RESEARCH ARTICLE

Photosynthesis and antioxidant activity in *Jatropha curcas* L. under salt stress

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ABSTRACT

Biodiesel is an alternative to petroleum diesel fuel. It is a renewable, biodegradable, and nontoxic biofuel. Interest in the production of biodiesel from *Jatropha curcas* L. seeds has increased in recent years, but the ability of *J. curcas* to grow in salt-prone areas, such as the Caatinga semiarid region, has received considerably meager attention. The aim of this study was to identify the main physiological processes that can elucidate the pattern of responses of *J. curcas* irrigated with saline water, which commonly occurs in the semiarid Caatinga region. This study measured the activity of the antioxidant enzymes involved in the scavenging of reactive oxygen species, which include catalase (CAT) and ascorbate peroxidase (APX), as well as malondialdehyde (MDA) levels. The levels of chlorophyll (Chl), carotenoids, amino acids, proline, and soluble proteins were also analyzed. The net carbon assimilation rate (P_N), stomata conductance (g_s), and transpiration rate (E) decreased with salt stress. The activities of CAT and APX were decreased, while H_2O_2 and MDA levels as well as electrolyte leakage were significantly increased in salt-stressed plants compared to the untreated ones. These observations suggest that the ability of *J. curcas* plants resist to salt stress is associated with the activities of protective enzymes and their defensive functions. However, our results indicate that the reactive oxygen species scavenging system is not sufficient to protect *J. curcas* leaves against oxidative damage caused by salt stress, and, therefore, it cannot be treated as a salt tolerant plant species.

Keywords: biodiesel, CAT and APX activities, *Euphorbiaceae*, H_2O_2 and MDA contents, salinity.

INTRODUCTION

The semiarid Caatinga region occupies the area between the Amazon (South of the equator) and the Atlantic rainforests. It covers 834,666 km^2 of Northeastern Brazil. A small number of crops is commercially cultivated in the Caatinga, and includes mainly beans, cassava, and corn. Some seed oil palms, such as oiticica (*Licania rigida*) and licuri (*Syagrus coronata*), are grown in this area, and theirs products have been industrialized. Unfortunately, the oil market of these plants has recently decreased followed a downward direction and low prices, a fact that has disappointed producers and decreased their cultivation in consequence. Other plants, such as the castor bean (*Ricinus communis*), cnidoscolus (*Cnidoscolus* sp.), and carnauba palm (*Copernicia prunifera*) were used as alternative plants choices for the biodiesel industry (Pinho et al., 2009). However, none of these species actually presents a viable source of material for biodiesel production (Oyagbemi et al., 2011).

The demand for vegetable oils as sources of raw material for biodiesel production has increased recently due to a number of factors, including increased petroleum price, which aims at reducing CO_2 emissions and fuel security. In addition, the demands on land, water and other resources that are required for the production of food for a growing-world population have increased (King et al., 2009). Thus,

the identification of other sources of oils from species that are adapted to semiarid regions of Brazil and other countries would make the production of biodiesel at competitive prices possible, allowing the sustainable production of biofuels in these areas using local raw materials (Oliveira et al., 2009).

Jatropha curcas L. belongs to the family *Euphorbiaceae* and thrives in many tropical and subtropical areas (King et al., 2009). There is little information available on the ecosystems where *J. curcas* naturally occurs (Behera et al., 2010). However, it is well-known that *J. curcas* is a drought-resistant plant (Zhang et al., 2007; Behera et al., 2010; Pompelli et al., 2010a), able to grow where most other crops cannot survive (Openshaw, 2000; Reubens et al., 2011). Its seeds contain 25 to 32% oil (Pompelli et al., 2010b), with yields of 1.5 tons of oil per hectare after five years of growth (Openshaw, 2000; Tiwari et al., 2007; King et al., 2009). Furthermore, *J. curcas* is well-adjusted to semiarid climate, although more humid environmental conditions result in better crop performance (Foidl et al., 1996; Openshaw, 2000; Tiwari et al., 2007; Maes et al., 2009; Achten et al., 2010; Behera et al., 2010; Pompelli et al., 2010a; Reubens et al., 2011). These promising characteristics of *J. curcas* have resulted in numerous plantation initiatives in the semiarid tropics (Behera et al., 2010). Although there are other sources of biodiesel, as a nonfood crop, *J. curcas* ranks first among all possible crops after considering the social, economic, and humanitarian aspects (Runge and Senauer, 2007; Achten et al., 2010; Behera et al., 2010; Reubens et al., 2011).

In areas with elevated evapotranspiration and reduced pluviometric indices, irrigation is frequently used in crop cultivation. This is the case of the Brazilian Northeastern semiarid Caatinga region (Pompelli et al., 2010b), where rainfall does not supply the water demands of most crops (300 to 1,200 mm y^{-1}). About 28% of all soils in Northeastern semiarid are affected by salts (Goes, 1978), and where the soil salt content is naturally high, the rainfall is unusually insufficient to leach the salt excess (Goes, 1978).

Drought and salinity are two major abiotic stresses that affect crop growth and yield in agricultural areas (Zhu, 2002), especially in tropical regions (Hernandez et al., 1995). Plants respond to salt through changes in several morphological, physiological, biochemical, and metabolic processes (Lee et al., 2001; Zhu, 2002). Salt stress can also trigger various interacting events, including inhibition of enzymatic activities in metabolic pathways, and decreased carbon-use efficiency and decomposition of proteinaceous and membrane structures (Hasegawa et al., 2000; Flagella et al., 2004; Garg and Singla, 2004).

Photosynthesis is one of the processes that is most affected by abiotic stress (Hasegawa et al., 2000). Reduced photosynthesis in salt-stressed plants may be caused by decreased CO_2 availability through increased resistance to CO_2 diffusion, from the atmosphere to the leaves or from the sub-stomatal cavity to carboxylation sites (Bernacchi et al., 2002). Photosynthetic rates are usually reduced in plants that are exposed to saline water, especially NaCl, but it is unclear whether these low rates are also responsible for the reduced growth observed in salt-treated plants. Moreover, there is no unified concept of the nonstomatal events that constrain photosynthesis. It is well-known that salt may result in impairments in photosystem II activity (Garg and Singla, 2004), chlorophyll (Chl) content (Sudhakar et al., 1997; Silva et al., 2010), and in the activities of key enzymes involved in the photosynthetic carbon reduction cycle (Guerrier, 1988).

Reactive oxygen species (ROS) occur naturally in most eukaryotic cells because energy metabolism depends on oxygen, though stresses enhance these molecules (Shigeoka et al., 2002). Due to high levels of phospholipid unsaturation in mitochondria and chloroplasts, these organelles are considered likely targets of damage induced by abiotic stresses (Nakano and Asada, 1981; Salin, 1991). In an attempt to alleviate such damage, plants developed two antioxidant systems, an enzymatic and a nonenzymatic one, to protect plant cells (Foyer et al., 1997; Polle, 1997). Two examples of the enzymatic scavenger system are catalase (CAT), (EC 1.11.1.6) and ascorbate peroxidase (APX) (EC 1.11.1.11). CAT is an enzyme present in all aerobic eukaryotes that participate in the dismutation of hydrogen peroxide (H_2O_2) into oxygen and water (Shigeoka et al., 2002). APX promotes dismutation of H_2O_2 and uses the oxidized ascorbate as electron acceptor, resulting in production of H_2O, O_2, and reduced ascorbate (Jin et al., 2006).

The objective of the present study was to evaluate the physiological and biochemical responses of *J. curcas* grown in various salt conditions. The relative importance of stomatal limitations to photosynthesis was determined in moderately salt-stressed plants. Therefore, we addressed the following questions: are *J. curcas* plant capable to resist irrigation supplemented by salt?; and do *J. curcas* plants present an antioxidative system capable of protecting plants against the salt stress?

MATERIALS AND METHODS

Plant material and experimental design: Experiments were conducted in a greenhouse located at Recife (8º02' S, 39º56' W, 15 m a.s.l) in Northeastern Brazil. Uniform *Jatropha curcas* L. seedlings with five leaf pairs each, obtained from seeds, were transplanted in February 2010 into 10 L pots, containing a mixture of soil and sand (2:1 v:v), in which they remained for five months. After this period, plants were irrigated daily with a 250 mL salt solution supplemented with 0, 0.7, 1.4, 2.1, 2.8, or 3.5 dS m^{-1} of electrical conductivity (EC) by 50 days. Hereafter, this will be referred to as T1 to T6 plants, respectively. Salt solutions was prepared with 1 M of NaCl, $CaCl_2$ 2 H_2O, and $MgCl_2$ 6 H_2O, considering the ratio 7:2:1 for Na:Ca:Mg described in Gurgel et al. (2003), as an approximation that represents the majority of available water sources for irrigating semiarid Northeastern Brazil. During the experimental period, maximum photosynthetic photon flux (PPF) reached more than 2,000 μmol m^{-2} s^{-1}. The youngest, fully expanded leaves corresponding to the third or fourth pair from the apex of the plagiotropic branches were sampled and measured in July, 2010. Throughout the experimental period, the plants were grown under naturally fluctuating conditions of temperature and relative humidity, which means that mean air temperature and air humidity were 29ºC and 66%, respectively (Figure 1). The pots were periodically randomized to minimize spatial variations within the greenhouse. A homogeneous group of 150-day-old plants was selected for analysis. The experiment used a completely randomized design with ten plants in individual pots per treatment combination as replicates. The experimental unit was considered to be one plant per container. The experiments were repeated three times with similar results each time.

Soil analysis: Every ten days, 10 g of soil were collected for all treatments, dried to a constant dry mass and combined with 25 mL of deionized water at 25ºC for 60 minutes. The supernatant solution was collected for pH and EC determinations, using pH and conductivity meter (conductivity meter model CD-4306, Lutron, Lutron Electronic Enterprise Co., Ltd., Taipei, Taiwan).

Photosynthetic measurements: Every two days, net carbon assimilation rate (P_N), stomatal conductance to water vapor (g_s), and transpiration rate (E) were measured with a portable open-flow gas exchange system (IRGA, LCI Pro+, ADC BioScientific Ltd, Hoddesdon, UK). Measurements were made from 0800 to 1000 h under 1,000 μmol photons m^{-2} s^{-1} of PAR provided to LED lamps and ambient CO_2 concentration (i.e 360 to 380 ppm). Water-use efficiency (WUE) was calculated as the P_N/E ratio. The leaf temperature (LT) was measured with the infrared gas analyzer (IRGA) during the gas exchange measurements.

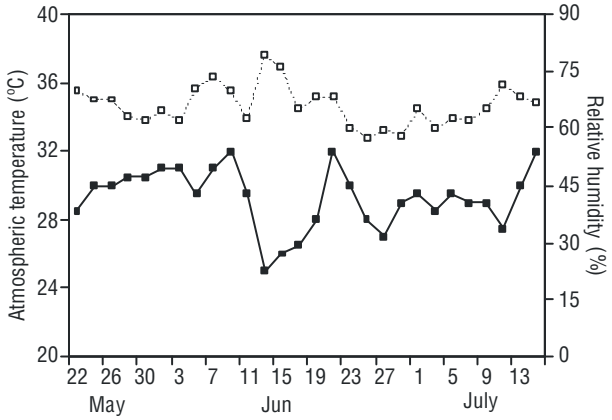

Figure 1. Time course of atmosphere temperature (closed symbols) and relative humidity (open symbols) from May to July 2010 at Recife, Northeastern Brazil.

Biochemical measurements: After 50 days of salt stress treatment, a fully expanded leaf from each plant was detached, immediately immersed in liquid nitrogen and stored in a freezer (-20ºC) for later use. The total soluble sugar content was determined using the phenol-sulfuric method (Dubois et al., 1956), and total free amino acids were measured using the ninhydrin reaction (Moore and Stein, 1954). The proline concentration was determined according to Bates et al. (1973), and the soluble protein concentration was determined using Bradford's (1976) method with bovine serum albumin (BSA) as a standard. Cellular damage was analyzed through H_2O_2 accumulation spectrophotometrically after a reaction with KI (Alexieva et al., 2001), malondialdehyde (MDA) accumulation, estimated as the amount of total 2-thiobarbituric acid-reactive compound (Cakmak and Horst, 1991); and electrolyte leakage, assayed immediately after leaf sampling by using the conductivity meter previously described (Alexieva et al., 2001). Key antioxidant enzymes, including APX (EC 1.11.1.11) and CAT (EC 1.11.1.6), were assayed exactly as described by Pompelli et al. (2010a). Chl and total carotenoids were extracted using 80% (v/v) aqueous acetone and quantified spectrophotometrically, according to Lichtenthaler, in 1987.

Leaf disks (each 1.3 cm in diameter) were collected with a cork borer from the same leaves used for P_N measurements and were used to determine the leaf relative water content (RWC), based on the method of Matin et al. (1989), with some modifications. Five disks per plant were collected immediately sealed in glass vials and quickly transported to the laboratory in an ice-cooled chest. Leaf disk fresh weights were determined after excision. Leaf turgid weights were obtained after immersion in deionized water for 24 hours at 25ºC. Then, the disks were quickly and carefully blotted dry with lint-free tissue paper before determining the turgid weights. Dry weights were recorded after drying the leaf samples in an oven for 48 hours at 80ºC (Matin et al., 1989).

Leaf area determination: Leaf areas were computed using an allometric method that was previously determined for this species (Pompelli et al., 2012).

Statistical analysis: The data were statistically analyzed using a fixed-model ANOVA, following a completely randomized design, and significant differences between treatments were determined using the Newman-Keuls' test at $p \leq 0.05$. Mean comparisons were performed using the Statistica 7.0 (StatSoft, Inc., Tulsa, OK, USA) program.

RESULTS

Soil pH and conductivity in all treatments during the experimental period are shown in Figure 2. Change in salt concentration in the soil did not significantly ($p \leq 0.05$) alter the soil pH, but the conductivity of the soil solution increased linearly with changes in salt concentration of the irrigation water.

The net carbon assimilation rate (P_N) decreased appreciably in all treatments in comparison to day 0. For instance, after 50 days of salt stress, it decreased by approximately 30, 57, 62, and 70% in T2, T3, T4, and T6 plants, respectively. Similarly, the stomatal conductance (g_s) and transpiration rate (E) decreased with increasing

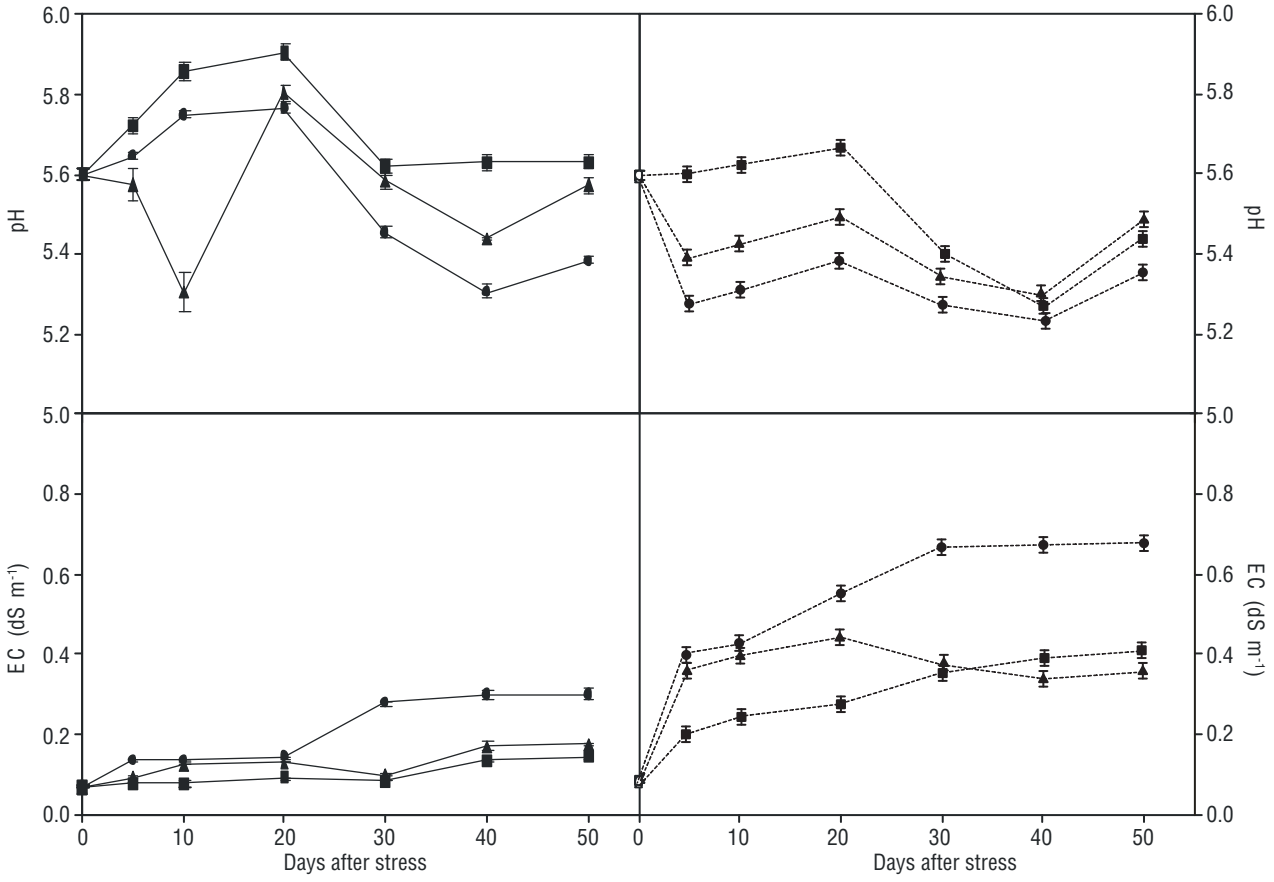

Figure 2. Effects of salt stress on soil pH and electrical conductivity (EC) on *Jatropha curcas* potted plants grown under 0 (■), 0.7 (▲), 1.4 (●), 2.1 (□), 2.8 (Δ) and 3.5 (○) dS m^{-1} of electrical conductivity in irrigation water. Data are expressed as means±standard error, n=10.

salinity. Compared to control plants, *E* values of the T2, T3, T4 and T6 plants decreased by 64, 73, 113, and 140%, respectively, after 50 days of salt stress (Table 1). The WUE change with increasing salt concentration, despite the weak influence on g_s (Table 1), is strong evidence that *J. curcas* has low WUE when irrigated with saline water.

The increase in salinity level has inverse relationship with leaf area (Figure 3). Thus, reductions occur in the area of energy capture and fixing of CO_2 per unit area (Table 1). The low rates of carbon assimilation were also caused by water deficits, inherent in osmotic stress, which may cause partial stomatal closure and decreases in g_s

Table 1. Effects of salt stress on the rate of net carbon assimilation (P_N), stomatal conductance (g_s), transpiration (E), and water use efficiency (WUE) in *Jatropha curcas* plants grown under 0 (T1), 0.7 (T2), 1.4 (T3), 2.1 (T4), 2.8 (T5), and 3.5 (T6) dS m^{-1} of electrical conductivity in irrigation water. Different capital letters denote significant differences between means for each parameters within each date, and different small letters denote significant differences for each parameter between means within each salt concentration ($p \leq 0.05$, Newman-Keuls' test). Data are expressed as means±standard error, n=10.

Treatment numbers	P_N (μmol CO_2 m^{-2} s^{-1})													
	Day 0		Day 5		Day 10		Day 20		Day 30		Day 40		Day 50	
T1	6.54±0.50	Ab	4.11±0.32	Ba	5.58±0.51	ABa	6.64±0.75	Aa	7.64±0.63	Aa	4.55±0.51	Ba	4.10±0.39	Ba
T2	6.07±0.58	Ab	2.78±0.41	Bab	5.03±0.36	Aab	5.17±0.27	Aa	5.14±0.55	Ab	3.07±0.27	Bab	2.88±0.29	Bab
T3	9.05±0.45	Aa	3.61±0.08	CDa	6.57±0.44	Ba	5.80±0.28	Ba	4.78±0.29	BCbc	2.59±0.35	Dab	1.78±0.18	Db
T4	7.35±0.82	Aab	1.21±0.21	Db	3.40±0.54	BCb	4.48±0.57	Bab	2.73±0.18	CDcd	3.63±0.29	BCab	1.94±0.21	CDb
T5	8.02±0.51	Aa	0.95±0.25	Db	1.47±0.38	Dc	5.14±0.54	Ba	3.43±0.45	Cc	1.99±0.26	Db	1.57±0.26	Db
T6	9.57±0.95	Aa	1.39±0.23	Cab	4.40±0.61	Bb	3.88±0.63	Bab	3.75±0.66	Bbc	2.08±0.36	Cb	1.26±0.21	Cb
Treatment numbers	g_s (mmol H_2O m^{-2} s^{-1})													
	Day 0		Day 5		Day 10		Day 20		Day 30		Day 40		Day 50	
T1	24.44±1.76	Ac	10.00±0.01	Cab	16.00±1.63	Ba	18.00±2.00	ABa	27.00±5.39	Aa	14.00±4.27	Ba	15.00±1.89	Ba
T2	26.67±1.67	Ac	12.50±1.64	Ba	16.25±1.83	ABa	14.44±1.76	ABab	21.00±5.47	Aa	13.75±3.24	ABa	7.14±2.86	Cb
T3	32.22±1.47	Aa	13.33±1.67	Ba	16.67±1.67	Ba	14.45±1.76	Bab	11.00±2.77	Cbc	7.50±2.50	Db	6.67±3.33	Db
T4	28.00±3.27	Ab	4.00±2.45	Cbc	10.00±2.58	Bbc	11.25±2.27	Bb	10.00±2.18	Bbc	8.89±2.61	Bb	6.00±4.00	BCb
T5	26.25±1.83	Abc	5.00±2.24	BCb	8.75±2.95	Bc	13.33±2.36	Bab	2.50±1.64	CDc	1.67±1.01	Dc	3.33±1.03	Cd
T6	34.29±2.02	Aa	2.00±1.95	Dc	12.50±1.64	Bb	10.00±2.67	Bb	14.00±4.00	Bb	14.29±6.49	Ba	5.00±1.05	Cc
Treatment numbers	E (mmol H_2O m^{-2} s^{-1})													
	Day 0		Day 5		Day 10		Day 20		Day 30		Day 40		Day 50	
T1	0.88±0.05	Bb	0.55±0.03	BCa	0.75±0.07	Ba	0.78±0.03	Ba	1.19±0.17	Aa	0.45±0.10	Ca	0.45±0.04	Ca
T2	0.88±0.06	Ab	0.40±0.07	BCa	0.65±0.05	ABab	0.63±0.05	ABa	0.68±0.13	Ab	0.33±0.11	Cab	0.16±0.07	Cb
T3	1.21±0.02	Aa	0.54±0.06	Ca	0.95±0.04	Ba	0.62±0.03	Ca	0.46±0.04	Cc	0.17±0.06	Dbc	0.12±0.08	Db
T4	1.12±0.06	Aab	0.03±0.02	Cb	0.32±0.08	Bcd	0.33±0.12	Bab	0.13±0.06	BCd	0.36±0.09	Bab	-0.06±0.06	Cbc
T5	1.15±0.04	Aab	0.07±0.07	Db	0.27±0.10	Cd	0.53±0.06	Ba	0.12±0.03	CDd	0.03±0.02	Dc	0.02±0.02	Dbc
T6	1.30±0.05	Aa	0.04±0.04	Cb	0.49±0.08	Bbc	0.35±0.08	Bab	0.08±0.08	Cd	0.01±0.05	Cc	-0.18±0.03	Cc
Treatment numbers	WUE (mmol mol^{-1})													
	Day 0		Day 5		Day 10		Day 20		Day 30		Day 40		Day 50	
T1	6.89±0.56	ABab	7.47±0.14	ABb	8.49±0.30	Aa	7.42±0.64	ABb	5.03±0.41	Bd	6.88±0.37	ABb	9.42±0.97	Ab
T2	7.07±0.29	Aa	6.57±0.24	Ab	7.05±0.38	Aa	7.62±0.30	Ab	6.53±0.36	Ad	7.86±0.60	Ab	6.25±0.38	Ac
T3	7.47±0.30	ABa	7.45±0.56	ABb	6.69±0.42	ABa	9.51±0.42	Ab	9.71±0.78	Abc	7.64±0.18	ABb	5.22±1.38	Bc
T4	6.43±0.60	Cb	10.63±0.99	BCa	8.80±1.43	Ca	12.05±0.38	Ba	10.86±1.49	Bb	11.76±0.34	Ba	16.26±1.29	Aa
T5	6.99±0.44	Ca	7.13±0.73	Cb	7.29±1.09	Ca	10.09±0.22	Bab	15.79±2.50	Aa	13.85±0.24	Aa	7.37±1.97	Cbc
T6	8.38±0.29	BCa	6.72±0.46	CDb	7.55±0.49	Ca	10.81±0.20	ABab	13.84±0.06	Aa	14.83±0.27	Aa	4.63±0.79	Dc

and *E* values (Table 1). Irrigation of *J. curcas* with NaCl induced leaf abscission and increased the LT (Figure 3). The negative correlation between EC and leaf area (Figure 3A) and the positive one between EC and LT suggest that salt affects stomatal conductance.

After irrigation with a large range of NaCl concentrations for 50 days, *J. curcas* displayed significant increases in amino acids and proline levels (Table 2). The highest amino acid and proline levels in leaf tissues were recorded in the most heavily stressed leaves, i.e., T5 and T6 leaves. The amino acid and proline levels were increased by 32 and 40%, respectively, in T5 plants compared to the nonstressed ones (Table 2). Conversely, the levels of total soluble sugars did not significantly differ between salt-stressed and untreated plants. The soluble protein content was differentially affected by the several salt treatments, resulting in a significant ($p \leq 0.05$) decrease in T2 to T4 leaves, but the other treatments produced no effects (Table 2). Of the organic solutes that were investigated in this study, the proline level was increased by the greatest amount with increasing salinity (Table 2). For instance, the proline level in leaves increased from 1.03 to 1.44 mmol kg^{-1} DW, when the salinity raised from 0 to 3.5 dS m^{-1}. Taken together, these results indicate that osmotic adjustment possibly occurs in leaves. However, the RWC was similar for all treatments (approximately 73%), even after 50 days of salt stress (Figure 4).

The changes in H_2O_2 and MDA content or electrolyte leakage in the leaves of *J. curcas* plants subjected to salt stress are shown in Table 3. The changes in H_2O_2, MDA and electrolyte leakage were proportional to the increases in stress intensity (salinity). Leaf electrolyte leakage, a membrane damage indicator, increased 44, 45, and 29% in T2, T4, and T6 leaves, respectively, while lipid peroxidation, measured by MDA accumulation, increased 42, 47, and 85%, respectively, compared to the control (T1) leaves. By the end of the study period (day 50), leaves from T6 plants exhibited a 36 mmol kg^{-1} DW and 260 mol kg^{-1} DW to H_2O_2 and MDA levels, representing 1.2- and 1.9-fold increases, respectively, compared to the control plants (Table 3).

Data analysis throughout the experimental period showed that plants subjected to salt stress experienced significant changes all the studied variables. For instance, a prominent increase in membrane damage was observed in parallel with decreases of CAT and APX activities (Table 4). The increased electrolyte leakage showed a positive correlation with H_2O_2 (r=0.446; $p \leq 0.01$) and MDA (r=0.361; $p \leq 0.05$) accumulation; however, a weak (i.e., nonsignificant) correlation

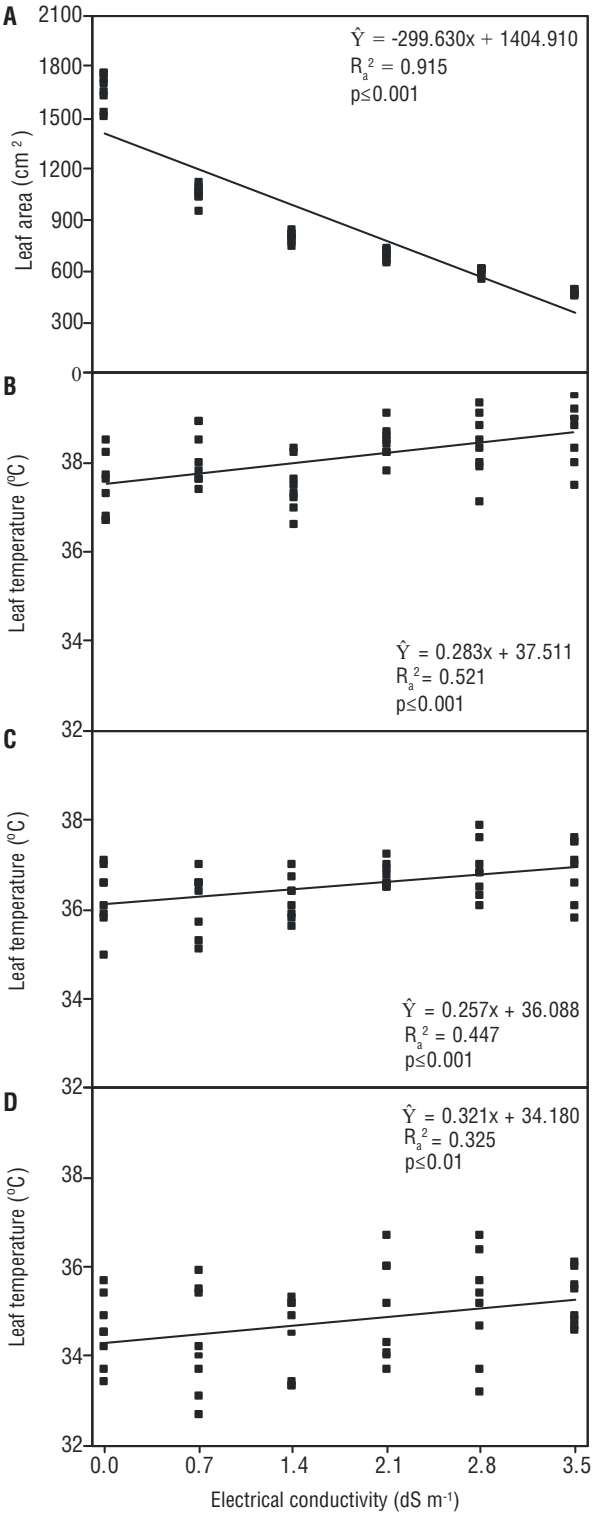

Figure 3. Electrical conductivity in relation to the leaf area (A) and leaf temperature in 5 (B), 30 (C), and 50 (D) days after salt stress in *Jatropha curcas* plants grown under 0, 0.7, 1.4, 2.1, 2.8 and 3.5 dS m^{-1} of electrical conductivity in irrigation water.

Table 2. The effects of salt stress on total soluble sugars, protein, amino acids, and proline in *Jatropha curcas* plants grown under 0 (T1), 0.7 (T2), 1.4 (T3), 2.1 (T4), 2.8 (T5), and 3.5 (T6) dS m^{-1} of electrical conductivity in irrigation water. Means followed by different letters in the column denote significant differences ($p \leq 0.05$, Newman-Keuls' test). Data are expressed as means±standard error, n=8.

Treatment numbers	Total soluble sugars (mmol kg^{-1} DW)		Soluble protein (g kg^{-1} DW)		Amino acids (mmol kg^{-1} DW)		Proline (mmol kg^{-1} DW)	
T1	852.62±60.45	ab	140.04±5.76	a	53.47±3.04	c	1.03±0.07	b
T2	799.19±37.72	ab	59.38±4.46	c	63.17±1.87	b	1.16±0.04	ab
T3	667.58±32.65	b	96.57±13.68	b	61.84±2.05	b	1.11±0.05	ab
T4	761.91±51.99	ab	104.51±9.27	b	64.04±2.01	b	1.11±0.02	ab
T5	757.80±35.16	ab	150.02±10.79	a	70.67±5.25	a	1.43±0.06	a
T6	1015.91±111.87	a	151.32±10.66	a	65.06±4.56	a	1.44±0.04	a

ns: non-significant.

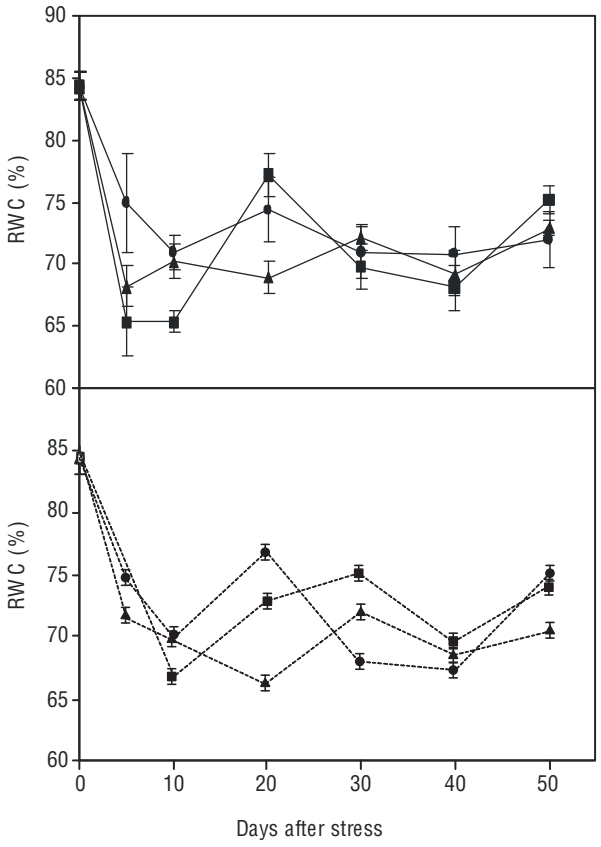

Figure 4. The effects of salt stress on the leaf relative water content (RWC) in *Jatropha curcas* plants grown under 0 (■), 0.7 (▲), 1.4 (●), 2.1 (□), 2.8 (Δ), and 3.5 (○) dS m^{-1} of electrical conductivity in irrigation water. Data are expressed as means±standard error, n=10.

Table 3. The effects of salt stress on hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and electrolyte leakage in *Jatropha curcas* plants grown under 0 (T1), 0.7 (T2), 1.4 (T3), 2.1 (T4), 2.8 (T5), and 3.5 (T6) dS m^{-1} of electrical conductivity in irrigation water. Means followed by different letters in the column denote significant differences ($p \leq 0.01$, Newman-Keuls' test). Data are expressed as means±standard error, n=8.

Trat No	H_2O_2 (mmol kg^{-1} DW)		MDA (μmol kg^{-1} DW)		Electrolyte leakage (%)	
T1	30.68±1.77	a	140.90±16.25	a	13.33±0.24	a
T2	39.25±3.54	b	199.96±7.17	b	19.15±1.27	b
T3	35.06±1.78	b	208.69±7.87	b	17.66±1.38	b
T4	39.83±4.41	b	206.51±8.61	b	19.36±0.74	b
T5	38.61±1.35	b	195.14±21.02	b	19.48±0.89	b
T6	36.23±0.84	b	260.40±39.94	c	17.14±0.68	b

was found between electrolyte leakage and CAT and APX activity levels, suggesting that the injuries induced in the plasmalemma by salt stress were, at least partly, a consequence of oxidative damage due to decreases in antioxidant enzymes activities. In T6 leaves, the activities of CAT and APX (expressed on a protein basis) decreased by 27 and 33%, respectively, after 50 days of stress compared to the enzyme activity of the control treatment. Together, these results indicate that the decrease in activity of antioxidant enzymes, leading to increased production of ROS, must have overwhelmed the capacity of salt stress adjustment of plants.

Table 4. The effects of salt stress on the activities of ascorbate peroxidase (APX) and catalase (CAT) in *Jatropha curcas* plants grown under 0 (T1), 0.7 (T2), 1.4 (T3), 2.1 (T4), 2.8 (T5), and 3.5 (T6) dS m^{-1} of electrical conductivity in irrigation water. Means followed by different letters in the column denote significant differences ($p \leq 0.05$, Newman-Keuls' test). Data are expressed as means±standard error, n=8.

Treatment numbers	CAT (U g^{-1} DW)*		APX (U g^{-1} DW)		CAT (U mg^{-1} protein)		APX (U mg^{-1} protein)	
T1	3.58±0.52	ns	122.89±6.21	a	0.11±0.01	ns	5.39±0.46	a
T2	3.29±0.26	ns	104.03±7.76	b	0.10±0.01	ns	4.33±0.53	b
T3	2.86±0.33	ns	119.02±3.79	a	0.10±0.01	ns	3.53±0.33	b
T4	3.75±0.47	ns	116.42±5.99	a	0.09±0.02	ns	3.67±0.75	b
T5	2.63±0.46	ns	120.65±8.65	a	0.12±0.01	ns	4.97±0.61	ab
T6	3.87±0.51	ns	100.69±2.85	b	0.08±0.01	ns	3.59±0.19	b

ns: non-significant. *One unit of CAT or APX was defined as the amount of enzyme required to oxidize 1 μmol of H_2O_2 or ascorbate per minute.

In this study, the irrigation of *J. curcas* plants with NaCl caused a significant ($p \leq 0.05$) decrease in total Chl (a+b), as seen in Table 5. In T6 leaves, the total Chl level was 3.10 g kg^{-1} DW, a decrease of 23% compared to control plants. Carotenoids, however, were not affected by salt stress; consequently, the Chl-to-carotenoids ratio was decreased in all treatments. We have showed that in these plants salinity led to Chl damage, which could ultimately result in leaf yellowing and abscission (Figure 3). Although changes in the Chl content were observed in response to salt stress (Table 5), they were not associated with a change in Chl quality, as evidenced by an average Chl a/b ratio (data not shown).

DISCUSSION

The P_N, g_s and E were negligible in salt-stressed leaves (Table 1), probably as a consequence of an ionic imbalance due to excess Na^+ and Cl^- in cells, as reported for *Atriplex nummularia*, which is a halophyte plant (Silveira et al., 2009). Physiological data indicate that Na^+ competes with K^+ for intracellular influx because these cations are transported by common proteins (Silveira et al., 2009). Whereas K^+ is an essential cofactor for many enzymes, Na^+ is not (Hasegawa et al., 2000). Similarly, the K^+ ion plays a central role in turgor maintenance and in the control of stomata opening control in plants that are under physiological or stress conditions (Finkelstein, 2010). Movement of salt into the roots and, consequently, into the shoots is a result of the transpirational flux, which is required to maintain the water status of the plant. When it is unregulated, transpiration can result in toxic levels of ion accumulation in shoots. An immediate response to salinity, which mitigates ion flux to the shoot, is a stomatal closure (Zhu, 2002; Garg and Singla, 2004; Praxedes et al., 2010), which can negatively affect photosynthesis (Praxedes et al., 2010). The water stress induced by salt accumulation promotes the lowering of the osmotic potential of the xylem and, consequently, the water potential that limits water absorption by plants. Plants subjected to osmotic stress, therefore, exhibit a rapid increase in stomatal resistance (Zhu, 2002). It is noteworthy that *Prosopis juliflora*, another species found in dry areas, has a high WUE, even when irrigated with saline water (Tomar et al., 2003), at a level that is generally 2.5 times higher than those found in *J. curcas* plants.

The data presented in Table 1 are consistent by the results obtained by Garg and Singla (2004) or Netondo et al.(2004) with chickpea or sorghum plants. These authors report that stomatal closure is a prime constraint to photosynthesis by limiting CO_2 flux into the leaves of salt-stressed plants. However, these findings contrast with the interpretations of Praxedes et al. (2010), who found no significant effects of salt stress on g_s, the transpiration rate or net photosynthesis in cowpea plants. Because of stomatal closure, CO_2 fixation is low while photosynthetic electron transport operates at a normal rate (Allen, 2005).

Table 5. Effects of salt stress on chlorophyll (a + b), carotenoids as well as on the ratio of Chl/Car in *Jatropha curcas* plants grown under 0 (T1), 0.7 (T2), 1.4 (T3), 2.1 (T4), 2.8 (T5), and 3.5 (T6) dS m^{-1} of electrical conductivity in irrigation water. Means followed by different letters in the column denote significant differences ($p \leq 0.05$, Newman-Keuls' test). Data are expressed as means±standard error, n=8.

Treatment numbers	Chlorophyll a + b (g kg^{-1} DW)		Carotenoids (g kg^{-1} DW)		Chlorophyll/ Carotenoids	
T1	4.03±0.19	ab	0.73±0.02	ns	5.48±0.23	a
T2	4.24±0.26	a	0.85±0.04	ns	5.11±0.25	b
T3	4.13±0.26	a	0.80±0.05	ns	4.79±0.15	b
T4	3.34±0.07	c	0.81±0.05	ns	4.03±0.23	c
T5	3.16±0.19	c	0.77±0.01	ns	3.99±0.29	c
T6	3.10±0.11	c	0.70±0.07	ns	4.10±0.34	bc

ns: non-significant.

Under these conditions, limited quantities of $NADP^+$ are available to accept electrons, so oxygen can function as an alternative electron acceptor (Egneus et al., 1975). Although this pseudocyclic pathway for electron transport provides additional ATP, it can result in the production of superoxide and H_2O_2 in cells.

The increase in LT is probably due to reduced evapotranspirational cooling, a result from drought-induced stomatal closure after salt stress. As stomata close in response to a water deficit, transpirational cooling ceases, leading to an increase in the LT (Jones, 2004; Long et al., 2006). While this physiological response to increasing water stress can help at preventing the development of lethal water deficits, it can also lead to lethal temperatures in warm and sunny conditions. The relatively lower LT of tolerant genotypes results from mechanisms that maintain a more favorable leaf water status and, hence, more open stomata and sustained transpirational cooling. Therefore, CO_2 influx toward chloroplasts may be sustained for longer periods, allowing greater photosynthetic rates and ultimately greater crop yields (Flagella et al., 2004). Thus, these results suggest that *J. curcas* has lower capacity to withstand irrigation with saline water, in contrast to cowpea genotypes (Praxedes et al., 2010).

One, probably universal, response to changes in the external osmotic potential is the accumulation of metabolites that act as compatible (Gillham and Dodge, 1986). With accumulation that is proportional to the change in external osmolarity within species-specific limits, the protection of structures and osmotic balance supporting continued water influx (or reduced efflux) are well-known functions of osmolytes. Their accumulation is thought to facilitate osmotic adjustment, by which the internal osmotic potential is reduced and may then contribute to salt and drought tolerance (McCue and Hanson, 1990; Delauney and Verma, 1993; Hasegawa et al., 2000; Chimenti et al., 2002; Zhu, 2002; HongBo et al., 2006; Yazici et al., 2007; Silveira et al., 2009). Compatible solutes are typically hydrophilic, which suggests that they could replace water at the surface of proteins, protein complexes or membranes, thus acting as osmoprotectants and, nonenzymatically, as low-molecular-weight chaperones. Compatible solutes at high concentrations can reduce the inhibitory effects of ions on enzyme activity and increase the thermal stability of enzymes and prevent the dissociation of enzyme complexes, e.g., the oxygen-evolving complex of PSII (Hasegawa et al., 2000).

Furthermore, the enzymes required for pathway extensions that lead to these osmolytes are often induced following salt and drought stresses. Proline accumulation provides an example. Under stress conditions, the imbalance between photosynthetic light capture and NADPH utilization in carbon fixation may alter the redox state and lead to photo-inhibition. Proline synthesis, which follows the transcriptional activation of NAD(P)H-dependent P5C-synthetase (P5CS), could provide an adaptive response whereby the regeneration of $NAD(P)^+$ could account for the observed protective effect (Delauney and Verma, 1993; Hasegawa et al., 2000; HongBo et al., 2006).

In this study, we showed that *J. curcas* plants exposed to NaCl had decreased protein levels and elevated ones of total soluble sugars and amino acids (Table 2). A decrease in protein synthesis in salt-stressed plants may simply be an outcome of decreased amino acid utilization in protein metabolism associated with the beginning of the salt-stress induced senescence process (Figure 3A). T6 leaves accumulated high levels of proline compared to control plants (Table 2). There is circumstantial evidence that salt stress-induced proline synthesis is an adaptive response since it may function as a nontoxic osmolyte, an osmoprotectant that occurs primarily in the cytoplasm, where it acts as an enzyme protectant (Delauney and Verma, 1993). However, there is evidence that proline accumulation is a symptom of salt stress-induced metabolic disorders rather than being involved in its alleviation (Viégas and Silveira, 1999; Silveira et al., 2009). As the accumulation of total soluble sugar, amino acids and proline did not affect the RWC in the leaves (Figure 4), we believe that *J. curcas* plants are strongly affected by the accumulation of salts, a fact that is supported by high levels of H_2O_2 and MDA synthesis (Table 3).

In fact, RWC is a key indicator of the degree of cell and tissue hydration, which is crucial for optimum physiological functioning and growth processes (Jamaux et al., 1997). In the present study, we showed that H_2O_2 levels increased approximately 30% in T4 and T5 leaves after a 50-day exposure of salt stress (Table 3). Our results are consistent with those of Hernandez et al. (1995), who reported an increased level of H_2O_2 in pea chloroplasts subjected to NaCl stress. However, the extent of the increase in H_2O_2 in the *J. curcas* plants in the present study was lower than the one found after short-term (3 d) exposure to 150 mmol L^{-1} NaCl in rice (Lee et al., 2001). MDA, which is a product of lipid peroxidation in plants that are exposed to adverse environmental conditions, is a reliable indicator of free radical formation in tissues. We note that in *J. curcas* plants grown in salt stress conditions, the MDA concentration was substantially increased (Table 3). For example, in T6 leaves, the MDA concentration was 1.9-fold higher than in control plants.

This finding suggests that oxidative stress is clearly established in the leaves of all salt-stress *J. curcas* plants, as indicated by increased MDA levels and lipid peroxidation. These findings were previously reported in other cultivated species (Dionisiosese and Tobita, 1998; Sudhakar et al., 2001). However, the present results are not consistent with the findings of Hernandez et al. (1995) in salt-tolerant pea plants. The excess H_2O_2 produced in peroxisomes and chloroplasts might diffuse to the cytosol and be converted to hydroxyl radicals via Fenton reaction (Moller et al., 2007), exacerbating lipid peroxidation (Noctor et al., 2002; Hernandez et al., 1995).

Under normal conditions, ROS are effectively scavenged by antioxidant systems. However, when plants are subjected to environmental stresses involving salinity, ROS production overcomes the antioxidant system capacity, and oxidative stress occurs, resulting in cytotoxic protein damage, DNA damage, and lipid peroxidation (Yazici et al., 2007). Decreases in the activities of the protective enzymes CAT and mainly APX were closely correlated with MDA accumulation, interacting as both cause and effect. On one hand, because of the reduced activities of these enzymes, free radicals, as H_2O_2 (Table 3), can accumulate and even exceed the injury threshold. On the other hand, MDA accumulation inhibits the activities of these enzymes (negative correlation, r=-0.259 to CAT and r=-0.526 to APX; $p \leq 0.05$), so their protective functions are lost, enhancing membrane injury. This finding shows that the ability of *J. curcas* plants to resist salt stress is associated with the activities of protective enzymes and their defensive functions. Miyake and Asada (1996) reported that low levels of H_2O_2 (2 μM) inactivate chlAPX within several seconds, when the level of ascorbic acid is too low for the APX catalytic cycle to operate. This phenomenon was previously reported in purslane (Yazici et al., 2007), jatropha (Silva et al., 2010), rice (Lee et al., 2001), pea (Moran et al., 1994), bean (Jebara et al., 2005), and the mangrove *Bruguiera parviflora* (Parida et al., 2004). CAT deactivation by salt stress may be due to the prevention of new enzyme synthesis (Feierabend et al., 1992) or CAT photo-inactivation (Polle, 1997).

Under severe stress conditions, CAT may function to remove H_2O_2 instead of APX to protect the stromal enzymes and photosynthetic apparatus in thylakoid membranes from oxidative stress. The activities of CAT were decreased at all concentrations of NaCl (Table 4), possibly due to the increase in H_2O_2 levels, as seen in Table 3 (Lee et al., 2001). Feierabend et al. (1992) have shown that under stress conditions inactivation of CAT is linked to H_2O_2 accumulation. Loss of CAT activity due to salt stress in cotton (Gossett et al., 1994), sunflower (Santos et al., 2001), and rice (Lee et al., 2001), as well as due to photo-oxidative stress in tobacco chloroplasts (Miyagawa et al., 2000), is consistent with our results for *J. curcas*. The inhibition of CAT and APX activities by salt stress may be a consequence of downregulated gene expression or degradation, denaturation and/or inhibition/inactivation of these proteins (Feierabend et al., 1992; Lee et al., 2001). Still, ongoing protein synthesis is required to maintain enzyme activity under conditions in which degradation exceeds re-synthesis and enzyme activity otherwise decreases (Cavalcanti et al., 2004). As shown in Table 2, the protein level was decreased as a result of salt stress. Downregulation of metabolic pathways would cause a substantial decline in photosynthesis because the antioxidant enzyme system would be swamped with a massive accumulation of H_2O_2. H_2O_2 production in the present study was approximately ten times higher than that reported for coffee plants submitted to high light or nitrogen deficiency conditions (Pompelli et al., 2010c), and approximately 100 times higher than the one reported for *Bruguiera parviflora*, a mangrove plant, which was subjected to salt treatment (Parida et al., 2004).

Chl is one of the first molecules to be negatively affected by water stress (Sarijeva et al., 2007; Pompelli et al., 2010a, b; Silva et al., 2010). The inhibitory effects of salt on Chl could be due to the suppression of specific enzymes, which are responsible for the synthesis of green pigments, an effect that depends on the biological processes and developmental stages of the plant and on the type and concentration of salt. The decrease in Chl may be attributed to increased chlorophyllase activity (Sudhakar et al., 1997). Several investigators have shown that Rubisco activity in leaves is inhibited by salinity, similar to the degradation of leaf Chl (Garg and Singla, 2004). The decrease in Rubisco activity caused by salt exposure may be attributed to the sensitivity of this enzyme to chloride ions (Seeman and Chritchley, 1985). If Chl could be degraded as a consequence of stress, then one possible cause of the decline in P_N could be a consequence of Chl photo-bleaching (Long et al., 1994).

These results revealed that salt stress can induce major changes in key physiological processes of *J. curcas* plants, as indicated by measurements of leaf gas exchange, metabolic pathways, osmoprotectors, membrane integrity, Chl and carotenoid contents, oxidative damage indicators, and the ROS-scavenging system. Our results indicate that the ROS-scavenging system is not enough to protect *J. curcas* leaves against oxidative damage caused by salt stress. As tolerant plants generally respond to abiotic stress, by increasing their antioxidant capacity to restore normal cellular equilibrium between productions and

scavenging of ROS, these data suggest that *J. curcas* is not tolerant to salt stress as it is to water deficit (Pompelli et al., 2010a). Then, the ability of *J. curcas* to produce ecologically and socioeconomically viable amounts of energy in barren/sodic land requires further exploration.

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