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Photosynthetic changes and protective mechanisms against oxidative damage subjected to isolated and combined drought and heat stresses in *Jatropha curcas* plants

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ABSTRACT

Photosynthetic changes and protective mechanisms against oxidative damage were evaluated in *Jatropha curcas* leaves subjected to drought and heat stresses, both individually and combined, in order to elucidate the synergistic and antagonistic mechanisms involved with these abiotic factors. Both the drought and heat stresses caused significant damage to the leaf membrane integrity and lipid peroxidation, and the combination of these stresses greatly enhanced these physiological disturbances. The leaf CO_2 assimilation rate, stomatal conductance and instantaneous carboxylation efficiency (P_N/C_I) were significantly decreased in all plants subjected to stressful conditions in comparison to unstressed plants (reference). In contrast, a reduction in photochemical activity was observed only in plants exposed to drought and drought + heat conditions. Catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) activities were stimulated only under heat stress, whereas APX activity was increased in all treated plants in comparison to the references. Moreover, the leaf H_2O_2 content was increased similarly under all studied stresses. However, the balance of reduced and oxidized ascorbate did not show significant differences between reference and stressed plants. Although *J. curcas* plants acclimated to the studied stresses, they did not present an efficient mechanism for protection against drought-induced oxidative stress, especially when at high temperatures. However, heat-treated plants triggered an efficient enzymatic antioxidant system of reactive oxygen species scavenging and an effective protection against photochemical damages. The combination of drought and heat most significantly impaired the photosynthetic assimilation of CO_2 and the photochemical activity. These results indicate that drought greatly disturbs photosystem II activity and oxidative metabolism and that these negative effects are strongly stimulated by heat stress. The data also evidence that the combination of heat and drought triggers an intricate response involving antagonistic and synergistic interactions.

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Introduction

Drought and heat are two major abiotic stresses that affect crop growth and yield in agricultural areas, especially in tropical regions. Although the isolated effects of those stresses on plant metabolism have been extensively studied, relatively little is known about the combined impact of drought and heat on biochemical processes. Both of these stresses occur simultaneously under field conditions in semi-arid regions and drought-stricken areas. Recent studies have revealed that the molecular and metabolic responses to the combination of drought and heat are unique and cannot be inferred or extrapolated from the plant responses to drought or heat stresses applied individually (Mittler, 2006).

Photosynthesis is one of the primary processes most affected by abiotic stresses (Liu and Huang, 2008). Under high temperatures, photosynthesis can be inhibited by impaired electron transport, reduced photochemical efficiency of PSII and inhibited Rubisco activity. In fact, decreased Rubisco activation is exhibited under moderate heat stress due to impaired Rubisco activase activity and is exacerbated with increasing temperature (Liu and Huang,

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; ASA, ascorbic acid; DHA, dehydroascorbate; ROS, reactive oxygen species; Ψ_w, leaf water potential; EL, electrolyte leakage; P_N, leaf CO_2 assimilation rate; g_S, stomatal conductance; C_I, intercellular CO_2 concentration; P_N/C_I, instantaneous carboxylation efficiency; $\Delta F'/F'_M$, actual quantum yield; $ETRS'_S$, apparent electron transport rate; NPQ, non-photochemical quenching.

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2008). On the other hand, reduced photosynthesis in drought-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 (Flexas et al., 2007). In addition, low photosynthesis under a water deficit may be imposed by changes in photosynthetic metabolism, such as a reduction in biochemical and/or photochemical activity (Lawlor and Cornic, 2002).

High temperatures are often accompanied by water deficits and stomatal closure under field conditions, which reduce CO_2 availability and may decrease the CO_2/O_2 ratio in chloroplasts (Foyer and Noctor, 2000). Once plants present low NADPH and ATP consumption due to reduced photosynthetic rates, the $NADP^+/NADPH$ ratio is also decreased and the photosynthetic electron transport chain becomes over-reduced. This condition facilitates electron flow to molecular oxygen (O_2) and superoxide radical ($O_2^{\bullet -}$) production by the Mehler reaction (Foyer and Noctor, 2003). Additionally, the photorespiration pathway may be enhanced when C3 plants are subjected to stressful conditions because it is a potential source of reactive oxygen species (ROS), especially hydrogen peroxide (Foyer and Noctor, 2000).

ROS are generated as natural products of plant cellular photosynthetic and aerobic metabolisms. At low concentrations, ROS can serve as signaling molecules in the redox signal transduction pathway of plants (Zhu et al., 1997). However, overproduction of ROS in plant cells under stress can damage cellular components, including DNA, proteins and membrane lipids (Mittler, 2002). Plants have evolved efficient antioxidant systems that can protect them from the damaging effects of oxidative stress (Asada, 1999). These mechanisms employ ROS-scavenging enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), as well as low molecular weight antioxidants, such as ascorbic acid (ASA) and glutathione (Noctor and Foyer, 1998).

Although there are several recent reports involving oxidative stress associated with abiotic factors, few studies have investigated combined stresses like drought and heat. Moreover, the results are frequently fragmentary and do not link oxidative responses with key processes like photochemistry and overall photosynthesis. Indeed, the control mechanisms of oxidative metabolism by isolated and combined abiotic stresses remain poorly understood, especially in terms of its relationship to photosynthesis and related processes. In other words, the current knowledge about this issue is still incipient and fragmentary (Mittler, 2006). This information is essential to crop breeding programs aiming to develop tolerant genotypes capable of displaying high yields under stressful conditions.

Jatropha curcas has been reported as a species that grows in marginal areas of semi-arid regions frequently subjected to dry and hot conditions, where most other crops are not able to survive (Francis et al., 2005). This species may have potential as an excellent model for the physiological and molecular mechanisms involved with crop resistance to combined abiotic stresses. In this study, we tested the hypothesis that *J. curcas* plants have efficient mechanisms of protection against oxidative stress and photochemical damages induced by drought and heat. We evaluated the leaf gas exchange, chlorophyll fluorescence and antioxidative response of *J. curcas* leaves subjected to isolated and combined drought and heat stresses.

Our results show that drought significantly impairs photochemical activity and oxidative metabolism and these negative effects are strongly stimulated by heat stress. The data also evidence that the combination of heat and drought triggers a complex response involving antagonistic and synergistic interactions. The importance of those physiological responses for plant resistance to abiotic stresses is discussed.

Materials and methods

Plant material and experimental conditions

The initial phase of the experiment was carried out under greenhouse conditions, where the mean air temperature varied between 24 (minimum) and 36 °C (maximum) with a mean temperature of 29 °C, a mean air relative humidity of 65%, a maximum photosynthetic photon flux density (PPFD) of 700 $\mu mol\, m^{-2}\, s^{-1}$ and a photoperiod of around 12 h. *Jatropha curcas* L. seeds were previously selected based on seed size and weight. Eight days after germination in sand substrate, the seedlings were each transferred to plastic pots (2 L) containing vermiculite. Plants were watered every 2 days with 250 mL of half-strength Hoagland and Arnon solution (1950), which was sufficient to reach 70% of the water-holding capacity of the vermiculite substrate. When plants were 23-day old, they were transferred to a growth chamber at 27 °C with a PPFD of 400 $\mu mol\, m^{-2}\, s^{-1}$. At this time, two treatments were imposed: well watered + 27 °C (the reference condition) and drought + 27 °C (drought). Drought was imposed by withholding water; after 5 days of treatment, the temperature was gradually elevated to 43 °C (4 °C h^{-1}) and plants were subjected to this temperature for 6 h. Two more conditions were defined at this time: well watered + 43 °C (heat) and drought + 43 °C (drought + heat). Before increasing the temperature (on the 5th day) and after the heat treatment, the leaf discs were harvested to determine the membrane damage (K^+ leakage) and relative water content. Then, the leaves were frozen in liquid nitrogen and stored at −80 °C until the biochemical determinations were performed.

Water status, electrolyte leakage (EL) and dry matter yield of leaves

The leaf water potential (Ψ_w) was evaluated immediately after sampling using the pressure chamber method (Scholander et al., 1965) at pre-dawn (Ψ_w, at 6:00 h) in leaves similar to those used for leaf gas exchange and chlorophyll fluorescence measurements. The leaf relative water content (RWC) was determined as previously described (Silveira et al., 2009). The leaf succulence (LS) was calculated by the equation (FW)/*A*, where *A* is the area of thirty leaf discs (diameter of 1.0 cm), as described by Silveira et al. (2009). EL in leaves was measured as previously described (Cavalcanti et al., 2004) and the leaf dry matter was obtained by drying the leaves in an oven at 75 °C for 48 h.

Leaf gas exchange and chlorophyll fluorescence

Leaf gas exchange was measured with an infrared gas analyzer – IRGA (LCi, ADC, Hoddesdonm, UK) operating in an open system and with an air flow of 200 mL min^{-1}. Measurements of leaf CO_2 assimilation rate (P_N), stomatal conductance (g_S) and intercellular CO_2 concentration (C_I) were taken and the instantaneous carboxylation efficiency (P_N/C_I) was calculated. Leaf temperature was measured with the IRGA during the gas exchange measurements. Plants grown at 27 °C had leaves at 29.5 °C (reference) or 31.4 °C (drought treatment), whereas plants grown at 43 °C had leaves at 46–47 °C regardless of the water treatment.

The chlorophyll fluorescence was evaluated with a modulated fluorometer (FMS2, Hansatech, King's Lynn, UK). Minimum (F_O), maximum (F_M) and maximum variable ($F_V = F_M - F_O$) fluorescence intensities were sampled under steady-state conditions in dark-adapted (30 min) leaves. In addition, measurements were also taken under light-adapted conditions, and are referred to as F'_O (minimum) and F'_M (maximum). The F'_O signal was measured after PSI excitation by far-red light. The fluorescence signal under light-adapted conditions before the saturation pulse is referred to as

F'_S and the variable fluorescence signal under light conditions is $\Delta F' = F'_M - F'_S$. The following photochemical variables were calculated: maximum (F_V/F_M) and actual ($\Delta F'/F'_M$) quantum yield of primary photochemistry; apparent electron transport rate ($ETR'_S = \Delta F' - F'_M \times PPFD \times 0.5 \times 0.84$) and non-photochemical quenching [$NPQ = (F_M - F'_M)/F'_M$] (Rohácek, 2002). For calculation of ETR_S, 0.5 was used as the fraction of excitation energy distributed to PSII and 0.84 as the fraction of incoming light absorbed by the leaves.

The ratio ETR'_S/P_N was calculated to estimate the use of electrons in other processes not related to the photosynthetic CO_2 uptake (Ribeiro et al., 2009). Therefore, an increase in ETR'_S/P_N indicates that more electrons are driven to other sinks (e.g., photorespiration), suggesting a stressful condition. Leaf gas exchange and chlorophyll fluorescence were measured simultaneously in fully expanded and mature leaves under a PPFD of 400 μmol m^{-2} s^{-1}. Measurements were taken after 5 days of treatment at 27 °C (well watered + 27 °C and drought + 27 °C), and repeated again after 6 h of heat treatment at 43 °C (well watered + 43 °C and drought + 43 °C).

Leaf hydrogen peroxide content and lipid peroxidation

Samples of fresh leaves (0.1 g) were powdered in liquid nitrogen and extracted with 100 mM potassium phosphate buffer (pH 6.4) containing 5 mM KCN, according to Cheeseman (2006). The reaction was carried out at 25 °C for 30 min and the absorbance was read at 560 nm. The H_2O_2 concentration was calculated according to a standard curve and expressed as μmol g^{-1} FW. Lipid peroxidation was determined by measuring the thiobarbituric acid-reactive substances (TBARS), according to Heath and Packer (1968). The TBARS concentrations were calculated using the molar extinction coefficient (155 mM^{-1} cm^{-1}) and are expressed as nmol g^{-1} FW.

Ascorbate content

The content of total ascorbate (ascorbic acid (ASA) + dehydroascorbate (DHA)) was determined by adding the leaf extract to a mixture of potassium phosphate buffer (pH 7.4) containing 2 mM DTT, 0.5% (w/v) N-ethylmaleimide, 10% (w/v) TCA, 45% (w/v) H_2PO_4, 4% (w/v) bipyridyl, and 5% (w/v) $FeCl_3$. The reaction was performed at 40 °C for 30 min, and the absorbance was read at 525 nm. The contents of ASA + DHA (total ascorbate) and ASA (reduced ascorbate) were estimated using L-ascorbate as a standard and are expressed as μmol g^{-1} FW. The oxidized ascorbate (DHA) content was obtained by subtracting the reduced fraction from the total ascorbate content (Kampfenkel et al., 1995).

Enzyme extraction and activity assays

Samples of frozen leaves (0.1 g) were macerated in liquid nitrogen and extracted with 100 mM Tris–HCl buffer (pH 8.0) containing 30 mM DTT, 20% (v/v) glycerol and 3% (w/v) PEG-6000 (Zimmermam et al., 2006). For superoxide dismutase (SOD) and ascorbate peroxidase (APX) extraction, the pH of the buffer was adjusted to 7.0 and 30 mM DTT was replaced by 1 mM ascorbate. The crude extract was centrifuged at 14,000 × *g* for 30 min at 4 °C and the supernatant was used as an enzymatic extract.

The activity of SOD (EC: 1.15.1.1) was determined by adding the leaf extract to a mixture containing 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM L-methionine, 2 μM riboflavin and 75 μM p-nitro blue tetrazolium chloride (NBT) in the dark. The reaction was carried out under illumination (using a 30 W fluorescent lamp) at 25 °C for 6 min. The absorbance was measured at 540 nm. One SOD activity unit (AU) was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction (Beauchamp and Fridovich, 1971), and the activity is expressed as AU g^{-1} FW min^{-1}. The APX activity (APX; EC: 1.11.1.1) was assayed after reaction of the extract in the presence of 50 mM potassium phosphate buffer (pH 6.0) and 0.5 mM ASA. The reaction was started by the addition of 0.1 mL of 30 mM H_2O_2, and the decreasing absorbance at 290 nm was monitored for 300 s (Nakano and Asada, 1981). The APX activity was estimated by considering the molar extinction coefficient of ascorbate (2.8 mM^{-1} cm^{-1}) and is expressed as μmol ASA g^{-1} FW min^{-1}. Catalase activity (CAT; EC: 1.11.1.6) was determined after the reaction of the extract in the presence of 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM H_2O_2. The reaction took place at 30 °C and the absorbance at 240 nm was monitored for 300 s (Havir and Mchale, 1987). The CAT activity was calculated according to the molar extinction coefficient of H_2O_2 (36 mM^{-1} cm^{-1}) and is expressed as nmol H_2O_2 g^{-1} FW min^{-1}.

Experimental design and data analysis

The effects of the water conditions (well watered and drought) and temperature (27 and 43 °C) were investigated in this study. Data were subjected to ANOVA procedures and the mean values (four replicates) were compared by the Tukey test at a confidence level of 0.05. The standard deviation is plotted in all figures. All changes are relative to a reference condition (well watered + 27 °C), and an overall evaluation of the physiological responses to stressful conditions was performed by plotting data in a radar graph.

Results

In this study, *J. curcas* plants were subjected to isolated and combined stresses of drought and heat. The drought, heat and drought + heat treatments caused decreased the leaf DW (dry weight) by 35%, 10% and 41%, respectively, in comparison with the well watered plants grown at 27 °C (reference plants, data not shown), but no stress visual symptoms were observed. The leaf water potential (Ψ_w) was significantly reduced ($p < 0.05$) only in the drought treatment, but remained similar to the reference in all of the other treatments (Fig. 1A). The relatively high leaf Ψ_w of drought-stressed plants (−1.05 MPa) was associated with a good leaf hydration status, as indicated by the high values of relative water content and leaf succulence, similar to those presented by the reference plants (data not shown).

The leaf electrolyte leakage (EL), a membrane damage indicator, increased by 15%, 28% and 122% in drought, heat and drought + heat treatments, respectively (Fig. 1B), while the lipid peroxidation, as measured by the accumulation of thiobarbituric acid-reactive substances (TBARS), increased by 100%, 50% and 200%, respectively, in comparison with the reference plants (Fig. 1C). These results indicate that the heat and drought interaction was negative or that adverse effects between the combination of drought and heat occurred in *J. curcas* leaves.

The leaf CO_2 assimilation rate (P_N) decreased appreciably in all treatments (86%, 41% and 89% in plants subjected to drought, heat and combined stresses, respectively (Fig. 2A)) by comparison with the reference plants. Similarly, the stomatal conductance (g_S) and instantaneous carboxylation efficiency (P_N/C_I) were also reduced due to drought and heat. Compared to reference plants, the g_S values decreased by 80%, 47% and 94%, while the P_N/C_I ratio was reduced by 84%, 35% and 82% in drought, heat and drought + heat treatments, respectively (Fig. 2B and C). The combination of drought and heat did not show interactive effects in leaf gas exchange measurements, and the drought effects predominated regardless of temperature. The effects of heat on these measurements were less pronounced than those of drought stress.

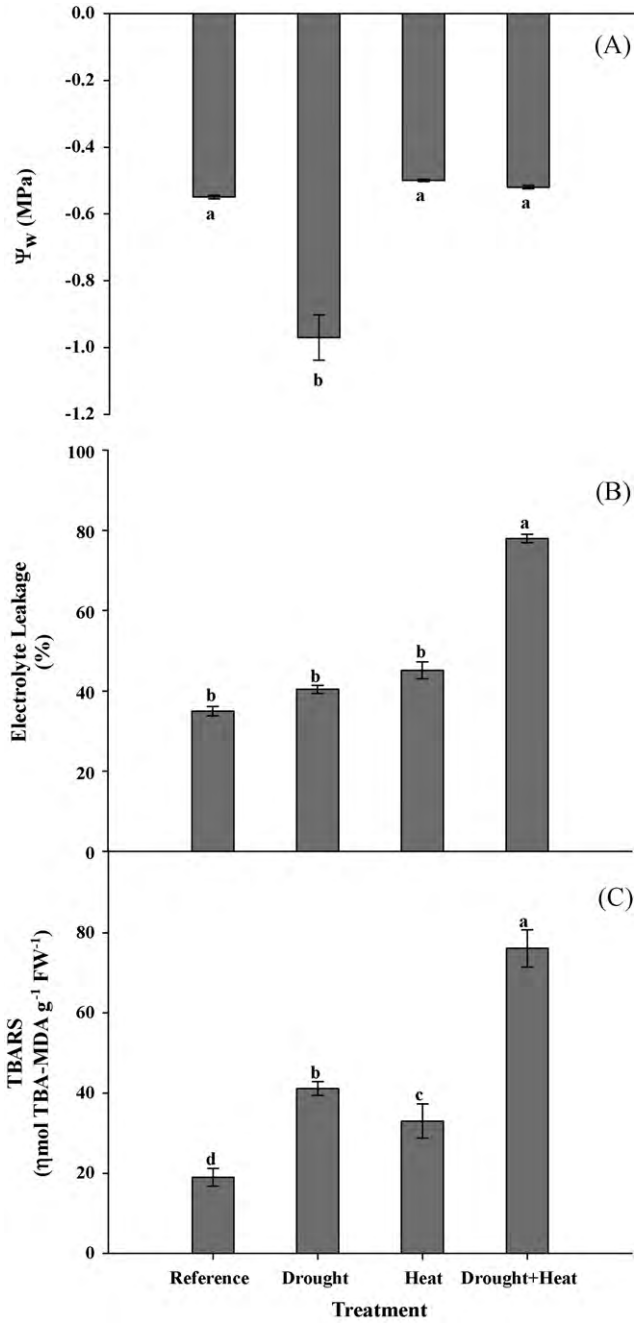

Fig. 1. Leaf water potential, Ψ_w (A), electrolyte leakage, EL (B), and lipid peroxidation and TBARS content (C) in *Jatropha curcas* plants subjected to isolated and combined stresses of drought and heat. Data are mean values of four replicates ± SD. The same letters are not significantly different to 0.05 by Tukey's test.

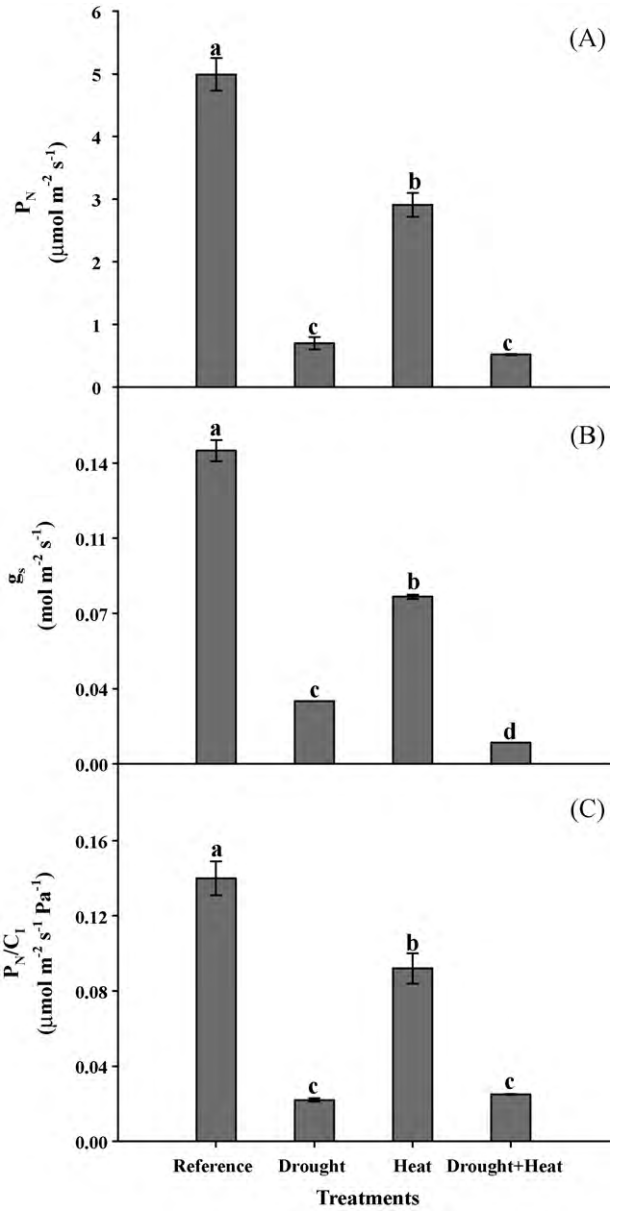

Fig. 2. Leaf CO_2 assimilation rate, P_N (A), stomatal conductance, g_S (B) and instantaneous carboxylation efficiency, P_N/C_I (C), in *Jatropha curcas* plants subjected to isolated and combined drought and heat. Data are mean values of four replicates ± SD. The same letters are not significantly different to 0.05 by Tukey's test.

The photochemical activity was evaluated by the actual quantum yield of primary photochemistry ($\Delta F'/F'_M$), non-photochemical quenching (NPQ) and apparent electron transport rate (ETR'_S). The $\Delta F'/F'_M$ and ETR'_S values decreased by 56% and 83% in plants subjected to drought and combined stresses, respectively (Fig. 3A and B). However, neither stressed nor non-stressed plants showed significant changes ($p > 0.05$) in F_V/F_M (data not shown), while the *qN* parameter significantly increased only in plants subjected to drought and combined stress, when compared to the references (Fig. 3C).

The ETR'_S/P_N ratio increased by ~50% by effects of heat and drought + heat treatments and 300% in the drought treatment (Fig. 3D). These data evidence a strong positive interaction (synergism) between drought and heat in terms of the allocation of the excess electrons from photosystem II for CO_2 fixation. Interestingly, the isolated heat treatment did not alter the majority of the evaluated photochemical parameters (Fig. 3). However, the damages caused on the photochemical apparatus by the drought stress were strongly intensified by heat (antagonistic effect).

Antioxidant enzymes, such as CAT, APX, and SOD, showed different patterns of responses to drought, heat and drought + heat treatments. Leaf CAT activity was stimulated by 38% with heat, but conversely was strongly inhibited by 60% and 36% in the drought and drought + heat treatments, respectively. These results suggest an antagonistic effect of the combined heat and drought treatment on the CAT activity (Fig. 4A). The APX activity was increased (by approximately 100%, 100% and 300% in plants subjected to drought, heat and combined stresses, respectively (Fig. 4B)) in all of the stressed conditions by comparison to the non-stressed plants. Therefore, a positive interaction between the two stress factors was

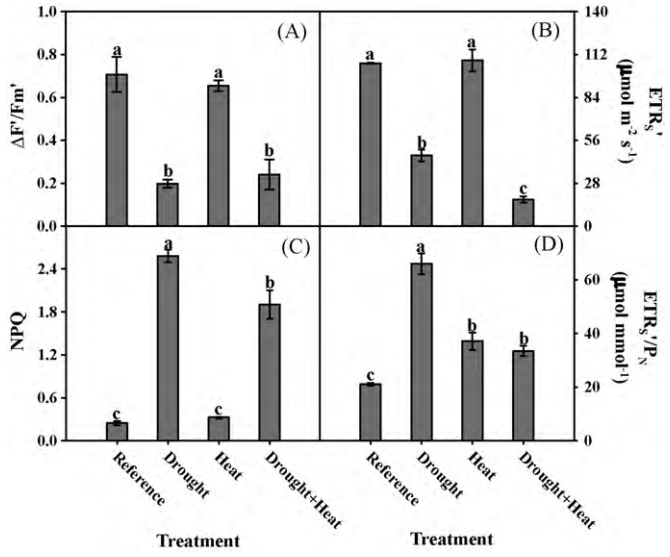

Fig. 3. Actual quantum yield of primary photochemistry, $\Delta F'/F'_M$ (A), apparent electron transport rate, ETR'_S (B), non-photochemical quenching, NPQ (C) and ratio between apparent electron transport rate and CO_2 assimilation rate, ETR'_S/P_N (D), in *Jatropha curcas* plants subjected to isolated and combined stresses of drought and heat. Data are mean values of four replicates ± SD. The same letters are not significantly different to 0.05 by Tukey's test.

verified. The SOD activity was stimulated 36% in plants undergoing the heat treatment; however, this activity was not affected by drought alone and was inhibited by 42% in plants subjected to combined stresses. These results suggest a strong negative interaction (antagonism) between heat and drought (Fig. 4C).

The leaf H_2O_2 concentration increased significantly in all of the stressed plants in comparison to the non-stressed ones (reference). This increase (~27%) was similar among the stress treatments (Fig. 5A), suggesting non-additive effects between drought and heat. By contrast, the total ascorbate concentration (ASA + dehydroascorbate (DHA)) decreased by ~13% in plants exposed to drought and heat. Non-significant changes were noted in plants subjected to combined stresses when compared with the reference (Fig. 5B). The leaf ASA/DHA ratio did not change in plants exposed to drought and drought + heat, but it was increased by 71% in plants under heat stress in comparison to the reference.

Data analysis through the radar plot (Fig. 6) showed that plants subjected to the combined drought + heat stress experienced the most significant changes in the studied variables. For example, a prominent increase in membrane damage (as indicated by increased TBARS and EL) was observed in parallel to higher APX activity. Moreover, under drought conditions, plants exhibited a higher activity of alternative electron sinks (high ETR'_S/P_N) as well as active mechanisms for dissipating excessive light energy (high NPQ) in comparison to other stress conditions. On the other hand, the maintenance of high photosynthetic rates (P_N) under heat stress caused lower ETR'_S/P_N values than those in drought-stressed plants (Fig. 6). However, these results do not mean that alternative electron sinks were deactivated due to high temperature.

As the *J. curcas* plants were subjected to conditions of high temperature for a reasonably long-term exposure (6 h), the photosynthetic machinery of this species likely became tolerant to the heat stress. Moreover, the drought pre-conditioning and simultaneous occurrence of drought did not improve the photosynthetic performance of *J. curcas* under heat stress. These results are not in accordance with other reports showing acclimatory responses to heat stress after previous drought conditioning (Srivastava and Strasser, 1996; Ribeiro et al., 2008).

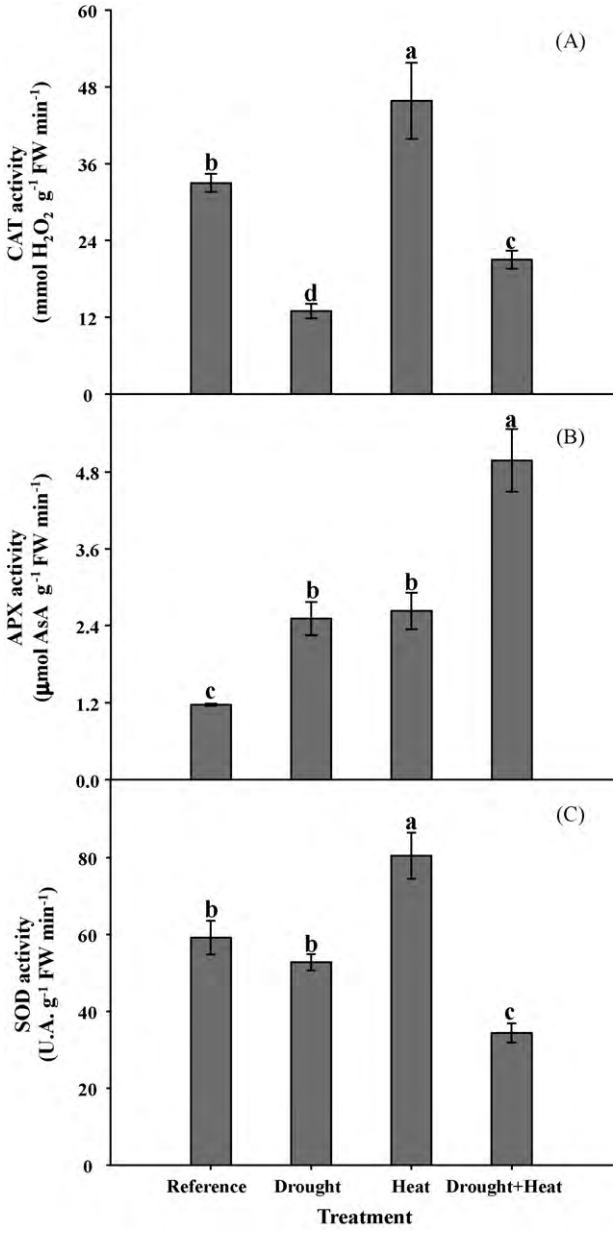

Fig. 4. Catalase activity, CAT (A), ascorbate peroxidase activity, APX (B), and superoxide dismutase activity, SOD (C), in *Jatropha curcas* plants subjected to isolated and combined stresses of drought and heat. Data are mean values of four replicates ± SD. The same letters are not significantly different to 0.05 by Tukey's test.

Discussion

This study reveals that drought, heat and drought + heat stresses were able to induce major changes in key physiological processes of *J. curcas* plants, as indicated by measurements of membrane integrity, leaf gas exchange, chlorophyll fluorescence, oxidative damage indicators and the ROS-scavenging system.

Decreases in the leaf CO_2 assimilation rate were partially attributed to stomatal (low g_S) and metabolic limitations (low P_N/C_I) under constraining conditions, mainly under drought stress (Fig. 2). The maintenance of an intercellular CO_2 concentration (data not shown) associated with the low leaf CO_2 assimilation rate provided additional evidence of non-stomatal limitation of photosynthesis in *J. curcas* under stressful conditions. However, reductions in g_S were not associated with low leaf water potential during heat treatment, suggesting an acclimatory response of

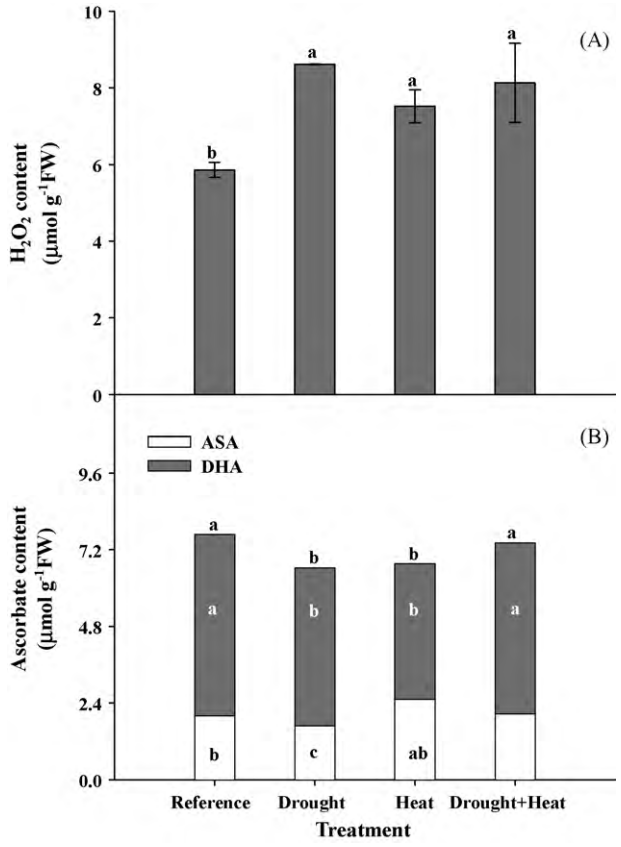

Fig. 5. Leaf contents of hydrogen peroxide, H_2O_2 (A), reduced (ASAred) and oxidized (ASAox) ascorbate (B) in *Jatropha curcas* plants subjected to isolated and combined stresses of drought and heat. Data are mean values of four replicates ± SD. The same letters are not significantly different to 0.05 by Tukey's test.

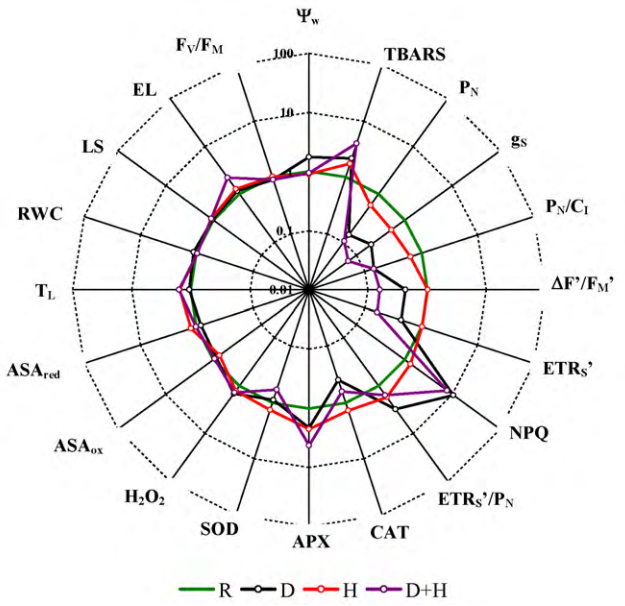

Fig. 6. Overall representation of *J. curcas* physiological responses to drought (D, black line), heat (H, red line) and drought + heat (D + H, violet line) stresses by a radar plot. Data refer to the relative changes of the following variables: leaf water potential (Ψ_w), lipid peroxidation (TBARS), leaf CO_2 assimilation rate (P_N), stomatal conductance (g_S), instantaneous carboxylation efficiency (P_N/C_I), maximum (F_V/F_M) and actual ($\Delta F'/F_M'$) quantum yield of primary photochemistry, apparent electron transport rate (ETR'_S), non-photochemical quenching (NPQ), ratio between apparent electron transport rate and CO_2 assimilation rate (ETR'_S/P_N), catalase activity (CAT), ascorbate peroxidase activity (APX), superoxide dismutase activity (SOD), leaf contents of hydrogen peroxide (H_2O_2), reduced (ASAred) and oxidized (ASAox) ascorbate, leaf temperature (T_L), relative water content (RWC), leaf succulence (LS) and electrolyte leakage (EL). The reference plants were grown without drought or heat stresses (R, green line). The *Y*-axis has a logarithmic scale. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

stomata to the increasing temperature itself or to an increasing air vapor pressure deficit (Ribeiro et al., 2004).

In addition to the changes observed in leaf gas exchange, photochemical activity was also affected by drought and combined stress but not by heat stress only. Indeed, the actual quantum yield of primary photochemistry and the apparent electron transport rate were strongly decreased by those stresses (Fig. 3). As ETR'_S may be considered as an overall index of photochemical activity, our data suggest that drought and drought + heat treatments caused significant deactivation of the electron transport chain in thylakoid membranes (Chagas et al., 2008). These changes are probably associated with damage caused on the primary electron acceptors of PSII (plastoquinone) due to reduced quinone accumulation (Foyer and Noctor, 2000), as revealed by the significant reduction in $\Delta F'/F'_M$ (Fig. 3).

Increases in NPQ were noticed when *J. curcas* plants were subjected to isolated drought stress or in combination with heat. High NPQ values indicate that the non-radiative energy dissipation mechanism is active and that a higher proportion of energy is lost as heat instead of being used to drive photosynthesis (Ribeiro et al., 2009). Such thermal dissipation of the excessive excitation energy is considered to be a photo-protective mechanism, which maintains the primary electron acceptors of PSII in an oxidative state and reduces the probability of photodamage (Souza et al., 2005). In fact, this mechanism is more active in drought-stressed plants (Srivastava and Strasser, 1996).

In addition, the highest ETR'_S/P_N ratios were found in *J. curcas* plants subjected to drought, suggesting that more electrons are driven to alternative sinks rather than to the CO_2 assimilation reactions (Ribeiro et al., 2009). Those alternative sinks probably provided additional protection to PSII activity, as $\Delta F'/F'_M$ and ETR'_S were less affected in drought-stressed plants when compared to those subjected to drought + heat. Drought stress caused the greatest effect on the leaf gas exchange and photochemistry in *J. curcas*. Moreover, simultaneous occurrence of heat + drought stress increased the negative effect of drought on the photochemical activity, as indicated by the lowest values of $\Delta F'/F'_M$ and ETR'_S observed in the plants subjected to drought combined with heat (Fig. 3).

The combined drought + heat treatment was most deleterious for the leaf gas exchange and photochemical activity, in accordance with the results for membrane integrity (EL) and lipid peroxidation (TBARS levels). The increased EL showed a positive correlation with TBARS accumulation, suggesting that the injuries induced in the plasmalemma by drought + heat stress were, at least in part, a consequence of oxidative damage. Numerous investigations have demonstrated that injuries caused by abiotic stresses on the plant cells are triggered in part by oxidative stress (Asada, 1999).

The SOD–APX–CAT system protects the photosynthetic machinery from oxidative damage in plants exposed to environmental stresses (Cavalcanti et al., 2004). SOD scavenges the $O_2^{\bullet -}$ generated by the electron transport chain in the chloroplasts and mitochondria, and the H_2O_2 produced by SOD activity is then eliminated by APX in different cell compartments (Shigeoka et al., 2002). In addition, CAT removes the H_2O_2 generated in the photorespiration pathway inside the peroxisomes (Mittler, 2002). In the current study, oxidative stress was clearly established in *J. curcas* leaves in all stress treatments, as indicated by increased lipid peroxidation (Fig. 1C). The intensity of lipid peroxidation was higher in plants

subjected to the combined stress, suggesting the development of major oxidative damages in leaf tissues. Cavalcanti et al. (2007) reported that TBARS accumulation in leaves is a good indicator of oxidative damage in plant tissues.

The CAT and SOD activities were stimulated by heat treatment, but were strongly inhibited in the combined stress conditions (Fig. 4B and C). The inhibition of the CAT and SOD activities by drought and by the combination of drought and heat may be a consequence of down-regulated gene expression or of degradation, denaturation and/or inhibition/inactivation of these proteins (Cavalcanti et al., 2007). For instance, a light-dependent decrease in total CAT protein and activity has been reported in response to salinity (Hertwig et al., 1992). Furthermore, ongoing protein synthesis is required to maintain CAT activity under conditions in which degradation exceeds resynthesis and the enzyme activity otherwise decreases (Cavalcanti et al., 2004). Conversely, the increased SOD and CAT activities in *J. curcas* leaves subjected to heat stress could be attributed to heat-induced up-regulation of gene expression and/or activation of protein isoforms.

In contrast to the CAT and SOD activities, the APX activity was increased in plants subjected to all stress treatments, but especially in the drought + heat combination. Several APX isoforms are widely distributed in almost all cell organelles, and abiotic stresses frequently induce increases in both the gene expression and APX total activity in order to compensate for deficiencies in CAT activity (Palatnik et al., 2002). However, the increase in total APX activity did not apparently compensate for the impaired CAT activity because H_2O_2 significantly accumulated in leaf tissue. The excess H_2O_2 produced in peroxisomes and chloroplasts might diffuse to the cytosol and be converted to hydroxyl radicals by the Fenton reaction (Møller et al., 2007). These are the most toxic ROS and are directly involved in lipid peroxidation (Foyer and Noctor, 2000).

Our results indicate that the ROS-scavenging system was not sufficient to protect *J. curcas* leaves against oxidative damages, especially those induced by the combined stresses of drought and heat, as confirmed by the high levels of lipid peroxidation and H_2O_2 accumulation (Figs. 1C and 5A). The hydrogen peroxide accumulated in leaves of *J. curcas* under stressful conditions may have been generated mostly by photorespiration, a process mainly located inside peroxisomes. This assumption is supported by the decreased CAT activity (Fig. 4) and increased ETR'_S/P_N ratios (Fig. 3D) under constraining conditions. Photorespiration is a major alternative electron sink in C3 plants (Osmond and Grace, 1995) that was probably stimulated by the stressful conditions within our study.

The ETR'_S/P_N ratios reached very high values (around 60), indicative of photosynthetic damage. In fact, we found ETR'_S/P_N around 10 in young citrus plants under non-stressful conditions during the growing season, and when well watered, these plants exhibited ETR'_S/P_N values around 21 in the winter season (Ribeiro et al., 2009). These changes were probably caused by impaired photosynthesis due to low temperature because of a reduced maximum rate of Rubisco carboxylation and maximum regeneration of RuBP, both dependent on the energy supply from the photochemical electron transport.

The data obtained from this study reveals the complexity of the relationships between photochemical damage and oxidative stress under conditions of isolated and combined drought and heat stresses. In fact, changes in the photosynthesis and in the antioxidative enzyme activities are not enough to explain some of the responses by *J. curcas* plants, especially the oxidative damage. As an isolated factor, drought is more stressful than heat, in terms of photosynthesis disturbances (CO_2 assimilation rate and photochemical activity) and oxidative damage. These more severe negative effects are associated with the highest loss of electrons from photosystem II to carboxylation reactions, inhibition of CAT and SOD activities and higher H_2O_2 accumulation.

Whereas drought alone greatly inhibited CAT, stimulated APX and maintained SOD activities in comparison with the reference, the heat treatment significantly stimulated the activity of all three enzymes. Collectively, this heat response is extremely favorable for H_2O_2 scavenging and superoxide radicals, especially in chloroplasts and peroxisomes. The maintenance of a favorable balance between SOD, APX and CAT is essential to avoid ROS accumulation, to preserve the photochemical apparatus, and to avoid significant oxidative damage (Guo et al., 2007), as exhibited in heat-treated plants (Figs. 3 and 1C). In fact, impaired photochemical activity and the abrupt increase in photorespiration are the main causes of ROS accumulation in leaves (Chagas et al., 2008).

Our data clearly suggest that the combined drought + heat condition induced the most negative changes in membrane integrity, photosynthetic assimilation of CO_2, photochemical activity and oxidative metabolism. In fact, we observed impaired photosystem II activity, markedly inhibited CAT and SOD activities and accumulated hydrogen peroxide. Unexpectedly, the intense increase in the total APX activity in leaves of plants subjected to the combined stress was insufficient to avoid significant oxidative damages or peroxide accumulation. The combination of stresses could trigger up-regulation of gene isoforms located mainly in the cytosol but not significantly affect the expression of the chloroplastic isoforms. The cytosolic APX isoforms are most important for maintenance of redox homeostasis in the plant cell (Shigeoka et al., 2002; Mittler, 2002).

Surprisingly, the total APX activity was significantly up-regulated, but the total ascorbate concentration and its redox balance were not altered under the combined stresses. These results suggest that the fraction of reduced ascorbate consumed as a substrate for APX activity was probably derived from newly synthesized ascorbate. Under these conditions, the oxidized ascorbate produced after the reaction of reduced ascorbate with hydrogen peroxide could be catabolized to produce two- and four-carbon products, such as oxalate and tartrate, which can accumulate at relatively high levels in plant cells (Noctor and Foyer, 1998). Thus, measurements of the concentrations of reduced and oxidized forms at specific times only reflect stationary conditions of the ascorbate redox balance.

Our results are in agreement with those reported by Mittler (2006), who observed that the simultaneous exposure of plants to different abiotic stress conditions results in the co-activation of different stress-response pathways. The response to combined stresses is quite different from the sum of the responses to individual abiotic factors, and the stress factors might display antagonistic or synergistic interactions with each other. In the present study, we observed that the combined heat and drought treatment triggers different types of responses (antagonism or synergism) for various processes (photosynthesis, enzymatic antioxidant response, and oxidative damages). However, in general, the combined effects of drought and heat are much more damaging to leaf tissues than those of the individual stresses. However, *J. curcas* young plants are more sensitive to individual drought stress as compared with the isolated heat treatment.

In summary, *J. curcas* plants subjected to the combination of drought and heat exhibited very different oxidative and photosynthetic (leaf gas exchange and photochemistry) responses from those triggered by the isolated stresses or the sum of both stress responses, thereby exhibiting a negative interactive response. Drought was more damaging in terms of oxidative stress and photosynthetic damage than heat stress. The heat treatment was less deleterious because plants exhibited an up-regulation in the activities of CAT, APX, and SOD and had a favorable redox balance between the reduced and oxidized forms of ascorbate. In conclusion, *J. curcas* plants exhibited acclimatizing mechanisms to the stresses studied; however, they did not present an efficient mech-

anism for protection against drought- and drought + heat-induced oxidative stress and were unable to avoid H_2O_2 accumulation and significant oxidative stress, especially under the combined stress conditions.

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