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Plasticity of photosynthetic metabolism in *Jatropha curcas* genotypes under water deficit

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ABSTRACT. With the advent of the Brazilian Government Biodiesel Program and the increasing demand for vegetable oils, *Jatropha curcas* has been emphasized as an alternative raw material. Given the known water stress tolerance of this species, we evaluated the plasticity of photosynthetic metabolism in nine genotypes of *J. curcas* under water stress (WS). The hypotheses that (1) the photosynthetic metabolism of *J. curcas* is plastic and changes towards crassulacean acid metabolism (CAM) according to water availability and (2) the plasticity of the metabolic alteration is variable among the different genotypes and is related to physiological adjustments, were tested. Water deficit led to 167 and 187% increase in phosphoenolpyruvate carboxylase (PEPC) activity in leaves collected at 4 and 16 h,, respectively, when compared to control plants. The accumulation of organic acids (malate and citrate) differed among genotypes and WS treatments. There was an average increase of 270% for plants submitted to WS, when citrate was compared to malate. There was an increase of 62% in total soluble sugar content and a decrease of 27% in starch content in plants under

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WS when compared to controls. Significant effects of genotype and watering regime were detected for carbon isotope composition of leaf biomass. We detected changes in photosynthetic metabolism towards low CAM levels, thus explaining the maintenance of efficiency of water use under water deficit.

Key words: Abiotic stress; Carbon isotopes; Euphorbiaceae; Organic acids

INTRODUCTION

Jatropha curcas (Euphorbiaceae), popularly known as physic nut is widely cultivated in many tropical and subtropical regions, being considered a promising crop for the production of biodiesel (Eijck et al., 2014). The oil content in the seeds can reach 50%; this oil has been used as a purgative andmany other medicinal uses, as well as in the manufacture of paints, soaps, as a lubricant and diesel fuel (Kumar and Sharma, 2008). Moreover, the residue of the oil extraction has a high protein content (60-65%), with potential to be transformed into food for birds, ruminants and fish (Jongschaap et al., 2007). Also, the cultivation of *J. curcas* offers the advantages of not competing directly with food production, of mitigating soil degradation and of recovering marginal or abandoned land (Kumar and Sharma, 2008). Although *J. curcas* has been widely reported as a drought-tolerant species, recent results have demonstrated negative effects of water deficit on several physiological and morphological characteristics (Arcoverde et al., 2011; Sapeta et al., 2013; Oliveira et al., 2016; Santana et al., 2015; Silva et al., 2016).

One well studied strategy for tolerance to drought and other factors such as salinity, temperature and luminosity, which has been reported to occur in green succulent-stem species is the transition from C_3 photosynthetic metabolism to crassulacean acid metabolism (CAM) under water deficiency conditions (Cushman and Borland, 2002; Dodd et al., 2002). Such metabolic plasticity would result in water conservation in arid environments as have been reported for several species, including some of the family Euphorbiaceae (Cushman and Borland, 2002; Dodd et al., 2002; Herrera, 2013).

CAM is also characterized by an intense daily carbon flow from carbohydrates, such as starch and sucrose for the synthesis of organic acids such as malate and citrate acids, which are stored during the dark period for use during the light period (Freschi et al., 2010a). There have been reports suggesting that, as in other species with green succulent stems, there is a low level of drought-induced CAM in *J. curcas* leaves (Maes et al., 2009; Rajaona et al., 2013). This hypothesis has been supported by recent results showing increased nocturnal acidification of foliar extracts in water deficit as compared to well-watered plants, as well as changes in the night leaf gas exchange diel (Winter and Holtum, 2015) in *J. curcas*. Although it has been suggested that the low level of CAM observed in *J. curcas* is related to stem carbon (Winter and Holtum, 2015) conservation, the implications of such metabolic plasticity are still under debate.

Carbon isotopic composition ($\delta^{13}C$) analysis has proven to be a valuable technique to be used both in long-term (seasonal) and daily carbon changes acquired directly through dark CAM reactions. The low percentage of ^{13}C in phosphoenolpyruvate carboxylase (PEPC) distinguishes the carbon in the plant material that is fixed by the reactions of the

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dark CAM from that fixed directly from atmospheric CO_2 by C3 metabolism (RUBISCO) (Griffiths, 1992).

We examined the amplitude of C3-CAM change induced by drought in leaves of different genotypes of *J. curcas* by means of the evaluation of overnight acidification of leaves tissues, as well as of physiological and growth changes. The hypotheses that (1) the photosynthetic metabolism of *J. curcas* is plastic and changes towards the CAM according to water availability and (2) the plasticity of the metabolic alteration is variable among the different genotypes and is related to physiological adjustments, were tested.

MATERIAL AND METHODS

Plant material and growth conditions

The experiment was conducted in a greenhouse on the campus of the State University of Santa Cruz (UESC), located in the city of Ilhéus, BA (14°47'00" S, 39°02'00" W). During the experiment, the photosynthetically active radiation, temperature and relative humidity of the air were monitored and recorded using, respectively, a quantum S-LIA-M003 and temperature/relative humidity S-THB-M002 sensors, connected to a HOBO Micro Station Data Logger H21-002 (Onset Computer Corporation, USA). Nine genotypes collected from different regions of Brazil (Table 1) to form the Germplasm bank of EMBRAPA were used. Seeds of *J. curcas* from the germplasm bank of Embrapa Agroenergia - Federal District were germinated in pots (five per pot), containing 12 dm³ of soil whose fertilization was carried out according to the chemical analysis.

Table 1. *Jatropha curcas* genotypes used in the experiment with the respective regions where they were collected to be included in the Germplasm Bank of EMBRAPA Agroenergia, DF, Brazil.

Genotypes	Region
121	Bom Jardim-RJ
124	Maranhão-MA
148	Candeias-BA
168	Minas Gerais-MG
222	Paraná-PR
215	São Francisco do Glória-MG
226	Água de Santa Bárbara-SP
298	Sidrolândia-MS
299	Rio Grande do Sul-RS

After 20 days of germination, only one plant was left per pot. The pots were covered with aluminum foil to avoid evaporation and heating of the soil and the water deficiency treatment was started and maintained for a period of 42 days.

The treatments consisted of two watering regimes measured as a percentage of the tank capacity (TC): control plants (100% TC) and plants submitted to water deficiency (50% TC). The plants were watered daily, always at 8 o'clock in the morning, at intervals of 24 h, with a water volume defined by the watering regime. The irrigation schedule led to soil matrix potential varying from -33.1 to -15.2 kPa (control) and from -207.0 and -89.9 kPa (water deficit). The soil moisture was determined by the gravimetric method and the soil matrix potential was estimated from the characteristic soil water retention curve.

Growth

All evaluations were performed 42 days after treatment (DAT). Plant growth was accessed by measuring height, diameter at collar, number of leaves and leaf area. Leaf area was estimated from values of length (P) and maximum width (L) of the leaves (Pompelli et al., 2012). Then, the aerial part (leaves and stem) and the roots were collected and cleaned. The roots were separated from the soil by dispersion with running water, using a 0.5mm mesh sieve. Subsequently, root images were captured with a digital camera (Sony DSC-H400). The images were saved in TIFF format and the *WinRHIZO* commercial software package was used for estimation of volume, length, total area and surface area of roots. Specific leaf area (SLA, the ratio between leaf area and dry mass) were estimated from five leaf discs (5 mm diameter) collected from the third completely mature leaf.

Leaf water potential

Leaf water potential (Ψpd) was measured before dawn (4:00 a.m.) and at noon (Ψmd). For this purpose, we used a pressure chamber PMS1000 (PMS Instrument Company, USA).

Water consumption and water use efficiency of biomass (WUE_b)

Water consumption in both treatments during the 62 days of the experiment was monitored through periodic weighing of the pots using load cells CSA/ZL e 100 (MK Control Instruments, Brazil) coupled to an automatic data collector model CR1000 (Campbell Scientific Inc., USA). Based on the biomass and water consumption data, water use efficiency of biomass was calculated as the ratio between the total dry biomass and the water consumed during the experiment, which was calculated from the daily replenishment of water in each treatment due to evapotranspiration.

Activity of PEPC

At the end of the experiment (42 DAT), leaf (4 h and 16 h) and stem (base and apex) samples were collected, immediately frozen in liquid nitrogen and stored in a freezer at -80°C. Protein extraction was performed on macerated leaf and stem samples, using a mortar and a cold pistil. After centrifugation at 14000 x g for 30 min, the supernatant was collected and used for the enzymatic activity assays. The amount of protein in the extract was determined by the Bradford (1976) method, the readings were performed using a microplate spectrophotometer (SpectraMax® Paradigm® - Multi-mode detection platform, Molecular Devices).

Approximately 0.02 g of lyophilized material (leaf or stem samples) was macerated in extraction medium containing 400 µL of 2 mM EDTA buffer, 5 mM β-mercaptoethanol, 1mM PMSF and 1% PVP-40 dissolved in 50 mM Tris-HCl pH 7.8. The extract was ultrasonicated (Ultrasonic processor Gex 130, 130 W) on ice until total tissue rupture, with pulses of 8 s at intervals of 10 s, and amplitude of 70%. The samples were then centrifuged for 15 min at 14,000 x g at 4°C. The activity of PEPC was measured in the supernatant by NADH extinction-induced decrease of absorbance at 340 nm to 300 s (Degl'innocenti et al.,

2002). The assay was performed in a reaction mixture containing 10 mM $NaHCO_3$, 0.3 mM NADH, 5 mM $MgCl_2$ and 33 mM and MDH 33 nkat dissolved in 50 mM Tris-HCl pH 7.8 buffer and enzymatic extract. The reaction was initiated by the addition of 20 mM phosphoenolpyruvate (PEP).

Titratable acidity

The quantification of the titratable acidity was performed in fresh leaf discs (5 mm). Three leaf discs were boiled in 10 mL of deionized water for 15 min, cooled and titrated with NaOH 0.01N after addition of 30 µL of phenolphthalein to estimate the H^+ corresponding to malate (pH 7.0) and 30 µL of bromothymol blue for citrate (pH 8.4).

Total soluble sugars and starch

The quantification of total soluble sugars (TSS) was performed by the anthrone reaction according to the methodology of Clegg et al. (1956). Readings of absorbance at 620 nm were obtained using a spectrophotometer (SpectraMAx® Paradigm® - Multi-mode detection platform, Molecular Devices), using as standard glucose and water mixture as blank.

For the starch determination, two consecutive extractions of the material were carried out, using the same extractor, at 80°C for 30 min. The quantification of starch was carried out by reaction with anthrone (0.2%), according to the method of McCready et al. (1950), and the samples were read in a spectrophotometer at a wavelength of 620 nm, using a glucose solution of 0 to 50 µg.mL^{-1} as a standard.

Carbon isotopic composition ($\delta^{13}C$)

Leaf and stem samples were dried and ground, and approximately 1 mg of the fine powder was packed into 3 mm x 35 mm tin capsules (Experimental Microanalysis Ltd., Okehampton, United Kingdom). The samples were then sent for analysis at Godwin Laboratory at the University of Cambridge, UK. The readings were obtained from a Costech elemental analyzer coupled to a mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) and the analyses were performed using linear $^{13}C/^{12}C$ two-point normalization software (Paul et al., 2007). Weighed samples of standards were analyzed at various points throughout the run allowing percentage carbon to be calculated for the batch of samples. Reference standards from IAEA in Vienna are also run at intervals throughout the sequence and these values are used to calibrate to the international standards for $^{12}C/^{13}C$ ($\delta^{13}C$ VPDB). Precision of analyses was +/- 0.5% for C, better than 0.1‰ for $^{12}C/^{13}C$

Experimental design and statistical analysis

The experiment was conducted in a completely randomized design, in a 2 x 9 factorial scheme, consisting of two levels of water availability and nine genotypes of *J. curcas*, with five replicates per treatment. The results were submitted to a 5% significance test, by factorial ANOVA and when indicated, average comparisons were carried out by

means of the Scott-Knott test at the same level of significance. The analyses were carried out with the aid of the SISVAR program (Ferreira, 2011).

RESULTS

The average values of minimum and maximum air temperature and of relative humidity measured during the experimental period were 20, 29°C and 75%, respectively. Photosynthetically active radiation integrated throughout the day was, on average, 12.1 mol photons m^{-2} day^{-1}, varying between 5.4 to 19.3 mol photons m^{-2} day^{-1}.

Growth

Except diameter of stem at collar, there were no significant differences among genotypes for growth variables. Diameter at collar was not decreased by water deficit only in genotype CNPAE 299 (Table 2). Moreover, water deficit led to a significant decrease of LA in genotypes CNPAE 124 (62%), 148 (61%), 215 (60%), 226 (55%) and 299 (53%). However, WS affected height across all genotypes, with average reductions of 74% when compared to the control plants. A significant decrease in leaf number was observed only in genotype CNPAE 215 (Table 2).

Table 2. Leaf area (LA), height (H), diameter at collar (D) and number of leaves (NL) of young plants of *Jatropha curcas* grown under full irrigation (control) or water stress (WS) for 42 days.

Genotypes		LA (cm²)	H (cm)	D (cm)	NL
121	Control	2515.8±214.8^{Aa}	50.8±2.2^{Aa}	2.44±0.0^{Aa}	18.6±0.8^{Aa}
	WS	1711.4±144.3^{Aa}	39.6±3.2^{Ab}	2.18±0.0^{Ab}	17.0±0.5^{Aa}
124	Control	2814.9±266.9^{Aa}	48.0±1.7^{Aa}	2.44±0.0^{Aa}	19.4±1.2^{Aa}
	WS	1742.5±160.3^{Ab}	33.8±2.9^{Ab}	2.04±0.1^{Ab}	14.8±0.8^{Aa}
148	Control	2723.4±604.9^{Aa}	51.0±4.6^{Aa}	2.32±0.0^{Aa}	20.4±1.1^{Aa}
	WS	1647.9±215.3^{Ab}	36.2±4.0^{Ab}	1.94±0.1^{Bb}	15.4±1.0^{Aa}
168	Control	2701.8±197.4^{Aa}	50.6±2.9^{Aa}	2.50±0.0^{Aa}	18.6±0.6^{Aa}
	WS	2141.2±188.3^{Aa}	40.4±3.0^{Ab}	2.25±0.0^{Ab}	16.0±0.7^{Aa}
215	Control	3313.8±578.7^{Aa}	54.8±3.9^{Aa}	2.44±0.1^{Aa}	26.0±5.1^{Aa}
	WS	1996.1±90.4^{Ab}	42.0±1.1^{Ab}	2.06±0.0^{Ab}	17.6±1.6^{Ab}
222	Control	2621.4±309.6^{Aa}	48.0±2.3^{Aa}	2.41±0.0^{Aa}	20.2±2.7^{Aa}
	WS	1819.4±132.3^{Aa}	37.4±2.0^{Ab}	2.06±0.0^{Ab}	16.2±1.0^{Aa}
226	Control	2504.1±299.2^{Aa}	48.7±4.5^{Aa}	2.33±0.1^{Aa}	21.2±3.3^{Aa}
	WS	1378.1±139.0^{Ab}	31.4±3.4^{Ab}	1.90±0.0^{Bb}	15.8±1.4^{Aa}
298	Control	2255.6±69.7^{Aa}	50.8±1.2^{Aa}	2.29±0.0^{Aa}	18.0±0.4^{Aa}
	WS	1783.8±228.4^{Aa}	34.5±2.9^{Ab}	1.80±0.0^{Bb}	16.4±1.3^{Aa}
299	Control	3422.5±495.9^{Aa}	47.4±2.3^{Aa}	2.24±0.1^{Aa}	20.4±2.7^{Aa}
	WS	1806.7±226.1^{Ab}	38.6±2.0^{Ab}	2.13±0.1^{Aa}	17.4±2.1^{Aa}

Values are mean (± standard error) of 5 replicates. Differences in lower case letters indicates differences between treatments within each genotype by the F-test and capital letters indicate significant differences among genotypes within each treatment by the Scott-Knott test.

While root volume of irrigated plants was significantly greater in genotypes 121 and 124 as compared to others, water deficit led to a significant decrease of volume in all genotypes (Figure 1A). No effect of genotype was observed in root length of irrigated plants, except in genotype CNPAE 222; a significant decrease of length was observed in WS plants of the other genotypes (Figure 1B). Significant decreases of 74.7, 75.2 and 53%

were observed in total root area of WS plants of, respectively, genotypes CNPAE 148, 226 and 299 when compared to their controls (Figure 1C). In addition, higher values of total root area were observed in irrigated plants of these same genotypes. No differences were found among genotypes for root surface area (Figure 1D). However, significant decreases of 28.5, 28.0 and 25.0% were detected in WS plants of genotypes CNPAE 124, 148 and 226, respectively, as compared to their controls.

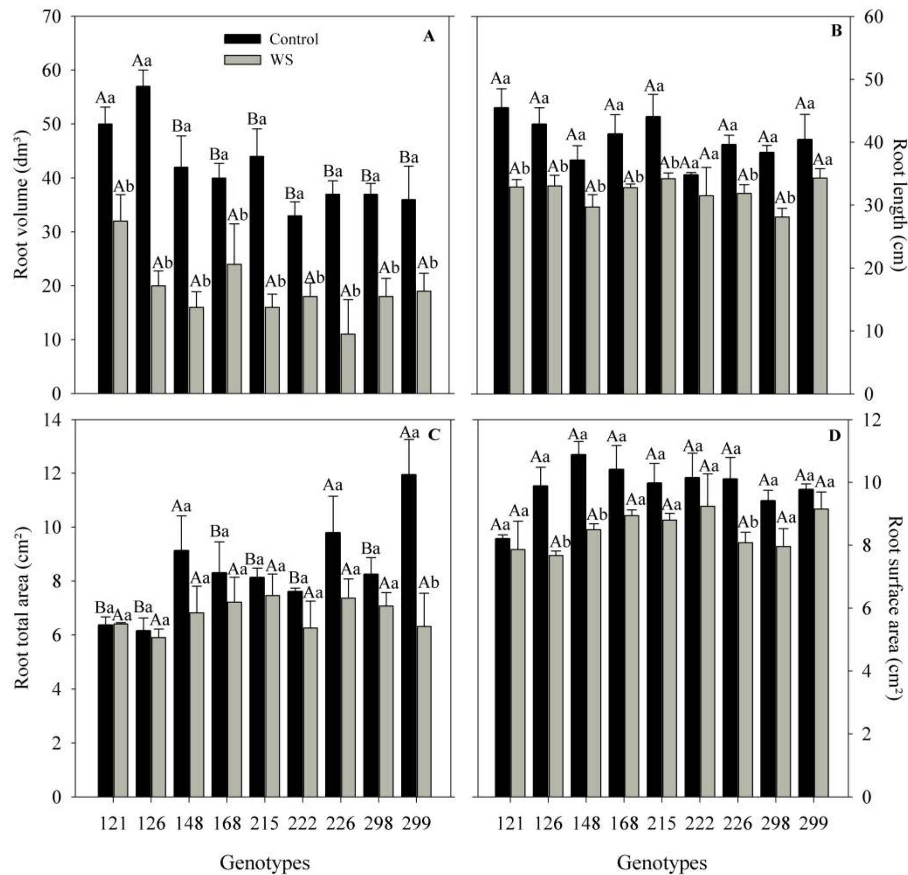

Figure 1. Volume (A), length (B), total area (C) and surface area (D) of roots in young plants of *Jatropha curcas* irrigated (control) or subjected to water stress (WS) for 42 days. Columns are means of 3-4 replicates and the bars represent the standard error of mean. Capital letters indicate comparison among genotypes within each treatment (control or WS) by the Scott-Knott test and lower case indicate comparison between water regimes by the F-test.

Specific leaf area (SLA) differed significantly ($P < 0.05$) among the genotypes and between the WS treatments (Figure 2). Interestingly, water deficit led to a significant decrease of SLA in genotypes CNPAE 168 (95%) and 226 (31%), to a significant increase in CNPAE 215 (170%), 222 (302%) and 299 (176%) and did not affect CNPAE 124, 148 and 298.

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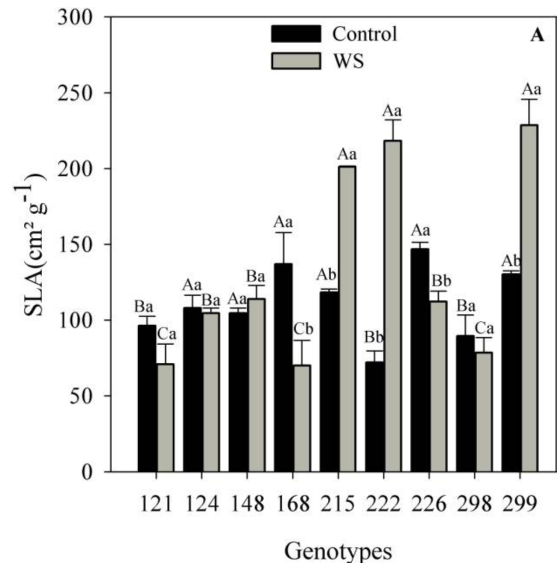

Figure 2. Specific leaf area (SLA) in *Jatropha curcas* subjected to water deficit (WS) for 42 days. Columns are means of four replicates and the bars represent the standard error of mean. Capital letters indicate comparison among genotypes within each treatment (control or WS) by the Scott-Knott test and lower-case comparison between water regimes by the F-test.

Water relations

No significant difference among genotypes and between watering regimes for pre-dawn leaf water potential (Ψpd) was observed in this experiment (Table 3). The same was observed for leaf water potential measured at noon (Ψmd), except for genotype CNPAE 222, in which under WS a significant decrease of Ψmd was observed. The average values of Ψmd for genotype CNPAE 222 were -0.83 and -1.37 MPa for control and WS plants, respectively.

Table 3. Values of water potential predawn (Ψam) and at midday (Ψmd) measured at 42 days after treatment in *Jatropha curcas*.

Genotypes	Ψpd (MPa)		Ψmd (MPa)	
	Control	WS	Control	WS
121	-0.47±0.08^{Aa}	-0.57±0.08 ^{Aa}	-1.00±0.05 ^{Aa}	-1.33±0.16 ^{Aa}
124	-0.43±0.06^{Aa}	-0.48±0.07 ^{Aa}	-1.03±0.03 ^{Aa}	-1.13±0.20 ^{Aa}
148	-0.32±0.04^{Aa}	-0.33±0.03 ^{Aa}	-1.07±0.06 ^{Aa}	-1.17±0.28 ^{Aa}
168	-0.32±0.04^{Aa}	-0.43±0.03 ^{Aa}	-0.87±0.06 ^{Aa}	-1.27±0.14 ^{Aa}
215	-0.33±0.06^{Aa}	-0.42±0.07 ^{Aa}	-1.27±0.03 ^{Aa}	-1.27±0.13 ^{Aa}
222	-0.33±0.06^{Aa}	-0.40±0.02 ^{Aa}	-0.83±0.12 ^{Aa}	-1.37±0.15 ^{Ab}
226	-0.27±0.04^{Aa}	-0.42±0.01 ^{Aa}	-0.75±0.10 ^{Aa}	-0.97±0.20 ^{Aa}
298	-0.35±0.07^{Aa}	-0.42±0.06 ^{Aa}	-0.78±0.20 ^{Aa}	-1.03±0.14 ^{Aa}
299	-0.35±0.08^{Aa}	-0.52±0.04 ^{Aa}	-0.87±0.06 ^{Aa}	-1.00±0.17 ^{Aa}

Mean values of three replicates (± standard error, n = 3). Lower case letters indicate significant differences between treatments within each genotype by the F-test and upper-case letters indicate significant differences by genotypes within each treatment by the Scott-Knott test.

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No significant differences among genotypes were detected for water use efficiency of biomass (WUEb) in irrigated plants (Figure 3). Water deficit led to an increase of WUEb in genotypes CNPAE 148, 226 and 298, being significant only in the last two.

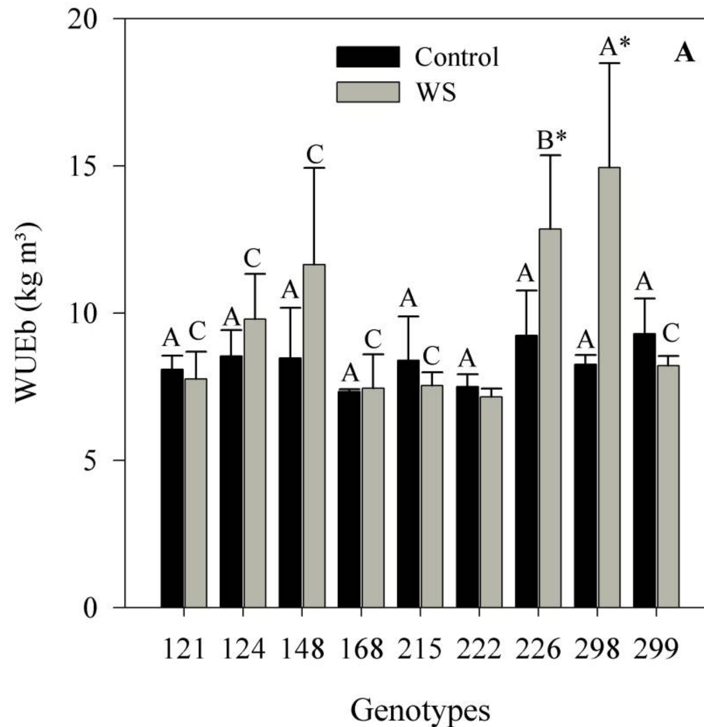

Figure 3. Water use efficiency of biomass (WUEb) in *Jatropha curcas* submitted to water deficit for 42 days. Columns are means of 3-5 replicates and the bars represent the standard error of mean. Capital letters indicate comparison among genotypes within each treatment (control or water stress - WS) by the Scott-Knott test and * comparison between watering regimes by the F test.

Activity of the PEPC

One of the tools used to examine expression of CAM was measurements of PEPC activity in leaf samples collected at two different times. Significant differences among genotypes, as well as between watering regimes were observed. Except for genotypes CNPAE 121, 222 and 299, higher activity was observed in WS plants at 16 h when compared to 4 h (Figure 4A, B).

Water deficit led to increases of 167 and 187% of PEPC activity, on average, for the samples collected respectively at 4 and 16 h. Moreover, PEPC activity was lower in the stem segments when compared to the leaves (Figure 4C, D). Significant increases of PEPC activity were observed in basal segments of stem in genotypes CNPAE 148 (111%), 222 (124%), 298 (308%) and 299 (168%) as a result of water

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deficit (Figure 4C). However, only non-significant effects of genotype and watering regimes were detected in samples of apical stem (Figure 4D).

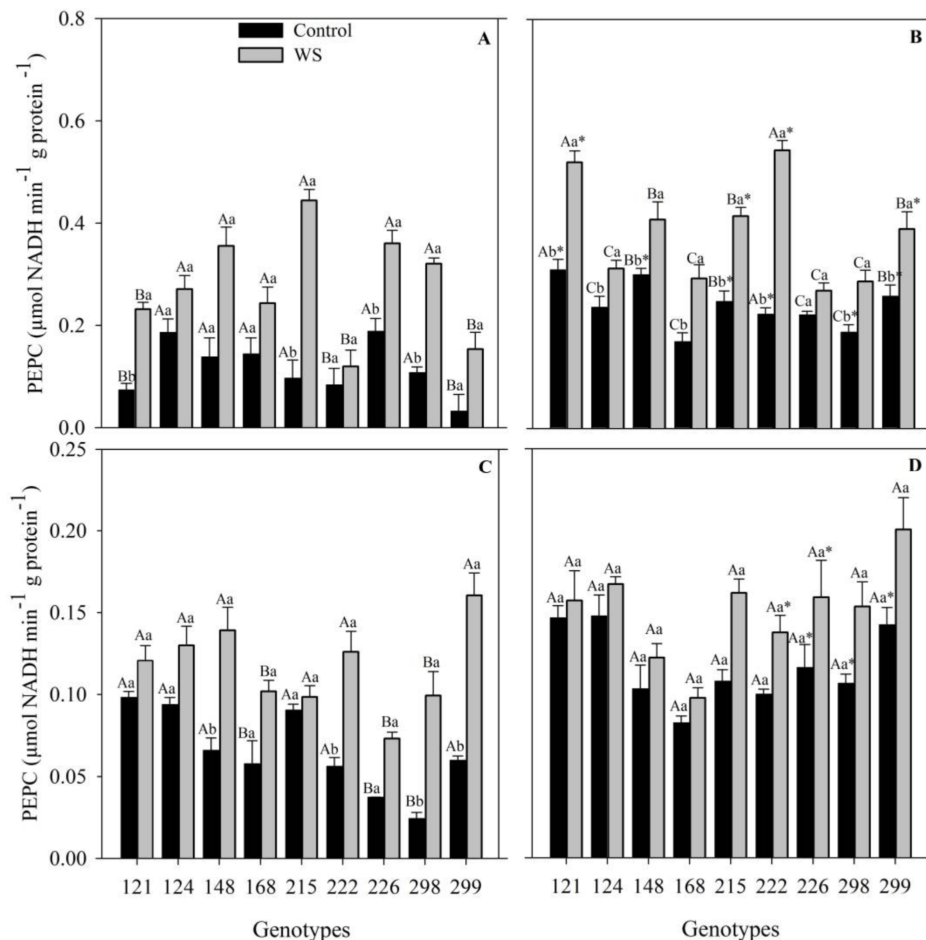

Figure 4. Activity of phosphoenolpyruvate carboxylase (PEPC) in leaves and stem of *Jatropha curcas* subjected to water deficit for 42 days. Leaf samples were collected at 4 h (A) and 16 h (B) and stem segments in basal (C) and apical (D) segments of main stem. Columns are means of four replicates and the bars represent the standard error of mean. Capital letters indicate comparison among genotypes within each treatment (control or WS) by the Scott-Knott test and lower-case comparison between watering regimes by the F-test. * indicates differences between the sampling times (AxB) and stem segments (CxD).

Biochemical determinations

Large differences in the accumulation of organic acids (malate and citrate) among genotypes and between WS treatments were observed. There was greater accumulation of citrate in relation to malate at 16 h (Figure 5A, B). In samples collected at 4 h, malate concentration increased significantly in genotypes CNPAE 121, 126, 226

and 299 (Figure 5C) and citrate concentration increased significantly in genotypes CNPAE 121, 168, 226, 298 and 299 (Figure 5D).

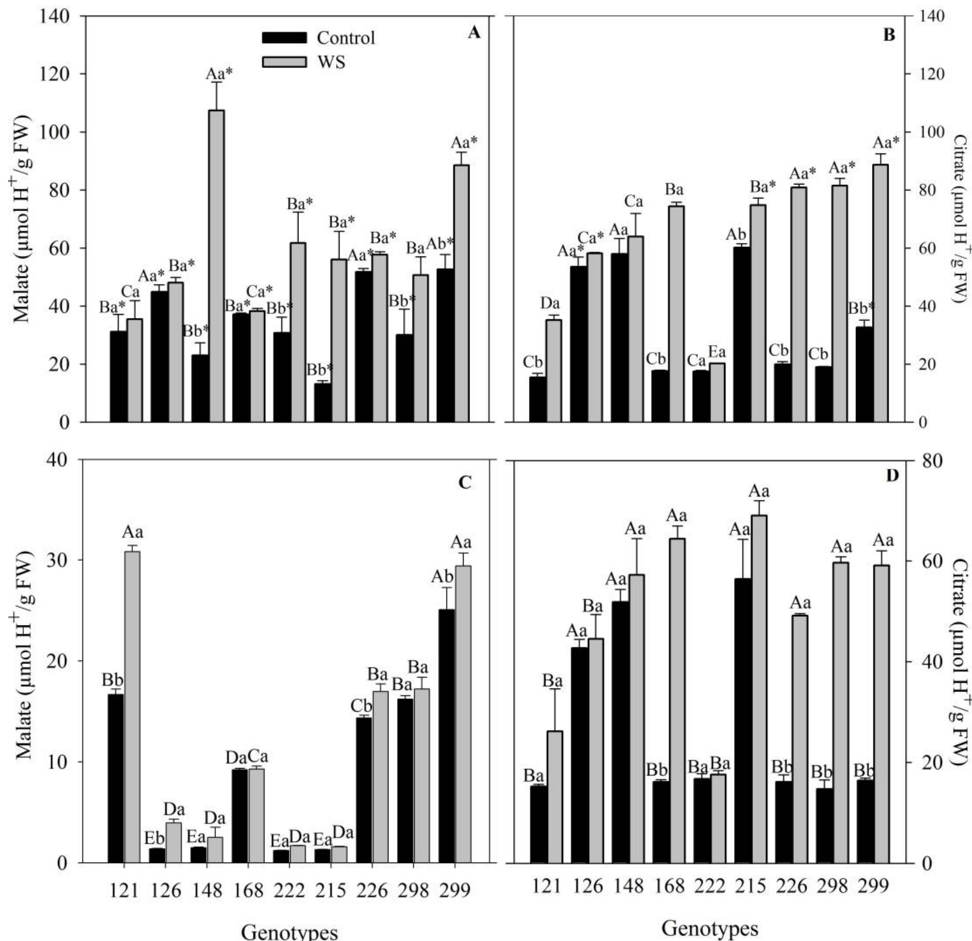

Figure 5. Concentration of malate (A, C) and citrate (B, D) in leaf samples of *Jatropha curcas* subjected to water deficit for 42 days. Samples were collected at 16 h (A, B) or 4h (C, D). Columns are means of 3-4 replicates and the bars represent the standard error of mean. Capital letters indicate comparison among genotypes within each treatment (control or WS) by the Scott-Knott test and lower-case comparison between watering regimes by the F-test. * indicates differences between the sampling times (AxC) and stem segments (BxD).

There was a significant difference among genotypes, as well as between watering regimes in terms of total soluble sugars (TSS) and starch (Figure 6A, B). Average increase of 62% of TSS and decrease of 27.5% of starch were observed in water deficit plants as compared to their controls.

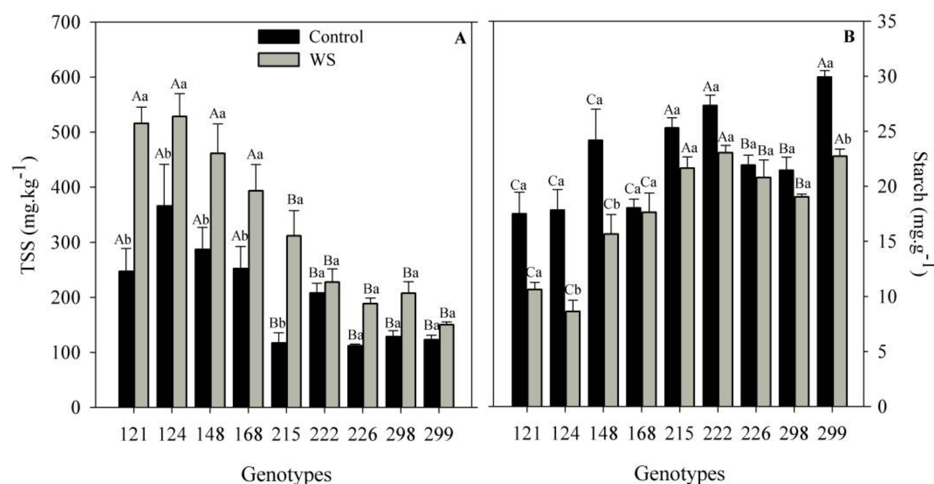

Figure 6. Concentration of total soluble sugars (TSS, A) and starch (B) in leaves of *Jatropha curcas* subjected to the water deficit for 42 days. Columns are means of four replicates and the bars represent the standard error of mean. Capital letters indicate comparison among genotypes within each treatment (control or WS) by the Scott-Knott test and lower-case comparison between watering regimes by the F-test.

Isotopic carbon composition ($\delta^{13}C$)

There were significant differences ($P < 0.05$) among genotypes for carbon isotope composition in the leaf biomass (Table 4). Lower (more negative) values of $\delta^{13}C$ were observed in genotypes 124, 148 and 168 in relation to the others and across the two water regimes. Only in genotype CNPAE 121 was it possible to detect a significant effect of water deficit, with a decrease of $\delta^{13}C$.

Table 4. Carbon isotope composition ($\delta^{13}C$) in leaves of *Jatropha curcas* subjected to water deficit for 42 days (WS).

$\delta^{13}C$ (‰) Genotypes	Control	WS
121	- 31.4±2.1^{Ba}	- 34.7±1.5^{Bb}
124	- 34.1±2.8^{Ca}	- 36.6±0.1^{Ca}
148	- 35.9±1.2^{Ca}	- 37.8±0.8^{Ca}
168	- 34.7±0.2^{Ca}	- 32.9±0.7^{Ba}
215	- 26.7±0.1^{Aa}	- 26.9±0.0^{Aa}
222	- 30.1±1.5^{Aa}	- 27.1±0.1^{Aa}
226	- 26.6±0.1^{Aa}	- 29.1±0.6^{Aa}
298	- 26.7±0.3^{Aa}	- 28.2±0.1^{Aa}
299	- 27.5±0.1^{Aa}	- 26.2±0.2^{Aa}

Values are means of three replicates (± standard error). Lower case indicate significant differences between treatments within each genotype by the F-test and capital letters indicate significant differences among genotypes within each treatment by the Scott-Knott test.

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DISCUSSION

There have been consistent proofs of the occurrence of a low level of drought-induced C_3-CAM change in leaves and stem of *J. curcas*, since the early reports based on high transpiration efficiency (Maes et al., 2009), as well as quantum yield of carbon assimilation lower than in other C_3 species (Rajaona et al., 2013). Other evidence includes, nocturnal increase of titratable acidity in samples of leaf and stem of water stressed plants, changes in gas exchange patterns during night period (Winter and Holtum, 2015).

Several mechanisms to avoid dehydration of plant tissues when they undergo water scarcity conditions were demonstrated. Among these, the reduction of leaf area (LA), which, in this study explains the increase in SLA in certain genotypes (215, 222 and 299). This reduction may be a mechanism to decrease the transpiratory surface and to maintain the tissues necessary for survival during the period of water scarcity. A negative effect of water deficit on leaf area has been reported by Santana et al (2015).

Water deficit is known to cause negative effects on cell expansion and photosynthesis, which in turn cause a reduction in plant growth (Zhu, 2001). Diameter at collar and height were also affected by WS, as have been demonstrated in other studies (Achten et al., 2010b; Santana et al., 2015). Significant decreases in height (20%) and diameter (10%) in plants of *J. curcas* submitted to a water deficit (40% of field capacity, considered by the authors as moderate) for 42 days have been reported (Achten et al., 2010b). In addition, decreases of 33.4 (height) and 14.6% (diameter) were reported in *J. curcas* grown under water deficit for 66 days (Santana et al., 2015).

The decrease in water availability (50% of TC) did not change the leaf water potential (Ψw) among the treatments, except for genotype 222 for Ψmd (Table 3). Control of Ψw, which practically does not vary with the decrease of soil water potential, is a main characteristic of plants with high water content in succulent stems, as *J. curcas* (Santana et al., 2015). Moreover, strategies for carbon gain in plants that present such "buffered" leaf water status might be associated to increased magnitude of CAM, through the accumulation of organic acids and PEPC activity, as observed here and elsewhere (Cushman and Borland, 2002, Griffiths et al., 2008, Davies and Griffiths, 2012).

The decrease of the SLA in some genotypes was due to drought-induced decrease in leaf expansion. The SLA serves as a useful indicator of how plants invest carbon and nutrients (dry biomass) in a particular area of the leaf that is intercepted by light (Vendramini et al., 2002). Species with lower SLA may have to resort to higher efforts for light interception (Poorter, 2009), a strategy that is common in species that inhabit environments where drought and/or nutrient limitations can hinder growth. Thus, a low SLA may be an indicative that predisposes an adjustment in the anatomy of *J. curcas* for a development of CAM in limited water habitats.

Major changes in PEPC activity, the main carboxylating enzyme in CAM, have been detected in leaf and stem samples of *J. curcas* in the present investigation The PEPC activity was more responsive to WS at the apex of stem when compared to the other samples, even though there were no significant differences among the genotypes and between WS treatments (Figure 4). There was an increase in foliar PEPC activity at dusk (16 h) in relation to 4 h. Decreased activity at the start of the day would result from the inhibition of malate which is decarboxylated in the cytosol. The increase of foliar activity at 16 h in relation to 4 h reached 91% when submitted to WS as compared to control plants. In

this case, acid accumulation may have started after 16 h and finished before 4 h. It is worthy note that, while the dark CO_2 fixation and the associated accumulation of malic acid may be unique to CAM, PEP carboxylation and accumulation and decarboxylation cycles of the malate anion are not, which may be important, for example, to the maintenance of charge balance during processes such as NO_3 reduction and changes in stomatal aperture (Martinoia et al., 2012). In addition, Freschi et al. (2010a) also demonstrated an increase in PEPC activity during the dark period in separate leaves of *G. monostachia*. In light of this, transcription, translation and post-translation of PEPC are likely to show variations during a daytime cycle.

The CAM can operate in four ways: obligatory *CAM* with nocturnal accumulation of organic acids and fixation of CO_2 during the day; facultative *CAM* with the fixation of CO_2 as in the C_3 metabolism (RUBISCO) under favorable environmental conditions with CAM behavior in unfavorable environmental conditions; CAM cycling, with daytime CO_2 fixation (no stomata opening at night) and nocturnal accumulation of this gas and of organic acids, and finally CAM idling characterized by a small nocturnal accumulation of organic acids and the closure of the stomata during the entire daytime cycle. In this last mode of operation, the species use CO_2 from respiration and photorespiration, which is recycled for the synthesis of organic molecules and for the maintenance of the photosynthetic apparatus (Freschi et al., 2010a). CAM cycling mode has been viewed as the first stage in the shift from C_3 to CAM that occurs as leaf age, or in response to water stress in facultative CAM species (Cushman and Borland, 2002). Many species perform only such initial stage of CAM, in which the recapture of respiratory CO_2 at night allows the maintenance of a positive carbon balance during frequent episodes of drought (Dodd et al., 2002).

In facultative species, CAM can be induced by environmental factors such as drought (Freschi et al., 2010a). The extent of CAM expression in *J. curcas* was also evaluated by analyzing the changes in titratable acidity (H^+) in the leaves, as expressed by the estimated content of malate and citrate (Figures 5). Titratable acidity (H^+) can differ more than 100-fold among species. In facultative CAM species, the values can range from 1000-1500 µmol H^+ g^{-1} FW in some species of *Clusia* at the onset of the dry season (Borland et al., 1992). Values around 150 µmol H^+ g^{-1} FW have been measured in inducible CAM species such as *Mesembryanthemum. crystallinum* (Davies and Griffiths, 2012).

Two main organic acids (malate and citrate) were estimated in the present experiment from the measurements of titratable acidity. The accumulation of citrate, as observed here in samples collected at 4 h, is intriguing, since the role of citrate accumulation in carbon or water balance during CAM is not yet clear (Lüttge, 2007). Citrate does not provide net gain in CO_2 just as malate does, but is more effective than malate by increasing the internal carbon concentration (Ci) during the day because the decarboxylation of 1 mol of citrate generates 3 mols of CO_2 in comparison to only 1 mol in the case of malate (Lüttge, 2006). However, the formation of citrate overnight would not result in a net gain in CO_2, as this is not produced from a carboxylation reaction and therefore its precursors have the same number of carbons than the acid (Lüttge, 2006). It is worthy note that, in addition to serving as a CO_2-storage medium, the organic acids accumulated at night have been suggested to be an osmotic adjustment component, aiming to maintain leaf water status, thus facilitating the absorption of water at dawn or helping to remobilize water from older leaves (Smith and Lüttge, 1985) or from the succulent stem, as suggested here for *J. curcas*. The drought-induced osmotic adjustment was reported in *J.*

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curcas as a component of the drought tolerance mechanism (Silva et al., 2016). However, the nocturnal accumulation of citrate was observed in *M. crystallinum*. (Freschi et al., 2010b).

The induction of CAM (the amount of malic acid accumulated during the dark period) has been related to leaf succulence (Cushman and Borland, 2002). In a specific *CAM cycling* species of Euphorbiaceae (*Euphorbia milii*), the average leaf ΔH^+ and succulence was 18 µmol.g^{-1} MF and 0.45 kg.m^{-2}, respectively with no match between changes in H^+ and changes in leaf succulence, which remained relatively constant during the dry treatment period (Herrera, 2013).

A previous study of *Guzmania monostachia* plants showed the CAM pathway modulation in response to changes in water availability (Freschi et al., 2010b). The authors observed remarkable levels of nocturnal H^+ accumulation in the apical portion of intermediate leaves of well-irrigated plants of this type of bromeliad, suggesting some level of CAM even when the water supply was abundant. In fact, the water deficit induced H^+ increase exclusively in the apical leaf portion, where most activities of CAM-related enzymes, such as PEPC, have also been observed

The quantification of $\delta^{13}C$ performed on leaves demonstrated that the evidences of CAM in *J. curcas* were relatively low, with values ranging from -26.2 and -37.8 ‰ for the plants of *J. curcas* submitted to WS. In a study with 23 facultative CAM species, the average, maximum and minimum $\delta^{13}C$ values were -23.9, -14.0 and -30.0 ‰ respectively, indicating that the variability in $\delta^{13}C$ values may lead researchers to classify a species as a C_3, facultative or constitutive MAC (Herrera, 2009). Moreover, several species of bromeliads were classified within a range of $\delta^{13}C$, where values more negative than −20‰ were typical of predominantly daytime carbon fixation via the C_3 pathway whereas values less negative than −20‰ indicated predominantly nocturnal fixation of carbon via the CAM pathway (Crayn et al., 2015). Present results put *J. curcas* in two groups, where a slight C_3-CAM metabolism for certain genotypes and purely C_3 for the others can be distinguished (Table 4).

Increase of total soluble sugars in all plants under WS, as observed here, can be explained by hydrolysis of the starch under stress induction, thus restricting the translocation of sucrose from leaves and inducing lower use of assimilates for growth (McCormick et al., 2009). According to Kramer and Boyer (1995), when subjected to drought, several species present a reduction in starch concentration to form soluble sugars, i.e. the decrease in starch concentration is accompanied by an increase in carbohydrate concentration. It can be inferred that the higher sugar content contributed to the protection against tissue dehydration, functioning as a cell membrane stabilizer and helping to maintain turgor. In addition, a higher production of soluble sugars may be required to maintain the pool of phosphoenolpyruvate and consequently the CAM activity.

CONCLUSIONS

Water deficit induced metabolic changes in the leaves of *J. curcas*, as indicated by acidification of the leaf extract, both at 4 h and at 16 h along with increase of PEPC activity. Moreover, high values of H^+ found in control plants suggest that some level of CAM may occur even under constant irrigation. However, the magnitude of CAM expression in *J. curcas* may be considered low. Severe reduction of growth, as observed here, suggest that

induction of CAM in *J. curcas* is more important for water conservation than for carbon acquisition, as demonstrated for another Euphorbiaceae (Herrera, 2013). Nevertheless, the matter still remains under debate as low level of CAM detected in *J. curcas* has already been related to the maintenance of the carbon balance (Winter and Holtum, 2015).

Genotypes 121, 148 and 299 can be considered more drought-tolerant than the others, mainly because of higher accumulations of organic acids, higher PEPC activity, higher TSS content and higher root growth.

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