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Photosynthesis and fluorescence responses of *Jatropha curcas* to chilling and freezing stress during early vegetative stages

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ABSTRACT

Jatropha curcas is a promissory species for biodiesel production. Chilling and freezing stress are major environmental constraints for its establishment as a result of the injury provoked on leaf photosynthetic apparatus. This study is aimed at evaluating the impact of chilling (40 h at 4 °C) and freezing (2 h at −1, −2 and −3 °C) on maximum leaf photosynthesis (A_{max}), in relation to stomatal conductance (g_s) and photochemical activity. Two similar experiments were conducted in pots outdoors; treatments were performed in climate chambers at the stage of four expanded leaves per plant, and then returned outdoors. Leaf gas exchange, water status and fluorescence variables were measured at 1 and 30 days after the end of the treatments (DAT). At 1 DAT, A_{max} and g_s were reduced up to 75% and 100% in chilling and freezing treatments, respectively. However, the intercellular CO_2 concentration (C_i) showed an inverse pattern, discarding a determinant role in A_{max} reductions. A lower efficiency electron use for photosynthesis was detected for plants subjected to chilling and freezing stress. The potential efficiency of PSII (Fv/Fm), chlorophyll content (Chl) and relative water content (RWC) were only affected by the lowest freezing treatments, while chilling and intermediate freezing plants showed an increase of the non photochemical quenching (NPQ). Leaf death occurred in the lowest freezing treatments, while several residual effects on A_{max}, g_s and electron transport rate (ETR) were also observed at 30 DAT in the survival plants. This work sheds light on the determinant processes involved in the depletion of photosynthesis by chilling and freezing injuries, revealing that low temperatures have persistent and detrimental effects on *J. curcas* crop establishment.

1. Introduction

Jatropha curcas L. is a perennial shrub belonging to the family of the Euphorbiaceae and native to tropical America. This species is a promising non-edible crop for biodiesel production as it satisfies the major specifications of biodiesel standards (Azam et al., 2005; Achten et al., 2008). Nowadays, it is being incorporated with relative success in some tropical and subtropical areas of Africa and Asia (Openshaw, 2000). This has made it possible to establish it as a crop in other sites around the world, although certain environmental conditions such as temperature regimes may set some limitations to the plantations.

J. curcas has been proposed as a perennial crop for marginal environments, due to its ability to grow under harsh conditions (e.g. scarce rainfall and poor soil), in which most of the traditional grain crops do not succeed (Silva et al., 2010b). In this respect, several contributions have been made in relation to the performance of this species when dealing with water shortage (Kheira and Atta, 2009; Achten et al., 2010; Silva et al., 2010a; Silva et al., 2010b; Wang et al., 2011; Kesava Rao et al., 2012; Silva et al., 2012a; Silva et al., 2012b; Fini et al., 2013; Sapeta et al., 2013), salinity (Kumar et al., 2008; Silva et al., 2010b; Díaz-López et al., 2012; Rajaona et al.,

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Abbreviations: DAT, Days after the end of the treatment; DAS, Days after sowing; T_c, Control treatment at outdoors everytime; T_b, Chamber control treatment subjected to continuous 15 °C during 48 h; T_4, Chilling treatment subjected to 4 °C during 40 h; T_{-1}, Freezing treatment subjected to −1 °C during 2 h; T_{-2}, Freezing treatment subjected to −2 °C during 2 h; T_{-3}, Freezing treatment subjected to −3 °C during 2 h.

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2012; Silva et al., 2012b; dos Santos et al., 2013), flooding (Gimeno et al., 2012) and nutrient deficiency (Wang et al., 2011; Fernandes Rodrigues et al., 2013; Rajaona et al., 2013). All these works have analyzed the potential of this species to cope with different abiotic stresses. However, just a few works attempted to unveil the effects of low temperatures on the physiological functioning of this species.

According to its tropical origin, *J. curcas* is vulnerable to low temperatures, especially at seedling (Yang et al., 2007; Andrade et al., 2008; Zheng et al., 2009; Windauer et al., 2012) and reproductive stages (Maes et al., 2009). Therefore, its cultivation in areas affected by low temperatures – both slightly-above and below zero degrees (i.e. chilling and freezing stress, respectively) – can be severely restricted. Low-temperature stress may impact on a number of plant physiological traits, being photosynthesis one of the processes most sensitive to this stress (Burke et al., 1976; Graham and Patterson, 1982; Pearce, 2001; Ruelland et al., 2009; Zheng et al., 2009). In this respect, the few available reports have shown negative impacts of chilling (Liang et al., 2008; Zheng et al., 2009) and freezing (Andrade et al., 2008) on photosynthesis in *J. curcas* seedlings associated to the damage of photosystem II (i.e. photoinhibition).

No information is available about the relative contribution of photochemical activity and stomatal conductance as determinants of photosynthesis depletion produced by low temperatures in *J. curcas*. The principal effect produced by stomatal closure in other species is due to the limiting CO_2 available for the Calvin cycle, and this is clearly evidenced by a depletion of the intercellular CO_2 concentration in stomatal cavity (C_i) (Taiz and Zeiger, 1998). Nevertheless, both photochemical and biochemical dysfunctions were found under severe drought, suggesting that similar processes could occur under low temperature stress. If this were the case, high C_i could be accumulated as consequence of internal impairments, independently of stomatal conductance. This paper addresses this issue.

Understanding photosynthetic responses of *J. curcas* seedlings to chilling and freezing stress is crucial in order to improve its management as a crop. Photosynthetic responses to low temperatures (immediate and after a recovery period) might define its chances to grow after a stress period, which is closely related with the success of crop establishment after plantation. Thus, the aim of this work is to make a contribution to these issues in seedling of *J. curcas* exposed to different low-thermal regimes, including both chilling and freezing. The involved hypotheses are H1) the lower photosynthesis produced after cold stress is associated not only with changes of photosystem II activity but also with a lower stomatal conductance, and H2) the effect of stomatal conductance on photosynthesis is produced by lowering CO_2 availability in stomatal cavity (C_i) for biochemical reactions.

2. Materials and methods

2.1. General experimental design

Two similar experiments were performed in the experimental garden of the Faculty of Agronomy of the University of Buenos Aires, Argentina (34°35'S, 58°29'W) in 2009 (Exp. 1) and 2010 (Exp. 2), respectively. *J. curcas* seeds were obtained from a plantation located in Siete Palmas, Formosa, Argentina (25°13'S, 58°17'W), which was originally established from seeds of native trees in 2007. Seeds were sown in 3/l pots (three seeds per pot) containing a mixture of local soil and sand (5:4 v:v), on January 8th, 2009 (Exp. 1) and February 2nd, 2010 (Exp. 2). Seedlings were subsequently thinned to one per pot. Pots were distributed along rows 0.3 m apart with 30 pots per row, following a completely randomized design ($n = 24$). In order to avoid nutrient deficiency, each plant was fertilized (5 g per pot) with nitrogen, phosphorus and potassium (15:15:15) at 20 days after sowing (DAS). Pots were hand-weeded and well-watered to maintain target plants without competition and good water status condition. Cipermetrine 25% (20 $cm^3\ hl^{-1}$) and Zineb 70% (20 $g\ hl^{-1}$) were applied every 15 days to prevent pest and fungal diseases. Mean daily temperature and radiation records were taken from an automatic meteorological station (Campbell 21X, Campbell Scientific, Logan, Utah) located 200 m away from the experimental site. Air temperature and photosynthetic active radiation (PAR) were monitored at hourly intervals, with a 083E sensor (Campbell Scientific, Logan, Utah) placed into a white shield and a Li-190 Quantum sensor (Li-Cor, Lincoln, Nebraska), respectively. Both sensors were placed at 2 m height.

2.2. Low temperature treatments

Treatments were applied when 50% of the seedlings reached the stage of four fully expanded leaves (48 and 51 DAS for Exps. 1 and 2, respectively), by transferring them to climate chambers (Sanyo MIR 253, Sanyo Biomedical, Illinois) with irradiance of 300 $\mu mol\ m^{-2}\ s^{-1}$ of photosynthetic photon flux density (PPFD). Climate chambers thermal regimes were manipulated to impose treatments: chilling simulation (T_4), which consisted of seedlings exposure to 4 °C for 40 h; and three freezing temperatures simulations (T_{-1}, T_{-2} and T_{-3}), which consisted of seedlings exposure to −1 °C, −2 °C and −3 °C for 2 h, respectively (Fig. 1).

Acclimating periods of 3 h and 12 h at 15 °C were allowed before imposition of low chilling and freezing treatments, respectively. Also, for freezing treatments, an additional period of 4 h at 4 °C was also considered for seedlings acclimation, thus avoiding thermal shock. At the end of treatments, seedlings were re-exposed to 15 °C for 4 h or 3 h (chilling and freezing, respectively), and moved to the experimental garden. T_{-3} treatment was only present in Exp. 1. Importantly, two groups of seedlings were considered as controls.

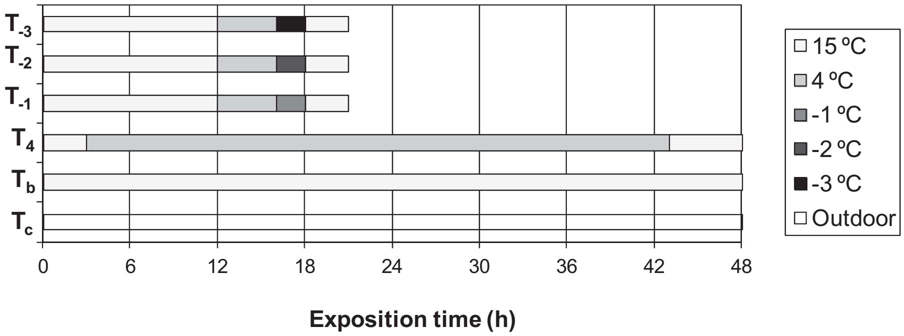

Fig. 1. Scheme of treatments arranged in the climatic chambers for the different treatments in the two experiments. Horizontal bars indicate the sequence of different temperature regimes. T_{-3} only for Exp. 1.

A first group remained growing under natural field conditions (T_c), and a second seedlings group was moved to a climate chamber and used as a chamber control treatment (T_b). The latter consisted of exposing seedlings to 15 °C for 48 h with the purpose of discarding any potential artefact associated to the growing conditions of the climate chambers. This allowed us to set comparisons among treatments and experiments in a more reliable way (see a detailed scheme of treatments in Fig. 1).

All measurements were made at 1 and 30 days after the end of the temperature treatments (DAT). This allowed us to separate and to analyse the immediate effects provoked by the treatments, from those associated with the recovery of seedlings one month after the stresses imposition.

2.3. Gas exchange and fluorescence measurements

In Exp. 1, photosynthetic rate under saturating irradiance (A_{max}) was measured using a Li-6200 Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA), using a 0.25 l chamber attached to a regulated portable red light power (QB1 205LI-670, Quantum Devices Inc., Barneveld, WI) at 1500 μmol m^{-2} s^{-1} PPFD. Stomatal conductance (g_s) was measured using a porometer model AP4 (Delta-T Devices, Cambridge, UK). Air temperature into the chamber during measuring periods was 34.5 ± 0.48 °C and 30.9 ± 0.19 (mean ± standard error, n = 30) for 1 and 30 DAT respectively.

In Exp. 2, A_{max}, g_s, and C_i were measured using a Li-6400 Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA) under 2000 μmol m^{-2} s^{-1} PPFD. Saturating light was provided by the 6400-40 leaf chamber fluorometer using a mix of 80% red and 20% blue light. Air flow, CO_2 concentration in the reference chamber and block temperature were controlled automatically by the equipment at 300 μmol s^{-1}, 400 μmol mol^{-1} (ppm) and 27 °C, respectively. Modulated fluorescence parameters for light acclimated leaves under outdoor conditions (Maxwell and Johnson, 2000; Lichtenthaler et al., 2005) were simultaneously measured with gas exchange parameters under steady 2000 μmol m^{-2} s^{-1} PPFD. Leaf acclimation into the chamber was ensured as each measurement was made after reaching stability with the environment of the sample cell. Thus, slopes with time (seconds) were (i) <1 for CO_2 concentration of the sample cell (CO_2S parameter, μmol mol^{-1}) and water concentration (H_2OS parameter, mmol mol^{-1}) and (ii) <0.5 for fluorescence signal (F parameter) and g_s (Cond parameter, mol m^{-2} s^{-1}).

Calculation of the overall parameters was made using the OPEN 5.2 software (Li-Cor Inc, Nebraska) of the equipment. Parameters measured simultaneously with those for gas exchange were estimated according with Maxwell and Johnson (2000). Electron transport rate (ETR), was calculated by the following equation:

$$\mathrm{ETR} = \Phi_{\mathrm{PSII}} \times \mathrm{PPFD_a} \times 0.5 \quad (1)$$

where Φ_{PSII} is the actual quantum yield of PSII, $\mathrm{PPFD_a}$ is the absorbed light (μmol photon m^{-2} s^{-1}) and 0.5 is a factor that accounts for the partitioning between PSII and PSI. Φ_{PSII} was estimated by:

$$\Phi_{\mathrm{PSII}} = \frac{(\mathrm{Fm'} - \mathrm{Ft})}{\mathrm{Fm'}} \quad (2)$$

where Fm′ is the maximum fluorescence intensity produced by a 1 s saturating flash (PPFD around 7000 μmol m^{-2} s^{-1}) and Ft is the fluorescence intensity at steady-state photosynthesis under a PPFD of 2000 μmol m^{-2} s^{-1}. Photochemical quenching (qP, the proportion of open PSII reaction centres) was estimated by the equation:

$$\mathrm{qP} = \frac{(\mathrm{Fm'} - \mathrm{Ft})}{(\mathrm{Fm'} - \mathrm{Fo'})} \quad (3)$$

where Fo′ is the minimum fluorescence intensity after a dark pulse of 6 s and a far red pulse of 10 μmol m^{-2} s^{-1} during 5 s (started 1 s before the light goes off). The intrinsic efficiency of PSII (Fv′/Fm′, the quantum efficiency if all PSII centres were open) was also estimated by the following equation:

$$\frac{\mathrm{Fv'}}{\mathrm{Fm'}} = \frac{(\mathrm{Fm'} - \mathrm{Fo'})}{\mathrm{Fm'}} \quad (4)$$

Additionally, maximum PSII (Fv/Fm) quantum yield was determined by an independent set of measurements after 30 min of acclimation in the dark (Cabrera-Bosquet et al., 2009) by:

$$\frac{\mathrm{Fv}}{\mathrm{Fm}} = \frac{(\mathrm{Fm} - \mathrm{Fo})}{\mathrm{Fm}} \quad (5)$$

where Fm is the maximum fluorescence intensity produced by a 1 s saturating flash (PPFD around 7000 μmol m^{-2} s^{-1}) under dark acclimation and Fo is the minimal fluorescence before the flash pulse. Mean Fv/Fm values for each treatment were used as inputs to estimate the non-photochemical quenching (NPQ) for the measurements on light-acclimated leaves by the equation:

$$\mathrm{NPQ} = \frac{(\mathrm{Fm} - \mathrm{Fm'})}{\mathrm{Fm'}} \quad (6)$$

For both experiment, leaf gas exchange and chlorophyll fluorescence were measured between 11:00 and 15:00 h on the youngest fully expanded leaf.

2.4. Chlorophyll and relative water content measurements

Chlorophyll content (Chl) of the youngest fully expanded leaves was estimated at midday using a portable chlorophyll meter (SPAD 502, Minolta Camera Co., Osaka, Japan). For each replicate (plant), chlorophyll content was the result of the average of 5 measurements on the same leaf. Relative water content (RWC) was also measured at midday following the methodology proposed by Barr and Weatherley (1962), which briefly consisted of three steps. First, disc samples (2 cm^2) from the youngest fully expanded leaf were cut, and immediately weighed to obtain their fresh weight (FW). Second, the same discs were allowed to float for 2 h in Petri dishes with distilled water and, after a gentle blotting to remove water excess, they were weighed to obtain turgid weight (TW). Finally, discs were weighed after drying them at 60 °C for 48 h until constant dry weight was reached (DW). Having these three parameters, the RWC was calculated using the following equation:

$$\mathrm{RWC}(\%) = \frac{(\mathrm{FW} - \mathrm{DW})}{(\mathrm{TW} - \mathrm{DW})} \times 100 \quad (7)$$

2.5. Statistical analyses

For each set of measurements made at 1 and 30 DAT, parameters were compared using one-way ANOVA. Tukey test comparisons were performed when significant differences between treatments were detected. Differences between years for mean temperature and PAR radiation under field conditions were analysed by Student's t-test.

A piecewise linear regression model was used to estimate the association between A_{max} and ETR (Feder, 1975), in order to detect changes in electron use efficiency for photosynthesis:

$$A_{max} = a + b\ \mathrm{ETR} + c\ (\mathrm{ETR} - d)\ \mathrm{if}\ (\mathrm{ETR} > d) \quad (8)$$

where a is the intercept, b and c are the different slopes of the linear regressions and d is a breakpoint of the function.

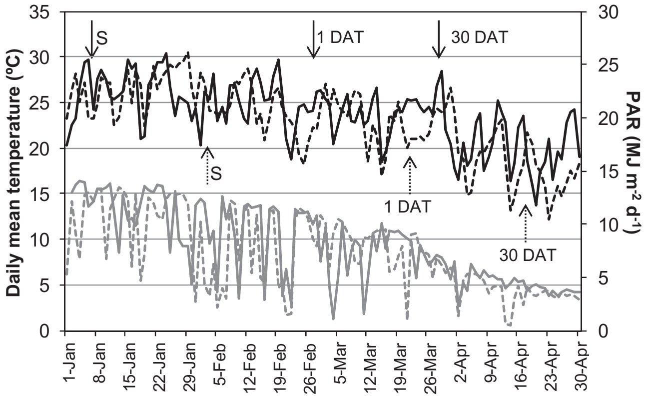

Fig. 2. Daily mean temperature (black lines) and daily photosynthetic active radiation (PAR, grey lines) for Exp. 1 (2009, solid lines) and Exp. 2 (2010, dashed lines), along the period when the experiments were arranged. Arrows indicate sowing date (S), the first measurement date at 1 day after the end of the treatment (DAT), and the second measurement date made at 30 DAT. Descendent and solid arrows for Exp. 1; ascendant and dotted arrows for Exp. 2.

3. Results

3.1. Environmental conditions outdoors

Similar environmental regimes were registered for the two seasons (January to April 2009 and 2010, Fig. 2). Average mean daily temperature was 23.7 ± 0.3 °C and 22.9 ± 0.3 °C and mean daily PAR radiation was 8.1 ± 0.33 MJ m^{-2} d^{-1} and 7.4 ± 0.33 MJ m^{-2} d^{-1} for 2009 and 2010, respectively, (mean ± standard error, average of 120 days). As expected, both temperature and PAR were higher in January and gradually decreased as autumn approached. When temperature and PAR field conditions were contrasted between experiments in the same ontogenic periods (from sowing to 1 DAT and 1 DAT to 30 DAT, Table 1), significant differences ($P < 0.0001$) were found for the overall growing period between years. In general terms, plants were subjected to higher temperature and PAR regimes in Exp. 1, respectively, reaching 4 °C and 3 MJ m^{-2} d^{-1} higher than Exp. 1, in the period between 1 and 30 DAT.

3.2. Photosynthesis and stomatal conductance

In both experiments, all cold treatments clearly reduced A_{max} and g_s values (Fig. 3). At 1 DAT, reductions reached 75% for T_4 in Exp. 2, while a drastic impact was found for freezing treatments, reaching null and negative values in T_{-3} and T_{-2} for Exps. 1 and 2, respectively. Thus, plants did not recover from these freezing treatments, determining the death of leaves and buds and the impossibility to be measured at 30 DAT.

In contrast, partial recoveries were observed at 30 DAT for T_4 and the intermediate freezing treatments of both experiments. In fact, the negative effects of the chilling and freezing treatments still persisted, although very low values were also found for controls in Exp. 1 with respect to those observed for 1 DAT.

As expected, no significant differences were detected between T_c and T_b treatments for both A_{max} and g_s, independently of time after treatment application, which indicated that environmental conditions of climate chambers did not provoke artefacts on the measured physiological responses.

3.3. Chlorophyll and relative water content

The response pattern observed for chlorophyll clearly differed from that described above for A_{max} and g_s. Chl values were around 35 and 45 Spad units for Exps. 1 and 2, respectively, for T_c, T_b, T_4 and T_{-1} treatments, although a similar response was found in T_{-2} treatment only in Exp. 1 (Fig. 4A and B). A drastic reduction, however, was produced by the coldest treatments in both experiments, reaching 75% in T_{-3} for Exp. 1 at 1 DAT. No changes in water status were observed for the coldest treatments either, with the exception of T_{-3} treatment in Exp. 1, showing a dramatic decrease to 20% (Fig. 4C and D). Similar trends were observed at 30 DAT for plants with survival leaves.

3.4. C_i and fluorescence parameters

The pattern observed for C_i at 1 DAT contrasts with that expected for a limiting effect produced by stomatal closure. Contrarily, the lower the photosynthesis and stomatal conductance, the higher the intercelular concentration. Thus, CO_2 concentration reached three-fold higher values in T_{-2} plants than those for T_c and T_b treatments (Fig. 5A). Neverthless, no significant changes were detected at 30 DAT, in spite of the persistence of detrimental effects of cold treatments on photosynthesis and stomatal conductance.

No injuries in Fv/Fm were detected at 1 DAT in T_4 and T_{-1} treatments, since values were near 0.8, being the same as for T_c and T_b controls (Fig. 5B). This pattern was similar to that observed for Chl units (Fig. 4B) and persisted at 30 DAT. Nevertheless, Fv/Fm was severely damaged in the coldest treatment T_{-2}, with a Fv/Fm around 0.2. In contrast, the pattern of Fv′/Fm′ was affected in both freezing treatments at 1 DAT (Fig. 5C). Thus, a drastic reduction of nearly 75% was observed for T_{-2}, a lower although significant effect was detected for T_{-1}, while a non significant tendency was also found in T_4 treatment. As a difference with that observed for A_{max} and g_s, total recovery was observed at 30 DAT in this variable.

Following a similar response to that observed for gas exchange parameters at 1 DAT, photochemical quenching was slightly reduced to 0.3 in T_4 and T_{-1} plants and highly reduced to 0.1 in T_{-2} treatment; while no significant differences were detected between T_c and T_b treatments, with values around 0.5 (qP, Fig. 5D). However, a total recovery was observed for this trait at 30 DAT for the survival treatments. The non- photochemical quenching (NPQ) followed an increasing response in T_4 and T_{-1} treatments at 1 DAT (Fig. 5E), as expected for the decreasing one observed in qP for these treatments (Fig. 5D). However, a great and unexpected reduction, near zero, was found for T_{-2}, in spite of the low qP also observed for this treatment. Finally, ETR responses showed a similar pattern to that for qP (Fig. 5F), but reduction significance still remained at 30 DAT in T_{-1} treatment.

Table 1
Mean daily temperature and PAR radiation for the periods between sowing (S) and the first measuring date made at 1 day after the establishment of the cold treatments (DAT), and between 1 DAT and the second measuring date, at 30 DAT. Average ± Standard errors. Different letters indicate significant differences between experiments for each variable and period ($P < 0.0001$).

	Mean daily temperature (°C)		Mean daily PAR (MJ m^{-2} d^{-1})	
	S-1 DAT	1 DAT-30 DAT	S-1 DAT	1 DAT-30 DAT
Exp. 1 (2009)	25.6 ± 0.38a	24.0 ± 0.35a	10.2 ± 0.45a	7.5 ± 0.44a
Exp. 2 (2010)	23.5 ± 0.38b	20.0 ± 0.60b	7.7 ± 0.44b	4.6 ± 0.34b

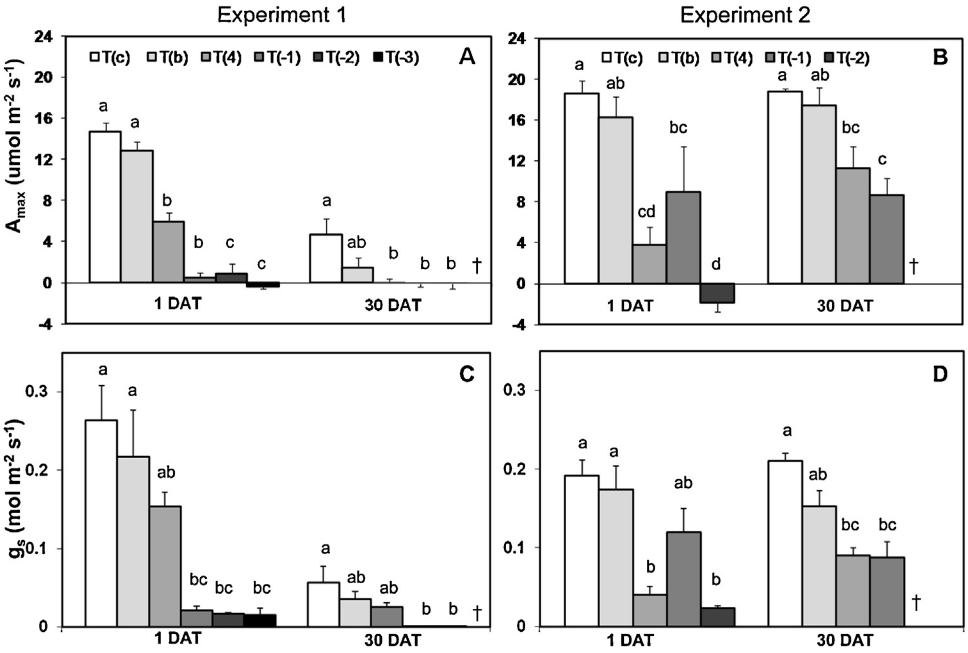

Fig. 3. Net photosynthesis rate at saturating irradiation (A_{max}, A and B) and stomatal conductance (g_s, C and D) for leaves from plants subjected to different temperature regimes, for Exp. 1 (A and C) and Exp. 2 (B and D), and for the two measurement dates made at 1 day after the end of the treatment (DAT) and 30 DAT. Values are means ± standard errors and different letters indicate significant differences ($P < 0.05$) for each variable and measurement date. † = Dead.

3.5. Fluorescence and gas exchange relationships

Statistical analysis of the relationship between A_{max} and ETR revealed a biphasic dynamics, estimated by a piecewise regression model (Fig. 6). In general terms, A and ETR increased linearly with an initial slope of 0.08 up to a threshold ETR of 108.4 μmol m^{-2} s^{-1}. This phase involved only plants subjected to cold treatments (T_4, T_{-1}, T_{-2}). After the ETR threshold, there was an increase of the slope to 0.11 where most of the fitted points corresponded to T_c and T_b treatments, with the exception of few points from T_4 and T_{-1} plants. Among cold treatments, T_{-2} plants presented the lowest ETR values and variability; while a high variability was observed for both T_4 and T_{-1} plants, with values covering not only the range before the ETR threshold but also the subsequent one.

The relationship between A_{max} and C_i of individual plants at 1 DAT showed two defined patterns (Fig. 7A). An increasing pattern according to that expected for a C3 species under limiting C_i (Taiz and Zeiger, 1998) involved all T_c and T_b plants and a fraction of those in T_4 and T_{-1} However, a lack of response of A_{max} under a wide range of C_i concentrations, was found for the overall T_{-2} plants and the

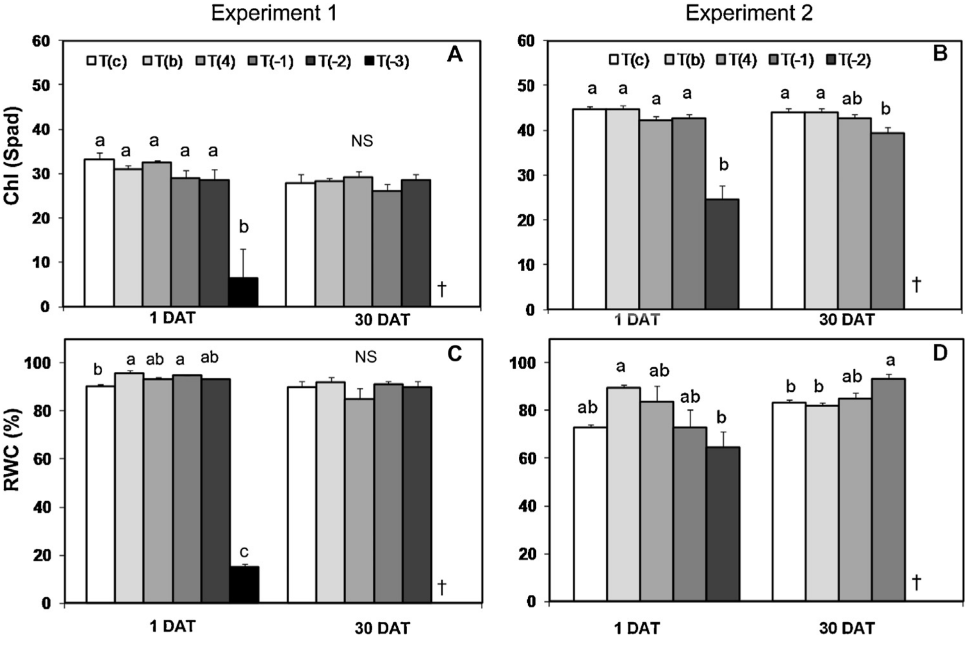

Fig. 4. Chlorophyll content (Chl, A and B) and relative water content (RWC %, C and D) for leaves from plants subjected to different temperature regimes, for Exp. 1 (A and C) and Exp. 2 (B and D), and for the two measurement dates made at 1 day after the end of the treatment (DAT) and 30 DAT. Values are means ± standard errors and different letters indicate significant differences ($P < 0.05$) for each variable and measurement date. † = Dead.

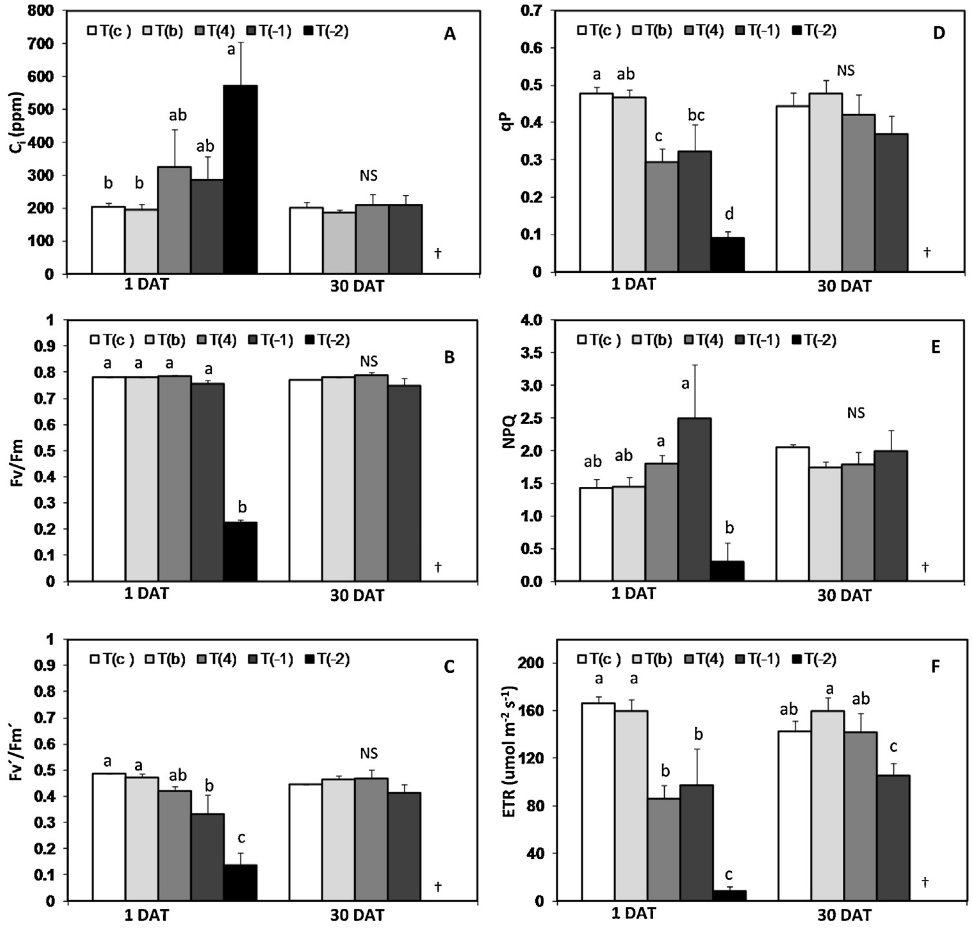

Fig. 5. Intercellular CO_2 concentration (C_i, A), maximum quantum yield of PSII (Fv/Fm, B), efficiency of excitation energy capture by open PSII reaction centres (Fv′/Fm′, C), photochemical quenching (qP, D), non-photochemical quenching (NPQ, E) and electron transport rate (ETR, F) made for leaves from plants subjected to different temperature regimes (Exp. 2) for the two measurement dates made at 1 day after the end of the treatment (DAT) and 30 DAT. Values are means ± standard errors and different letters indicate significant differences ($P < 0.05$) for each variable and measurement date. † = Dead.

remained T_4 and T_{-1} ones. Again, two different patterns were also observed for the relationship between C_i and g_s (Fig. 7B). A typical saturating response was observed for all T_c and T_b plants and a fraction of the T_4 and T_{-1} ones. On the other hand, a high C_i variability was detected around a narrow g_s range below 0.05 mol m^{-2} s^{-1} for all T_{-2} plants and the remaining fraction of T_4 and T_{-1} ones. Similar patterns, although attenuated, were observed at 30 DAT (Fig. 7C and 7D).

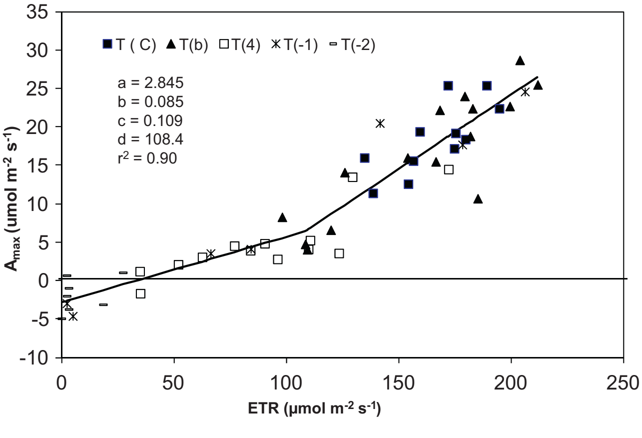

Fig. 6. Net photosynthesis rate at saturating irradiation (A_{max}) as a function of the electron transport rate (ETR), measured in leaves of plants subjected to the different treatments. Regression fitting is based on the piecewise linear regression model described in Eq. (8). Estimated parameters were shown inside the box. Exp. 2.

4. Discussion

Our results revealed that photosynthetic rate under saturating irradiance (A_{max}) was affected by both chilling and freezing regimes, although the impact was more intense in the latter. The effect was directly associated not only with fluorescence parameters measured under light acclimated leaves (qP, Fv′/Fm′, ETR) but also with reductions in stomatal conductance, in agreement with our first hypothesis. Leaf photosynthesis values of non-stressed plants were in general terms similar to others found for this species in previous research (Fukuzawa et al., 2012; Ranjan et al., 2012; Santos Matos et al., 2012). Other traits such as chlorophyll, relative water content and Fv/Fm were only reduced in the most severe freezing treatments (T_{-3} and T_{-2} for Exps. 1 and 2, respectively), in agreement with previous reports that found reductions in chlorophyll content only under severe water stress in *J. curcas* (Pompelli et al., 2010) and other species (Martínez-Ferri et al., 2004; Jaleel et al., 2009; Anjum et al., 2011). Although previous research has shown a negative impact on Fv/Fm produced by the exposition of *Jatropha* seedling under 4 °C during a similar period than that of our experiments (Liang et al., 2008; Zheng et al., 2009), other works revealed a rapid recovery of Fv/Fv, measured 24 h after the end of chilling stress in soybean (Tambussi et al., 2004) and at the end of the day under moderate drought stress in the studied species

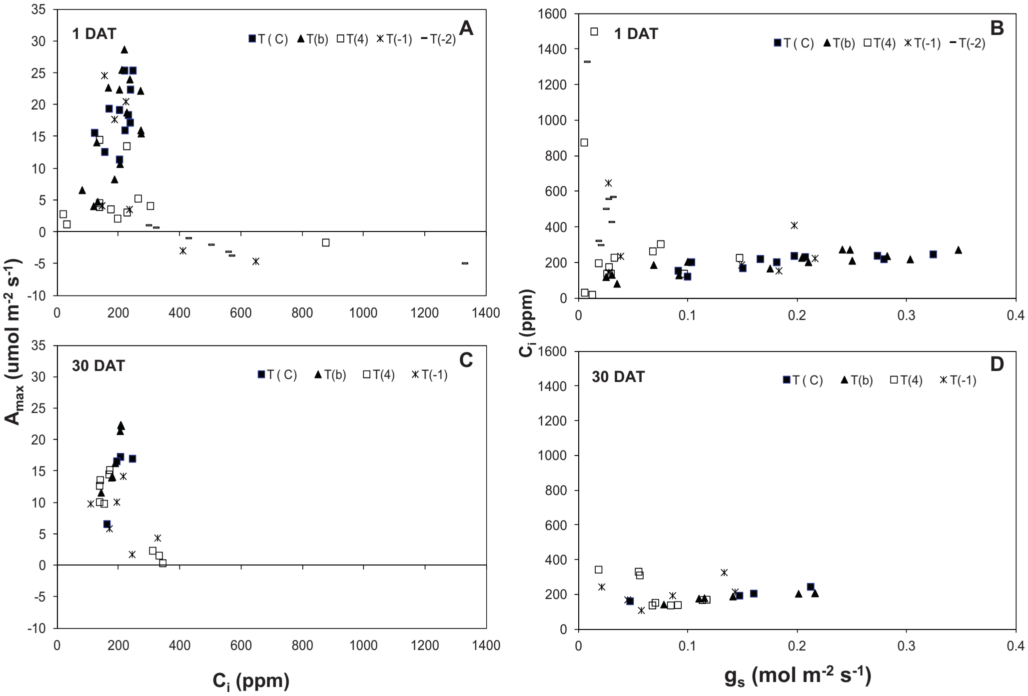

Fig. 7. Net photosynthesis rate at saturating irradiation (A_{max}) as a function of the intercellular CO_2 concentration (C_i) (A, C) and C_i as a function of stomata conductance (g_s) (B, D) measured in leaves of plants subjected to the different treatments at 1 DAT (A, B) and 30 DAT (C, D). Exp. 2.

J. curcas (dos Santos et al., 2013). This process of dynamic recovery from photoinhibition could occur under chilling and less intense freezing treatments of our experiments, discarding any effects on photosynthesis 24 h after the recovery. Further research is needed to corroborate this possibility.

However, the results clearly revealed that reductions of A_{max} were not determined by the lower stomatal conductance because, in contrast with our second hypothesis, this was not linked with lower internal CO_2 concentrations of stomatal cavity (C_i, Fig. 5A and Fig. 7). Thus, limitations in C_i only explained the variability in A_{max} among plants not exposed to low temperature treatments (T_c and T_b), while the absence of such relationship for cold treatments (T_4, T_{-1} and T_{-2}) indicates the presence of other limitations related to changes in several fluorescence parameters (Maxwell and Johnson, 2000). Therefore, although we cannot discard an effect of low temperatures over the mechanisms involved in stomatal closure, our results clearly reject a causal effect of g_s on photosynthesis, because C_i availability was high under low stomatal conductance. The lack of a causal effect of g_s on photosynthesis contrasts with other works conducted under drought stress in *J. curcas* (Sapeta et al., 2013) and other species (Meyer and Genty, 1998 and 1999; Sánchez-Rodriguez Perez and Martínez-Carrasco, 1999). Nevertheless, the higher C_i concentrations found in cold treatments under very low g_s are in agreement with those reported by Gimeno et al. (2012) in *J. curcas* subjected to flooding and salinity (which highlighted the importance of the non-stomal factors in the depletion of photosynthesis) and Flexas et al. (2006) in several species subjected to severe drought. In this way, it is known that high CO_2 concentrations into the stomatal cavity cause stomatal closure (Nobel, 2009). So, our results suggest that part of the lower g_s produced by cold stress might be a consequence (instead of a cause) of the depletion in photosynthesis. This hypothesis merits further experimental investigation, in order to separate direct and indirect effects of low temperatures over stomatal closure.

The negative impact of cold treatments on photochemical quenching (qP) might be a reflection of a higher closure of the reaction centres and light saturation, as a consequence of other diffusion limitations such as a lower mesophyll conductance, because it might limit CO_2 availability for the Calvin Cycle (Flexas et al., 2006). However, our results clearly allow us to conclude that depletion on photosynthesis produced by low temperature treatments is also modulated by phototochemical and other biochemical impairments apart from C_i limitation. First, lower Fv′/Fm′ values indicate that cold stress injury on PSII efficiency under light is independent of the open proportion of reaction centres (Maxwell and Johnson, 2000). Second, the lower initial slope of the A_{max}-ETR relationship (Fig. 6) provides evidence that, when exposed to cold stress, *Jatropha* plants could increase electron transport to alternative electron sinks, such as photorespiration, nitrogen metabolism and electron donation to oxygen (Mehler reaction; Maxwell and Johnson, 2000), in agreement with previous research (Fryer et al., 1998). This lower efficiency represents a reduction of 22% of the electron use efficiency for net photosynthesis, revealing that alternative electron sinks triggered by low temperatures might be highly relevant. This also suggests the possibility that certain processes like the above mentioned would be commonly produced by both chilling and freezing exposures.

Nevertheless, we found other responses revealing specific effects for chilling and freezing processes. Hence, only the lowest freezing treatments (T_{-3} and T_{-2} for Exps. 1 and 2, respectively) showed injuries of potential PSII quantum yield (Fv/Fm, Fig. 5), chlorophyll and relative water content (Fig. 4) at the moment of photosynthesis measurements, with probable generation of active oxygen species and antioxidant components (Fryer et al., 1998; Maxwell and Johnson, 2000; Liang et al., 2008). On the other hand, only in plants exposed to survival cold treatments (T_4 and T_{-1}, Exp. 2) an increased non photochemical quenching was detected (NPQ, Fig. 5), suggesting a probable role of the expression of the xanthophyll cycle in heat dissipation (Adams et al., 1999) as a response to chilling stress. Thus, different mechanisms could be involved in photosynthesis depletion, depending on the nature and intensity of cold stress. In our work, constitutive injury to chlorophyll (measured as chlorophyll content and Fv/Fm) was not detected in chilling and non lethal freezing treatments, at least when measured 24 h after the end of the treatments. If it eventually occurred, a rapid recovery should be performed, turning its effect on outdoor photosynthesis negligible. In addition, it is expected (Maxwell and Johnson, 2000; Lichtenthaler et al., 2005) that the increase of non photochemical quenching in these treatments could occur at expenses of the unaffected PSII potential (Fig. 5B).

Although results were very consistent between the two experiments conducted in this work, the threshold freezing temperature for triggering lethal effects slightly differed between them (−3 °C and −2 °C for Exps. 1 and 2, respectively). A possible explanation is related to the different thermal and radiation regimes outdoors to which the two experiments were subjected, both before and after the establishment of the low temperature treatments (Fig. 2; Table 1). Nevertheless, a higher temperature threshold would be expected in the former, considering that the lower temperatures could have produced a positive acclimation response, according to previous references (Iba, 2002; Hao et al., 2009; Ruelland et al., 2009,; Ao et al., 2013; Wisniewski and Gusta, 2013). In addition, previous research reported minimum lethal temperatures of seedlings between −3 °C and −4 °C in this species (Andrade et al., 2008), suggesting that the threshold freezing temperature for triggering lethal effects would be dynamic and dependent on the interaction with other environmental signals such as freezing duration and acclimating responses (Wanner and Junttila, 1999).

Finally, the negative effects of the non lethal chilling and freezing treatments remained, although attenuated, until at least one month after the end of the treatments, revealing an important residual effect. The depletion in $A_{\max}$ and g_s observed in T_c and T_b plants in Exp. 1 at 30 DAT is unclear, but fortunately this did not avoid detecting the persistence of the residual effects in the cold treatments. Although no significant differences were detected for the most of the fluorescence parameters, photosynthesis, stomatal conductance and ETR remained lower in these treatments, with respect to those for outdoors and controls. However, the lack of a positive association between $A_{\max}$ and g_s with C_i still remained (Fig. 7C and D), suggesting the persistence of the photochemical limitations as determinant factors. The large residual effect produced by exposure of *J. curcas* seedlings to relatively brief periods of cold stress could represent a serious limitation for the development of seedlings by nurseries and a successful crop implantation during the early vegetative stage. In addition, genetic variability and resistant germoplasm for the effect of night chilling have been identified in the studied species (Zheng et al., 2009), which open the possibility for future research to determine if the identified traits in this work are involved in such differences.

5. Conclusions

Several mechanisms are involved in the photosynthesis impairment produced by low temperatures on *J. curcas* plants during their early vegetative stages. Both chilling and freezing effects were related to a higher closure of reaction centres and an increase of electron transport to alternative sinks other than photosynthetic. Only lethal freezing regimes produced stable depletions in water status and constitutive components such us chlorophyll content and the potential PSII efficiency (Fv/Fm). Under chilling and other non lethal freezing regimes, reductions of up to 75% of photosynthesis were associated to a higher non photochemical quenching attributed to heat dissipation. Although stomatal conductance was always linked to the drop in photosynthesis, this association might not be due to a causal effect but a consequence of the higher CO_2 concentration in the stoma. Residual effects were observed up to one month after the end of the cold treatments, suggesting that local low temperature regimes should be considered when planning sowing date, considering their detrimental effects on plant growth and, hence, the establishment of the crop.

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