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IS *Annona emarginata* CAPABLE OF ACCUMULATE ESSENTIAL HEAVY METALS WITHOUT DAMAGES IN THE METABOLISM?[1]

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ABSTRACT- This study aimed to evaluate if ionic strength variation causes differential accumulation of heavy metals in plants of *Annona emarginata* (Schltdl.) H. Rainer var. '*terra-fria*', modifying gas exchange and dry matter production. The seedlings were cultivated under different ionic strengths of the 'Hoagland and Arnon's' nutrient solution (75% *I*, 50% *I* and 25% *I*). At 180 days after the application of the treatments, the effects of the essential heavy metals on the physiological parameters and foliar ionic concentration were assessed. The leaf gas exchanges and vegetative growth were affected by the variation in the essential heavy metals, with positive effects on the plants that were treated with 75%*I*. The seedlings that were grown under 75% *I* presented higher concentrations of Zn (24.2mg kg^{-1}). On the other hand Fe (453mg kg^{-1}) e Mn (803.5mg kg^{-1}) accumulated more under 25% *I*, but gas exchanges and vegetative growth were reduced, compared to 50%*I* and 75%*I*. We can conclude that the ionic strength variation alters gas exchange and the dry matter accumulation, causing differential accumulation of Fe, Mn and Zn in *A. emarginata*, occurring inverse relationship between increased ionic strength and Fe and Mn content, besides directly relationship to the Zn content.
Index terms: Heavy metals; ionic strength; mineral nutrition; photosynthesis.

A *Annona emarginata* É CAPAZ DE ACUMULAR METAIS PESADOS ESSENCIAIS SEM CAUSAR DANOS AO SEU METABOLISMO?

RESUMO- Este estudo teve como objetivo avaliar se a variação da força iônica (*I*) provoca acúmulo diferencial de metais pesados, em plantas de *Annona emarginata* (Schltdl.) H. Rainer 'var. *terra-fria*', modificando as trocas gasosas e produção de matéria seca. As mudas foram cultivadas sob diferentes forças iônicas da solução nutritiva (75%*I*, 50%*I* e 25%*I*). Aos 180 dias após a aplicação dos tratamentos, foram avaliados os efeitos dos metais pesados essenciais sobre os parâmetros fisiológicos e concentração iônica foliar. As trocas gasosas e crescimento vegetativo foram afetados pela variação das concentrações dos metais pesados essenciais, com efeitos positivos sobre as plantas que foram tratadas com 75%*I*. As mudas que foram cultivadas em 75%*I* apresentaram maiores concentrações de Zn (24,2mg kg^{-1}). Por outro lado, o Fe (453mg kg^{-1}) e Mn (803,5mg kg^{-1}) foram acumulados em maiores concentrações na solução com 25% *I*, mas as trocas gasosas e o crescimento vegetativo foram reduzidos comparados com 50%*I* e 75%*I*. Podemos concluir que a variação da força iônica altera as trocas gasosas e o acúmulo de matéria seca, causando acúmulo diferencial de Fe, Mn e Zn em A. emarginata, ocorrendo relação inversa entre o aumento da força iônica e teor de Fe e Mn, além de relação direta ntre a força iônica ea concentração de Zn.
Termos para indexação: fotossíntese; força iônica; metais pesados; nutrição mineral.

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INTRODUCTION

The term “heavy metals” refers to a group of elements with metallic properties, which present high density (higher than 3.5 to 7g cm^{-3}) and high atomic number (higher than 20g cm^{-3}), for example, As, Cd, Cr, Cu, Pb, Hg, Ni, Mn, Mo, Zn. Heavy metals are usually absorbed by plants, but some of them, in high concentrations, are toxic (CHANDRASEKARAN; RAVISANKAR, 2015; MUSTAFA and KOMATSU, 2016; STEFANOWICZ et al. 2016).

Regarding the role of plants in biological systems, heavy metals are classified as essential or non-essential. Essential heavy metals (E.H.M.) are those, which are required in smaller amounts for vital physiological and biochemical functions of plant, e.g. B, Cu, Fe, Ni, Mn and Zn (HÄNSCH; MENDEL, 2009; ALI et al. 2013; O’CARRIGAN et al. 2014). Several of these elements are redox-active, making them essential as catalytically active cofactors in enzymes, while others have enzyme-activating functions or fulfill a structural role in stabilizing proteins, in this manner they are crucial for growth, DNA transcription, nitrogen fixation, seed germination, flowering, photosynthesis, resistance to biotic and abiotic stresses (BITYUTSKII et al. 2014; BOLDINGH et al. 2016; NAVARRO-LEÓN et al. 2016).

Despite the significant role of the elements are toxic at high concentrations for plants and animals. Environmental pollution by heavy metals has become a serious problem worldwide. Unlike organic substances, heavy metals are essentially non-biodegradable and therefore accumulate in soils and waters, posing a risk to environmental and human health. In soil, heavy metals cause toxicological effects, which may decrease the numbers and activities of living organisms (CHEN et al. 2015; SFAKIANAKIS et al. 2015; CHOWDHURY et al. 2016). Soils worldwide, especially the rainforest soils in southeastern Brazil, have been contaminated as a result of civil, agricultural and industrial activities (VAMERALI et al. 2010). Even though there is an urgent need for a knowledge-based effort, the information about the effect of with hydrocarbons, heavy metals, and other chemical substances of forest woody plants is not enough (ÜBELHÖR et al. 2014; VILLACÍS et al. 2016).

The Annonaceae family has been proven to be an excellent proxy for tropical rainforests. The distribution center of the large family Annonaceae is the Amazon region, the Guianas and southeastern Brazil, occurring in upland forests, wetlands and savannas (CHATROU et al. 2012). This family has species with fundamental importance in the ecological restoration of degraded forests (INFANTE MATA et al. 2011; WHEELER et al. 2016). Furthermore, some authors have reported *Annona emarginata* (Schltdl.) H. Rainer have tolerance to flooding and, used as a rootstock, provide adaptability to atemoya in different environments and soils (ALMEIDA et al. 2010). This species is native to the South-American and, in Brazil, is found with naturally occurring, preferably at locations above 950m altitude in South and Southeast regions of ‘poor’ nutrient availability (KAVATI, 2013).

The aim of this study was to evaluate if ionic strength variation of essentials mineral elements causes differential accumulation of essentials heavy metals in plants of *Annona emarginata* (Schltdl.) H. Rainer var. ‘*terra-fria*’, modifying gas exchange and dry matter production. Furthermore, is relevant the understanding about how this species develops in soils with high availability of essential mineral elements. This information may elucidate new possibilities of use of this species as a rootstock in areas that are traditionally unutilized for commercial fruit production.

MATERIAL AND METHODS

Plant material and treatments

This experiment was conducted under greenhouse conditions at the Botany Department, Instituto de Biociências, Universidade Estadual Paulista (Unesp), Botucatu-SP, Brazil (48º24’35”W, 22°49’10”S). *A. emarginata* seeds were obtained from trees in the municipality of São Bento do Sapucaí-SP, Brazil (45°44′ 11″ W, 22°41′18″S) and were germinated in polystyrene trays that were filled with previously autoclaved vermiculite. Approximately 4 months after emergence, the young plants were transplanted into plastic trays (1 young plant per pot) with a 4.5L (4.5dm^3) capacity and cultivated in greenhouse (20–32ºC, UR 65-75%).

The applied treatments consisted of varying ionic concentrations (ionic strength, *I*) of complete nutrient solution of Hoagland and Arnon (1950) nº2, which were formed with subsequent dilutions in demineralized water up to a concentration of 75% or ¾ Hoagland and Arnon (1950) nº 2, i.e., 75% *I* conductivity electrical (1.50 ± 0.2mS cm^{-1} EC) and successively to 50% or ½ Hoagland and Arnon (1950) nº 2, i.e. 50% *I* (1.00 ± 0.2mS cm^{-1} EC) and 25% or ¼ Hoagland e Arnon (1950) nº2, i.e. 25% *I* (0.5 ± 0.02mS cm^{-1} EC) (Table 01). During experimental period, nutrient solution were monitored daily for pH, which was adjusted between 5.5 to 6.5. using KOH and/or HCl (both 0.1M) and kept under constant aeration.

Leaf gas exchange

Gas exchange was measured with an infrared CO_2 and water vapor analyzer (LI-6400, Li-Cor Inc., Lincoln, NE, USA). Four plants of each treatment were measured at 180 days after the application of treatments (DAAT), between 09:00 a.m. and 11:00 a.m., on the second mature and fully expanded leaf starting from the apex. The photosynthetic photon flux density (PPFD) was standardized through the use of a light-emitting diode that was coupled to a photosynthesis chamber and emitted 1500μmol m^{-2} s^{-1} (BARON et al., 2013). The reference CO_2 concentration and air temperature that was used during the evaluation was the ambient value, which ranged from 390.9 ± 1.1μmol mol^{-1} of air and 27.5 ± 1ºC (average ± SE, *n*=20, representing 4 treatments × 5 replications).

The net CO_2 assimilation rates (A_{net} , μmol CO_2 m^{-2} s^{-1}), transpiration rates (*E*, mol H_2O m^{-2} s^{-1}), stomatal conductances (g_s, mol H_2O m^{-2} s^{-1}), and intercellular CO_2 concentrations (C_i, μmol CO_2 mol air^{-1}) were evaluated. The water-use efficiency (*WUE*, μmol CO_2 (mol $H_2O)^{-1}$) was determined by the relationship between the net assimilation rate and the transpiration rate (*A*/*E*), and the apparent carboxylation efficiency (A/C_i) was determined by the relationship between the net CO_2 assimilation rate and the intercellular CO_2 concentration.

Growth parameters

By the end of the experiment (180 DAAT), five young plants of each treatment were harvested, washed with deionized water, dried with paper towels, and separated into roots, stem and leaves. The evaluated parameters were the leaf area, height, and number of leaves per seedling. The leaf area was measured using an automatic Leaf Area Meter LI-3100® (Li-Cor, USA). The height values were obtained using a ruler. The plant material was dried at 65°C, until achieving a constant mass, after this procedure, the material was weighed in order to obtain the biomass dry weight.

Chemical composition

After drying and weighing of the plant material, were milled in a Wiley mill to pass through a 20 mesh screen so that they could be analyzed chemically.

The levels of Zn, Cu, Fe, Mn and B in the leaves were quantified in the Laboratory of Plant Mineral Nutrition, Soil and Environmental Resources Department, Faculdade de Ciências Agronômicas, Universidade Estadual Paulista (Unesp), Botucatu *campus*, São Paulo, Brazil. Based on these results, calculations to ensure unit adequacy were performed namely for each mineral element accumulated, according to Malavolta et al. (1997).

Statistics

The experiment was carried out on a completely randomized design with three treatments (ionic strength, *I*) with five replicates, eight seedlings each. The chemical composition and dry weight results were subjected to an analysis of variance (ANOVA), and the means were compared by Tukey's Test ($p \leq 0.05$). To determine the homogeneity of the treatment variances were performed Levene's Test. The SAS®9.2 statistical software was used.

RESULTS

The leaf gas exchanges in *A. emarginata* were clearly affected by the variation in the mineral elements, therefore 75% *I* solution caused positive effects on the different physiological indexes, except *WUE* (Figure 01, Tables 02 and 03), in the plants. The plants that were subjected to treatments with 25% *I* decreased net assimilation rate (A_{net}) and apparent carboxylation efficiency (A/C_i) compared those grown with 75% *I*, which exhibited the highest values of A_{net} (Figure 01a). However, the stomatal conductance (g_s) and transpiration (*E*) of the plants that were grown with 25% *I* were not different from those in 50% *I* (Figure 01b and d).

In plants grown with 25% *I* we observed highest concentrations of Fe and Mn and lower Zn concentration in relation to 75% *I,* however, not statistically different from 50%*I*. The different treatments did not change the concentrations of Cu. The concentrations of Mn and B were higher in plants grown in 50% *I* compared with 75% *I*, however, not statistically different from 25%*I* (Table 02). The plants were grown with 50% and 75% *I* showed a greater number of leaves and total dry mass. However it was not observed different leaf area and height in plants of the treatments (Table 03).

DISCUSSION

Variations in the nutrient solution ionic strength affected the gas exchange, biomass accumulation and ion concentration in the leaves of *A. emarginata*.

The plants grown in 75% *I* accumulated Zn and show increase in net assimilation rate (A_{net}), apparent carboxylation efficiency (A/C_i) and dry matter. In chloroplasts, zinc-dependent enzymes provide several major functions, like as proteolytic activities depend on Zn, that repair processes of photosystem II, by changing photo-damaged D1 protein (IBRAHIM; RAMADAN, 2015; FU et al. 2015).

On the other hand, the low accumulation of Zn in plants grown in 25% *I* could explain reduction of the net assimilation rate by disturbing the activity of carbonic anhydrase (CA; carbonate hydrolyase, E.C. 4.2.1.1). In this enzyme the Zn is the active site, holding prime importance in plants for carbon assimilation and photosynthesis (RASCIO, 2002). Hence Zn might play a very important role in protecting CA from ROS-mediated oxidation and modifications to its structural conformation, due to its association as a co-factor of the enzyme (ARAVIND; PRASAD, 2005). Net assimilation rate (A_{net}), as much as apparent carboxylation efficiency (A/C_i) it may have not only a consequence of the reduction in Zn, but, also, the reduction of macronutrients (N, P, K, Ca, Mg and S), when supplied in 25% *I*, as observed by Baron et al. (2013).

There was an inverse relationship between the absorption of Fe and Zn, comparing plants grown in the solutions 75%*I* and 25%*I*. Which is evidenced by higher concentrations of Fe in leaves of plants grown in 25% *I* and lower in leaves of plants grown in 75% *I*. On the other hand, lower concentrations of Zn in leaves of plants grown in 25% *I* and higher in leaves of plants grown in 75% *I*. Fe deficiency decreases the uptake or immobilization in the roots and is caused by increased levels of Zn because they are antagonists (SIEDLECKA, 1995). Fe toxicity damage is generally associated with the formation of degrees of chlorosis, and therefore the induction of antioxidative enzymes, such as ascorbate peroxidase, and Fe-binding proteins, such as ferritin, represent an important cellular defense mechanism against iron toxicity damage (HÄNSCH; MENDEL, 2009; GAMA et al. 2016; MAI; BAUER, 2016), which may have contributed to reducing gas exchange, when the plants were grown in 25% *I*.

Wild plant species, which are adapted to conditions of low soil fertility, are classified as slow-growing plants, type I (plants that maintain high ionic levels on dry matter), or pioneer plants, type II (fast-growing plants), and these process a low mineral supply have upgraded root/shoot ratio (CHAPIN, 1980). Baron et al. (2013) also showed that the species *Annona emarginata* (Schltdl.) H. Rainer var. '*terra-fria*' does not require the maximum availability of mineral nutrients from the Hoagland and Arnon's nutrient solution to achieve high primary productivity. The electrical conductivity (EC) increased of the solution can cause negative effects in the photosynthetic electron transport chain and, consequently, on the photosynthetic rate (WORTMAN et al. 2015), reducing biomass production (ROOSTA; HAMIDPOUR, 2011). In addition, 50% *I* and 75% *I* showed a higher apparent carboxylation efficiency and biomass production.

According to Gough e Marrs (1990) there are few information comparing the ionic strength of natural vegetation soils and intensive agriculture soils. Ionic strength of soil solutions from tropical soils range from about 0.005 and 0.002 mmol L^{-1} (EDMEADES et al. 1985), this results are comparable with the ionic concentration of the 25% *I* nutrient solution used in this experiment. However, the concentrations of cations and anions in nutrient solution are direct proportional to the levels of ionic strength, on other words, low concentration of cations and anions are present in low levels of ionic strength.

In summary, the species under study can survive in environments with poor soil and present slower metabolism. On the other hand, when growing in fertilized soil conditions, the metabolism will be accelerated with higher development. That way, *A. emarginata*, that commonly occur in poor soils, has a potential to accumulate essential heavy metals, in special Fe e Mn, but with plant metabolism reduced. It is worth mentioning that the highest ionic strength may be used without impairing the plant metabolism, since it does not accumulate heavy metals (Fe and Mn) or accumulated (Zn) without damages.

In conclusion, the ionic strength variation alters gas exchange and the dry matter accumulation, causing differential accumulation of Fe, Mn and Zn in *A. emarginata*, occurring inverse relationship between increased ionic strength and Fe and Mn content, besides directly relationship to the Zn content.

TABLE 1 - Nutrient composition of different ionic strengths (*I*) of complete nutrient solution of Hoagland and Arnon (1950) nº 2 used for the cultivation of *A. emarginata*.

Used compounds	Stock Solution ($g L^{-1}$)	Treatments ionic strength (*I*) ($mM L^{-1}$)		
Macronutrients (M) (1)		25% *I* or '1/4' ($mmol L^{-1}$)	50% *I* or '1/2' ($mmol L^{-1}$)	75% *I* or '3/4' ($mmol L^{-1}$)
$NH_4H_2PO_4$	115.0	0.12	0.24	0.47
KNO_3	101.1	0.84	1.68	2.52
$Ca(NO_3)_2.4H_2O$	236.1	0.97	1.95	3.90
$MgSO_4.7H_2O$	246.5	0.51	1.02	2.03
Micronutrients		25% *I* or '1/4' ($\mu mol L^{-1}$)	50% *I* or '1/2' ($\mu mol L^{-1}$)	75% *I* or '3/4' ($\mu mol L^{-1}$)
(1) H_3BO_3	2.86	2.95	5.90	11.8
$MnCl_2.4H_2O$	1.81	1.86	3.71	7.42
$ZnSO_4.7H_2O$	0.22	0.22	0.45	0.91
$CuSO_4.5H_2O$	0.08	0.08	0.16	0.33
$H_2MoO_4.H_2O$	0.02	0.02	0.04	0.08
Fe Solution(2)		25% *I* or '1/4' ($mmol L^{-1}$)	50% *I* or '1/2' ($mmol L^{-1}$)	75% *I* or '3/4' ($mmol L^{-1}$)
Fe-EDTA	26.1	0.07	0.05	0.11
$FeSO_4.7H_2O$	24.9	0.06	0.05	0.10

(M) = Molar; (1) = dilution in distilled water and volume completed to 1 L; (2) = dilution in 700 mL distilled water containing 268 mL NaOH (40g/L) and volume completed to 1 L.

TABLE 2- Variantion of foliar essentials heavy metals concentration in leaves of *A. emarginata* plants cultivated in nutrient solution during 180 DAAT.

$mg kg^{-1}$	Ionic strength (*I*) 25% *I*	50% *I*	75% *I*	*Pr >F*	*C.V.*
Zinc (Zn)	15.8 ± 0.3b	19.5 ± 1a	24.2 ± 4a	0.0134*	16.60
Copper (Cu)	3.5 ± 0.1a	4.7 ± 0.1a	3.75 ± 0.2a	0.7113*	55.59
Iron (Fe)	453.0 ± 87a	306.0 ± 26ab	168.5 ± 15b	0.0062*	30.01
Manganese (Mn)	803.5 ± 35a	824.2 ± 47a	205.75 ± 53b	<.0001*	12.88
Boron (B)	122.2 ± 12ab	137.5 ± 14a	94.50 ± 5b	0.0356*	16.60

Means ± S.E. followed by the same letter are not significantly different at $^*P \leq 0.05$ according to Tukey's Test. The number of replicates per treatment was n=5 ($mg kg^{-1}$).

TABLE 3- Growth and biomass parameters of *A. emarginata* plants cultivated in nutrient solution during 180 DAAT.

Variables treatments	Ionic strength (*I*) 25% *I*	50% *I*	75% *I*	*Pr > F*	*C.V. (%)*
Leaves (unit)	64.96 ± 6.1b	79.52 ± 15.3ab	102.48 ± 2.8a	0.0293	23.44
Height (cm)	19.30 ± 0.6a	23 ± 2.3a	21.34 ± 2.0a	0.3083	17.13
Leaf area (cm^2)	0.40 ± 0.1a	0.58 ± 0.0a	0.64 ± 0.1a	0.0596	27.26
Dry matter mass (g)	0.66 ± 0.1b	1.16 ± 0.0a	1.14 ± 0.2a	0.0041	21.42

Means ± SE followed by the same letter are not significantly different at $*P \leq 0.05$, according to Tukey's Test. The number of replicates per treatment was n=5 ($g kg^{-1}$).

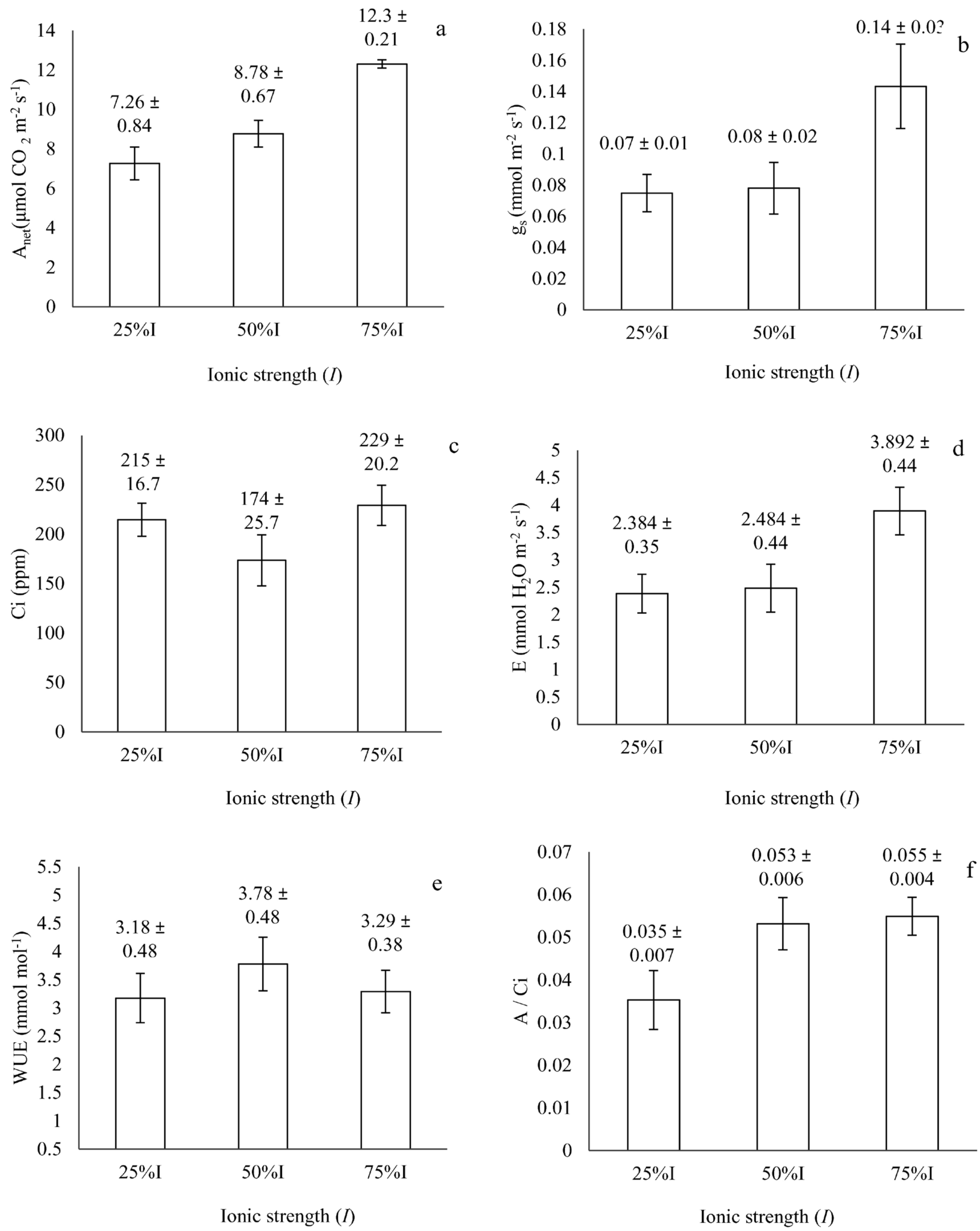

FIGURE 1- (a) Net assimilation rate (A_{net}); (b) stomatal conductance (g_s); (c) transpiration (E); (d) water-use efficiency (WUE); (e) internal carbon concentration in the substomatal chamber (C_i); and (f) apparent carboxylation efficiency (A/C_i) of *A. emarginata* plants that were cultivated in nutrient solution for 180 DAAT.

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AUTHORS CONTRIBUTIONS

Daniel Baron and Gisela Ferreira conceived and designed research, conducted experiments, analyzed data and wrote the manuscript; Carmen Sílvia Fernandes Boaro assisted in the gas exchanges and growth parameters data analyses and statistical analysis; Ana Claudia Macedo and Amanda Cristina Esteves Amaro conducted the gas exchanges measurements and assisted in the literature review and the data analyses. All authors read and approved the manuscript.

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