the authors, this contributed to a decreased production of fresh and dry matter by the plants.

When colonized with the two species of fungi, *H. suaveolens* presented structures characteristic of mycorrhizal colonization in all treatments regardless of salt dose, including arbuscules, hyphae, and vesicles (data not shown). Colonization was null in non-inoculated plants, showing that the soil sterilization method was effective (Figure 4A).

The evaluation of antioxidant activity (% AA) in the aqueous extract of *H. suaveolens* leaves showed that all treatments presented DPPH sequestering activity, with DPPH sequestration above 80% (Figure 4B), indicating that the species already has a complex antioxidant defense system regardless of stress. This system involved the activity of enzymes and non-enzymatic metabolites, which, jointly, eliminate ROS and reduce damage (BARBOSA et al., 2014).

The level of toxicity generated by salinity is a worldwide agricultural and environmental problem. In this sense, our study showed that stress severity may have affected AMF development (AGGARWAL et al., 2012). The necrotic points visualized in the plants treated with NaCl may be related to ion accumulation, since excess ions result in morphological changes (smaller leaves and fewer leaves) and even cell death. However, it is worth mentioning that despite the evidence of oxidative stress, the plants had higher dry biomass content when inoculated with *C. etunicatum* in the most severe stress scenario, suggesting an attenuation of the effects of stress, which corroborates with the literature on the performance of this fungus in relation to better absorption of water and nutrients, better nutrition, greater contents of reserve substances, and greater vegetative development of plants when considering physiological mechanisms (SILVEIRA et al., 2002; FERNANDES et al., 2019).



## CONCLUSION

Osmoregulatory mechanisms and antioxidant activity did not prevent MD due to the severity of salt stress, which negatively interfered with the development of *H. suaveolens*. However, the fungus *C. etunicatum* improved the physiological performance of plants experiencing salt stress.

## DECLARATION OF CONFLICT OF INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## AUTHORS' CONTRIBUTIONS

MCGS participated in the data collection, designed the experiments, data analysis wrote the manuscript, MBM participated in the interpretation, review the article and provided editorial advice; MSA, MAV and SSS realization of analyses physiological and biochemistry interpretation and analyzed data; CCA guided every step of the work and participated in the drafting and review of the project and of the article.

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