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Application methods of hydrogen peroxide in soursop seedlings irrigated with saline water

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Abstract

Plant acclimatization to salt stress with the application of hydrogen peroxide occurs through the activation of the antioxidant defense system. In this perspective, this study aimed to evaluate the contents of photosynthetic pigments and the photochemical efficiency of soursop seedlings under irrigation with saline waters and different methods of application of hydrogen peroxide. The study was performed under plant nursery conditions, in a 5 x 4 factorial arrangement, referring to five conductivity levels of the irrigation water (0.6, 1.2, 1.8, 2.4 and 3.0 dS m^{-1}) and four methods of application of hydrogen peroxide (without H_2O_2 application, application via seed soaking, application via foliar spraying, and application via seed soaking and foliar spraying). Irrigation water salinities up to 1.4 and 1.1 dS m^{-1} caused an increase in the synthesis of chlorophyll b and carotenoids, respectively. Irrigation water salinities from 0.6 dS m^{-1} reduced the synthesis of chlorophyll a and total chlorophyll of the soursop seedlings. The application of 20 μM of H_2O_2 via seed soaking results in the increase of the contents of chlorophyll a, b, total, and maximum and variable fluorescence of the soursop seedlings. Seed soaking with hydrogen peroxide increased the initial fluorescence of chlorophyll in the plants irrigated with 1.2 dS m^{-1}.

Keywords: *Annona muricata* L., acclimatization, salt stress

Introduction

Soursop (*Annona muricata* L.) is an Annonaceae species of significant importance in terms of production and cultivated area in Brazil, especially in Southern Bahia. The interest for this fruit species has grown due to its several food and pharmaceutical uses, preventing a series of chronic-degenerative disorders (Dauda et al., 2018). However, soursop cultivation in the semiarid region has been limited due to the salinity of the water used in irrigation (Silva et al., 2018).

The semiarid region of Northeastern Brazil is characterized by the variability in the spatial and temporal distribution of rainfall, which favors water scarcity and the increase of water and soil salinity, compromising the whole productive cycle of the crops. Usually, when sensitive plants are subjected to salt stress, they may present physiological and biochemical modifications due to the toxic, osmotic, and nutritional effects caused by salt excess (Santana Junior et al., 2020).

Furthermore, plant response to salinity is complex, and the efficiency of the physiological and biochemical mechanisms, besides the molecular adjustment required for their survival in saline environments, will depend on the species, genotype or cultivar, concentration, and composition of salts in the irrigation water or soil solution (Rodrigues et al., 2019; Bráz et al., 2019).

Secondly, salinity also induces oxidative stress due to the accumulation of reactive oxygen species (EROs), such as hydrogen peroxide (H_2O_2) and free radicals, superoxide (O_2^-), and hydroxyl (OH^-), which in high concentrations can cause oxidative damage to the lipids of the membrane, proteins, and nucleic acids. However, plant acclimatization through the application of H_2O_2 in adequate quantities can activate the defense system of the plants, promoting metabolic changes that are responsible for the increase in their tolerance to new

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stress exposure, such as salt stress (Nunes et al., 2019; Gohari et al., 2019).

Some studies were performed to demonstrate the efficiency of the exogenous application of H_2O_2 in crop acclimatization to salt stress (Bagheri et al., 2019), although, in the literature, there are still no reports on the recommended method of application of this product in agriculture.

In that perspective, this study aimed to evaluate the contents of photosynthetic pigments and the photochemical efficiency of soursop seedlings irrigated with saline waters and distinct methods of application of hydrogen peroxide.

Material and Methods

The experiment was performed in a protected environment (plant nursery) in the period from April to September 2019, at the Center of Technology and Natural Resources of the Federal University of Campina Grande (CTRN/UFCG), located in the municipality of Campina Grande, Paraíba, at the geographic coordinates 07^0 15'18" S latitude, 35^0 52'28" W longitude, and mean elevation of 550 m.

The experimental design was in randomized blocks, in a 5 x 4 factorial arrangement with four replications, whose treatments resulted from the combination of five levels of electrical conductivity of the irrigation water – ECw (0.6, 1.2, 1.8, 2.4 and 3.0 dS m^{-1}) and four methods of application of hydrogen peroxide - H_2O_2 (M1= without H_2O_2 application, M2 = application via seed soaking, M3 = application via foliar spraying, and M4 = application via seed soaking and foliar spraying). The salinity levels used in this research were based on studies performed by Silva et al. (2019b). The H_2O_2 concentration used was 20 μM, as established in the research performed by Silva et al. (2018).

The soursop seedlings were irrigated with waters prepared though the addition of sodium chloride (NaCl) dissolved in the supply water (ECw = 0.4 dS m^{-1}) from the municipality of Campina Grande, Paraíba, considering the relationship between ECw and salt concentration ($mmol_c$ L^{-1} =10*ECw dS m^{-1}), according to Richards (1954). After the preparation and calibration of the ECw, the saline waters were stored in plastic recipients with a 120 L capacity, which were properly sealed to avoid evaporation.

The concentration of hydrogen peroxide (H_2O_2) used was 20 μM, obtained through the dilution of H_2O_2 in distilled water. Before sowing, the seeds of treatments M2 (application by soaking) and M4 (application by soaking and foliar spraying) were subjected to a pre-treatment with hydrogen peroxide, in which they were soaked for 36 hours.

The polyethylene bags with capacity for 2 dm^3 used in the experiment were filled with 2.6 kg of a mixture of soil (84%), sand (15%), and humus (1%). The soil presented a sandy-loam texture and was originated from the municipality of Lagoa Seca, Paraíba, collected in the 0-20 cm depth (A horizon), whose chemical and physical properties are exhibited in Table 1.

Table 1. Chemical and physical characteristics of the substrate used in the experiment.

Chemical characteristics									
pH (H_2O) (1:2, 5)	O.M.	P	K^+	Na^+	Ca^{2+}	Mg^{2+}	$Al^{3+} + H^+$	ESP	ECse
	%	(mg kg^{-1})	................................($cmol_c$ kg^{-1})............................					(%)	(dS m^{-1})
5.90	1.36	6.80	0.22	0.16	2.60	3.66	1.93	1.87	1.0
Physical characteristics									
Size fraction (dag kg^{-1})			Textural class	Water content (kPa)		AW	Total porosity %	AD	PD
Sand	Silt	Clay		33.42	1519.5			(kg dm_{-3})	
				....	dag kg^{-1}	....			
73.29	14.21	12.50	FA	11.98	4.32	7.66	47.74	1.39	2.66

O.M. – Organic matter: Walkley-Black wet digestion; Ca^{2+} and Mg^{2+} extracted wifh 1 M KCl at pH 7,0; Na^+ and K^+ extracted with 1 M NH_4OAc at pH 7,0; Al^{3+} e H^+ extracted with 0.5 M^1 calcium acetate at pH 7,0; ESP – Exchangeable sodium percentage; ECse – Electrical conductivity of the saturation extract; SF – Sandy loam; AW – Available water; AD - Apparent density; PD- Particle density.

The soursop cultivar Morada Nova was chosen for possessing a genetic material preferred by the producers of the Northeast region, constituting the majority of the commercial orchards in Brazil since it presents a higher number of favorable traits, such as high yield, better fruit quality, and lower susceptibility to the fruit borer (*Cerconota anonnela* Sepp.) (Costa et al., 2016).

Before sowing, the soil moisture content was elevated to field capacity using respective water, according to the treatments. After sowing, irrigation was daily performed by applying in each bag a sufficient water volume as to maintain soil moisture close to field capacity. The volume applied was determined according to the water requirement of the plants, estimated by the water balance: water volume applied minus the volume drained in the irrigation of the previous day; also, a leaching fraction of 0.10 was applied every 15 days (Ayers & Westcot, 1999).

In each bag, three soursop seeds were sown at a 3 cm depth, equidistantly; thinning was performed 40 days after sowing, aiming at allowing only one plant per bag, which was the most vigorous one.

Fertilization with nitrogen (100 mg N kg^{-1} of soil), potassium (150 mg K_2O kg^{-1} of soil), and phosphorus (300 mg P_2O_5 kg^{-1} of soil) was performed via topdressing, based on the recommendation by Novais et al. (1991). The contents of 0.58 g of urea, 0.65 g of potassium chloride, and 1.56 g of monoammonium phosphate, equivalent to 100, 150, and 300 mg kg^{-1} of the substrate of N, K_2O, and P_2O_5, respectively, were applied in four equal applications via fertigation, every 15 days, with the first application performed 15 days after sowing (DAS). The need for micronutrients was supplied through the foliar spraying, at 60, 75, and 90, 105, 120, and 135 DAS, of 2.5 g L^{-1} of a solution with the following composition: N (15%); P_2O_5 (15%); K_2O (15%); Ca (1%); Mg (1.4%); S (2.7%); Zn (0.5%); B (0.05%); Fe (0.5%); Mn (0.05%); Cu (0.5%); Mo (0.02%).

At 80, 95, 110, and 125 DAS, the plants of treatments M3 (foliar spraying) and M4 (seed soaking and foliar spraying) were subjected to applications of H_2O_2 (20 µM). The applications were performed at 5:00 p.m. by spraying the abaxial and adaxial leaf surfaces to obtain a complete wetting of the leaves, using a backpack sprayer.

The initial (Fo), maximum (Fm), and variable fluorescence (Fv), as well as the quantum efficiency of FSII (Fv/Fm), and the content of chlorophyll a, b, total, and carotenoids were determined. At 150 DAS, the photochemical efficiency was measured in leaves that were pre-adapted to the dark, using leaf tweezers, for 30 minutes, between 7:00 and 8:00 a.m., in the middle leaf of the intermediate productive branch of the plant, using a modulated fluorometer, model OS5p, produced by Opti Science®.

The photosynthetic pigments were quantified according to the laboratory method developed by Arnon (1949), by which plant extracts are obtained from disc samples from the third mature leaf of the plant apex. These extracts were used to determine the contents of chlorophyll a, b, total, and carotenoids in the solutions using a spectrophotometer at the absorbance wavelengths (ABS) of 470, 646, and 663 nm, using equations 1, 2, and 3, as follows:

Chlorophyll a (Chla) = (12.21 x ABS663) – (2.81 x ABS646) Eq. (1)

Chlorophyll b (Chlb) = (20.13 x ABS64) – (5.03 x ABS663) Eq. (2)

Carotenoids (Car) = [(1000 x ABS470) – (1.82 x Chla) – (85.02 x Chlb)]/198. Eq. (3)

The values obtained for the tests of chlorophyll a, b, total, and carotenoids in the leaves were expressed in mg g^{-1} FM (fresh matter).

The data obtained were subjected to analysis of variance by the F-test at 0.05 and 0.01 of probability, in the cases of significance, linear and quadratic polynomial regression analyses were performed for the water salinity levels; for the methods of application of H_2O_2, the means were compared by Tukey's test at 0.05 of probability, using the statistical software SISVAR ESAL (Ferreira et al., 2014).

Results and Discussion

The analysis of variance revealed a significant effect of the water salinity levels on the contents of chlorophyll a, b, total, and carotenoids of the soursop plants. Regarding the methods of application of hydrogen peroxide, a significant difference is only observed for chlorophyll b. There was no significant effect of the interaction between factors (NS x MA) on the measured variables.

The irrigation with waters of different salinity levels significantly affected the content of chlorophyll a of the soursop seedlings, and according to the regression equation (Figure 1A), a linear decrease of 14.75% is verified per unit increment of the ECw, resulting in a reduction of 44.25% in the plants irrigated with the ECw of 3.0 dS m^{-1} compared with those subjected to the water salinity of 0.6 dS m^{-1}. The reduction in the synthesis of photosynthetic pigments may be related to the activation of the chlorophyllase enzyme, occurring in stressed plants, and being the main factor related to the reduction of the photosynthetic pigments. Furthermore, the damage caused to the pigments promotes biochemical alterations that can limit the photosynthetic activity, which may result in oxidative damage to the cells (Monteiro et al., 2018). In a study conducted by Silva et al. (2019a), evaluating water salinity in the synthesis of soursop photosynthetic pigments, the authors verified that salt stress caused a reduction of 15.2% in the synthesis of chlorophyll a in soursop plants, with a ECw increment from 0.7 to 3.5 dS m^{-1}, at 120 days after sowing.

According to the means comparison test for the chlorophyll a (Figure 1B), it was verified that the application of 20 µM of H_2O_2, through seed soaking (M2), different statistically from the remaining application methods (M1, M3, and M4), with an increase of 287.8 µM g^{-1} FM compared to the plants subjected to treatment

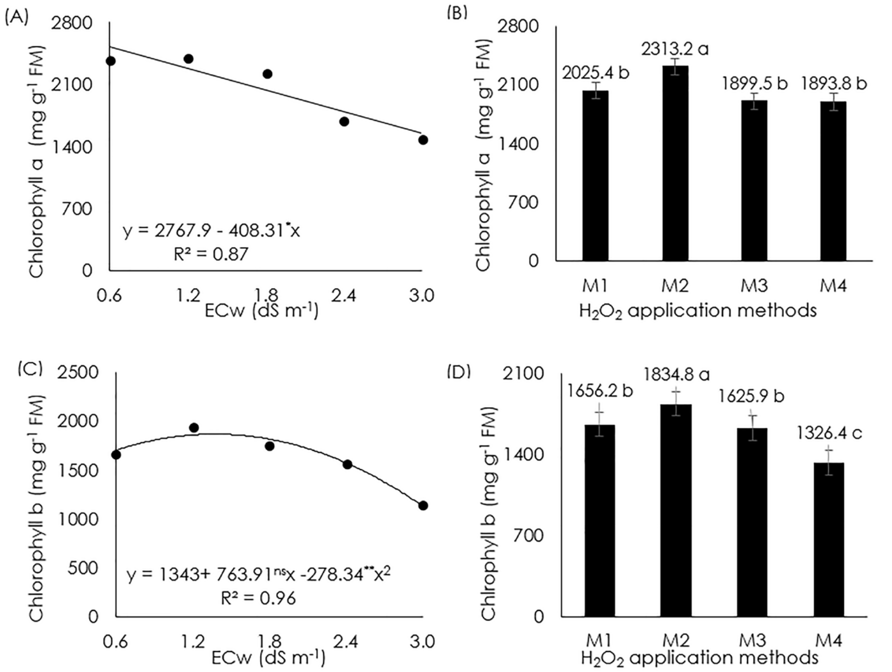

Figure 1. Chlorophyll a and b of plants of the soursop cv. Morada Nova as a function of the electrical conductivity of the irrigation water – ECw (A and C) and methods of H_2O_2 application (B and D), at 150 days after sowing (DAS). M1= without H_2O_2 application, M2 = application via seed soaking, M3 = application via foliar spraying, and M4 = application via seed soaking and foliar spraying; means followed by the same letter do not differ statistically from each other by Tukey's test ($p<0.05$).

M1 (without H_2O_2 application). Resembling the plants of the M2 method, the plants of the M4 method were also subjected to seed soaking, although obtaining a lower value of chlorophyll a. This probably occurred because they were subjected to two absorption methods (soaking + spraying), and the amount of hydrogen peroxide assimilated may have exceeded the adequate amount for good plant development (Carvalho et al., 2011).

On the other hand, the positive effect of H_2O_2 in the synthesis of chlorophyll, especially in the plants that received it via seed soaking, can be explained by the fact that this process was initiated since the hydration of the seed, considering the role performed by hydrogen peroxide, which involves interactions with germination-controlling hormones, O_2 production for mitochondrial respiration, and metabolic activity, aiding in the overcoming of tegument dormancy, allowing higher water absorption, besides contributing to the decomposition of germination inhibitors (Oliveira Junior, 2017), and assisting in the protection of the seedlings to biotic and abiotic stress. Several processes are initiated in the soaked seeds, such as genetic and biochemical modifications, constituting a 'stress memory' that can be expressed during other plant development phases (Santos et al., 2019).

Regarding the synthesis of chlorophyll b, the regression equation (Figure 1C) reveals that the increase in the electrical conductivity of the irrigation water caused a quadratic effect, whose maximum estimated value was 1,866.9 mg g^{-1} FM, obtained when the plants were irrigated with 1.4 dS m^{-1}. There was a reduction from this level, reaching the minimum value of 1,129.67 mg g^{-1} FM in the soursop plants irrigated with 3.0 dS m^{-1}.

Lima et al. (2019) verified a reduction in the content of chlorophyll b in cashew plants with the increase in the salinity levels of the irrigation water, and attributed this to the adaptation capacity of the plants to salt stress, leading to the saving of energy due to the lower capture of luminous energy and, consequently, the reduction in photo-oxidative stress.

Resembling the results observed for chlorophyll a (Figure 1B), the means comparison test (Figure 1D) allowed verifying that the application of H_2O_2 via seed soaking (M2) resulted in a higher synthesis of chlorophyll b, significantly differing in relation to the plants that did not receive the application of hydrogen peroxide (M1), that received via foliar spraying (M3) and the ones subjected to the application via seed soaking + foliar spraying (M4). This result may be related to the lower absorption efficiency by the leaf (M1 and M3) due to cutin, present on the surface of the leaves, which determines the penetration capacity of the compounds, whereas the application via seed soaking was more efficient in absorbing the hydrogen peroxide. It is also noted that the foliar spraying of H_2O_2 began at 80 days after sowing, which allows inferring that the H_2O_2 may have had a late action compared to the method via seed soaking, with the previous exposure to H_2O_2 possibly resulting in more effective acclimatization to the salt stress conditions (Silva et al., 2016).

The total chlorophyll content of the soursop seedlings was significantly affected by the irrigation water salinity (Figure 2A). It is observed that the increase in the water electrical conductivity provided a quadratic effect on the total chlorophyll content, with the maximum value (4,119.74 mg g^{-1} FM) being reached when the plants were subjected to irrigation with 1.1 dS m^{-1}. Pereira Filho et al. (2019), in their study, verified a reduction in the chlorophyll content of plants subjected to high salinity levels, which may characterize an indication of oxidative stress, probably resulting from the photo-oxidation of pigments associated with the degradation of chlorophyll molecules, considering that salt stress causes imbalances between the production and removal of reactive oxygen species, leading to a series of negative implications for metabolic mechanisms associated to photosynthetic processes. Silva et al. (2017), in a study with soursop under salt stress conditions, also verified a reduction in the chlorophyll contents in soursop plants due to the increase in the ECw.

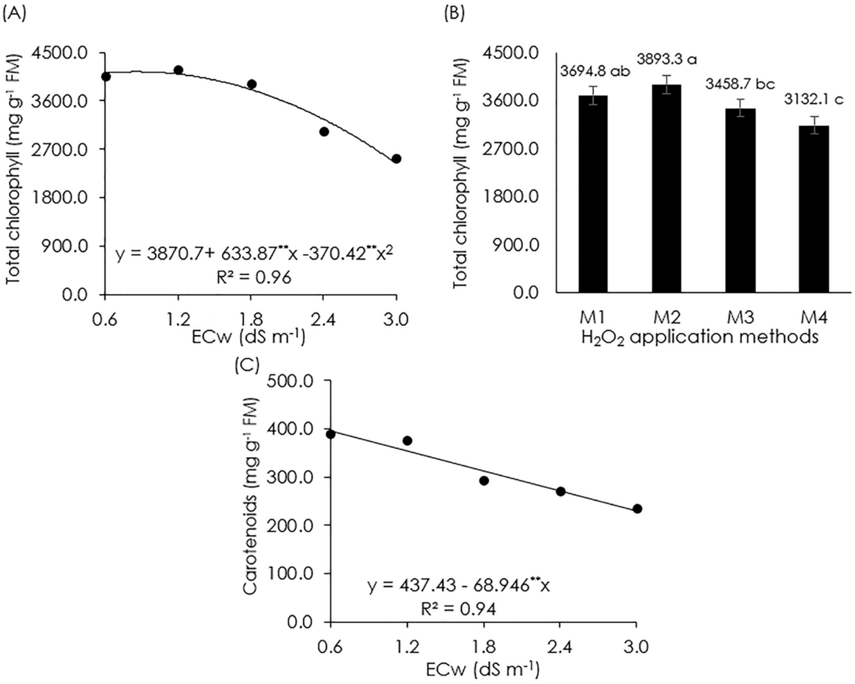

Figure 2. Total chlorophyll and carotenoids of seedlings of the soursop cv. Morada Nova as a function of the electrical conductivity of the irrigation water - ECw (A and C) and total chlorophyll content as a function of the application methods of H_2O_2 (B), at 150 days after sowing (DAS). M1= without H_2O_2 application, M2= application via seed soaking, M3= application via foliar spraying, and M4= application via seed soaking and foliar spraying; means followed by the same letter do not differ statistically from each other by Tukey's test ($p<0.05$).

The total chlorophyll content was statistically different in the soursop seedlings subjected to 20 µM of hydrogen peroxide via seed soaking (M2) and via foliar spraying (M3), and, according to the means comparison test, the seedlings under the M2 method had a 11.16% increase in relation to the seedlings under the M3 method (Figure 2B). The limitations of leaf spraying with H_2O_2 may be related to physical barriers, such as the epidermis and the cuticle layer, which become thicker in plants subjected to salt stress, a fact that contributed to the lower foliar absorption of H_2O_2 (Arias et al., 2018).

According to the regression analysis (Figure 2C), the content of carotenoids in the soursop seedlings was reduced by 15.76% with each unit increase in irrigation water salinity, that is, there was a reduction of 47.28% in the content of carotenoids in the plants irrigated with the ECw of 3.0 dS m_{-1} in relation to the plants grown under the highest water salinity (0.6 dS m^{-1}).

Sousa et al. (2017) observed a reduction in the content of carotenoids in Citrus species with the increase in the electrical conductivity of the irrigation water, and attributed it to the fact that salt stress leads to a reduction in the production of photosynthetic pigments, inducing the degradation of β-carotene, causing a decrease in the content of carotenoids.

The analysis of variance revealed a significant interaction between the factors (NS x MA) for the initial fluorescence of the soursop seedlings. The water salinity levels significantly affected only the Fo. In turn, the application methods of hydrogen peroxide provided a significant effect for the maximum and variable fluorescence in the soursop seedlings at 150 DAS.

When analyzing the interaction between the distinct application methods of hydrogen peroxide and the electrical conductivities of the irrigation water (Figure 3A), it is noted that there was no significant difference between application methods in the plants irrigated with 2.4 dS m^{-1}. However, the plants subjected to the M2 method (seed soaking) and irrigated with 1.2 dS m^{-1} presented an increase of 62,92, and 70% in the initial fluorescence compared with the application methods M1, M3, and M4. As for the plants irrigated with 1.8 dS m^{-1}, it is observed that the Fo of the plants subjected to the M1 and M2 methods differed statistically from methods M3 and M4. In turn, the plants irrigated with 3.0 dS m^{-1} exhibited a higher Fo value (886) when subjected to the M4 method (soaking + spraying).

In general, plants subjected to salt stress tend to present an increase in the Fo, evidencing the occurrence of structural alterations in the photosynthetic pigments, thus compromising the efficiency of the excitation energy from the light-collecting antenna and causing damage to the reaction centers of the FSI (Tatagiba et al., 2014). Unlike the results found in this research, Veloso et al. (2020) verified that the exogenous application of 20 μM of hydrogen peroxide promoted a reduction in the Fo of guava seedlings even at the highest salt level (3.0 dS m^{-1}), in which they showed that adequate concentrations of H_2O_2 could bring benefits to the plant, especially for the initial fluorescence of chlorophyll.

According to the means comparison test for the maximum and variable fluorescence (Figures 3B and 3C), it is noted that the plants whose seeds were soaked in hydrogen peroxide (M2) obtained highest values of maximum and variable fluorescence of 3,418.6 and 2,577.3, respectively. In this manner, it may be inferred that there was efficiency in the photoreduction of quinone A and in the flow of electrons between the photosystems, promoting an adequate activity of the PSII in the membrane of the thylakoid, directly influencing the flow of electrons between the photosystems.

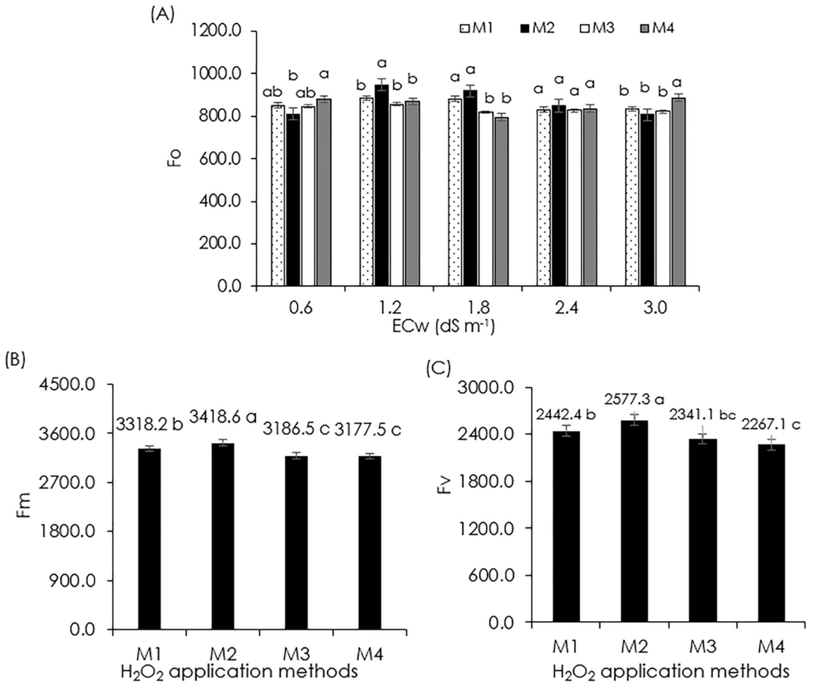

Figure 3. Initial fluorescence (Fo) of seedlings of the soursop cv. Morada Nova as a function of the electrical conductivity of the irrigation water - ECw (A) and maximum and variable fluorescence as a function of the application methods of H_2O_2 (B and C), at 150 days after sowing (DAS). M1 = without H_2O_2 application, M2 = application via seed soaking, M3 = application via foliar spraying, and M4 = application via seed soaking and foliar spraying; means followed by the same letter do not differ statistically from each other by Tukey's test (p<0.05).

The increase in the variable fluorescence of the seedlings subjected to previous exposure to H_2O_2 (M2) is an indicator that the photochemical activity of the leaves was not damaged since the increase of the Fv depicts the capacity of the rootstocks in transferring the energy from the electrons released by the pigment molecules for the formation of NADPH, ATP, and reduced ferredoxin (Fdr), thus increasing the CO_2 assimilation capacity in the biochemical phase of photosynthesis (Dias et al., 2018).

Conclusions

The irrigation water salinity up to 1.4 and 1.1 dS m^{-1} led to an increase in the synthesis of chlorophyll b and carotenoids, respectively. In turn, the irrigation with waters with electrical conductivity from 0.6 dS m^{-1} reduced the synthesis of chlorophyll a and total in plants of the soursop cv. 'Morada Nova', at 150 days after sowing.

There are no damages to the quantum efficiency of the FSII in the soursop plants irrigated with waters with electrical conductivity up to 3.0 dS m^{-1}.

The application of 20 μM of hydrogen peroxide via seed soaking results in an increase in the contents of chlorophyll a, b, total, and maximum and variable fluorescence of the soursop seedlings.

The water salinity of 1.2 dS m^{-1} and the application of H_2O_2 via seed soaking causes a higher initial fluorescence in the soursop plants.

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