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 promising 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\(_{\text{max}}\)), 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\(_{\text{max}}\) 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\(_{\text{max}}\) reductions. A lower efficiency electron use for photosynthesis was detected for plants subjected to chilling and freezing stress. The potential efficiency of PSII (F\(_v\)/F\(_m\)), 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\(_{\text{max}}\), 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.

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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 its establishment.

*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.,...
CO2 concentration in stomatal cavity and this is clearly evidenced by a depletion of the intercellular space of the plant (Gimeno et al., 2012; Silva et al., 2012b; dos Santos 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; Rueolland 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 CO2 available for the Calvin cycle, and this is clearly evidenced by a depletion of the intercellular CO2 concentration in stomatal cavity (Ci) (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 Ci 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 CO2 availability in stomatal cavity (Ci) 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 cm3 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 µmol m⁻² s⁻¹ of photosynthetic photon flux density (PPFD). Climate chambers thermal regimes were manipulated to impose treatments: chilling simulation (Tk), 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 $15 \, ^\circ C$ 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 ($A_{\text{max}}$) was measured using a Li-6200 Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA), using a 0.251 chamber attached to a regulated portable red light power (QBI 205SLI-670, Quantum Devices Inc., Barneveld, WI) at 1500 $\mu$mol 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 $\pm$ 0.48 $^\circ C$ and 30.9 $\pm$ 0.19 (mean $\pm$ standard error, $n = 30$) for 1 and 30 DAT respectively.

In Exp. 2, $A_{\text{max}}$, $g_s$, and $C_i$ were measured using a Li-6400 Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA) under 2000 $\mu$mol 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 $\mu$mol 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 $\pm$ 0.48 $^\circ C$ and 30.9 $\pm$ 0.19 (mean $\pm$ standard error, $n = 30$) for 1 and 30 DAT respectively.

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 $^\circ C$ for 48 h until constant dry weight was reached (DW). Having these three parameters, the RWC was calculated using the following equation:

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

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 $A_{\text{max}}$ and ETR (Feder, 1975), in order to detect changes in electron use efficiency for photosynthesis:

$$A_{\text{max}} = a + b \times \text{ETR} + c \times (\text{ETR} - d)$$

where $a$ is the intercept, $b$ and $c$ are the different slopes of the linear regressions and $d$ is a breakpoint of the function.
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. 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 Amax and gs values (Fig. 3). At 1 DAT, reductions reached 75% for T4 in Exp. 2, while a drastic impact was found for freezing treatments, reaching null and negative values in T1 and T2 for Exp. 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 T4 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 Tc and Tb treatments for both Amax and gs, 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. Chlorthyll and relative water content

The response pattern observed for chlorophyll clearly differed from that described above for Amax and gs. Chl values were around 35 and 45 Spad units for Exp. 1 and 2, respectively, for Tc, Tb, T1, T2, and Tc-T1 treatments, although a similar response was found in T2 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 T3 for Exp. 1 at 1 DAT. No changes in water status were observed for the coldest treatments either, with the exception of T3 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. Ci and fluorescence parameters

The pattern observed for Ci 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 intercellular concentration. Thus, CO2 concentration reached threefold higher values in T2 plants than those for Tc and Tb treatments (Fig. 5A). Nevertheless, 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 Tc and T1 treatments, since values were near 0.8, being the same as for Tc and Tb 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 T3-1, 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 T2-1, a lower although significant effect was detected for T1, while a non significant tendency was also found in T4 treatment. As a difference with that observed for Amax and gs, 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 T4 and T1 plants and highly reduced to 0.1 in T2 treatment; while no significant differences were detected between Tc and Tb 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 T4 and T1 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 T2-1, in spite of the low qP also observed for this treatment. Finally, the ETR responses showed a similar pattern to that for qP (Fig. 5F), but reduction significance still remained at 30 DAT in T3-1 treatment.

Table 1

<table>
<thead>
<tr>
<th>Mean daily temperature (°C)</th>
<th>Mean daily PAR (MJ m⁻² d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-1 DAT</td>
<td>1 DAT-30 DAT</td>
</tr>
<tr>
<td>Exp. 1 (2009)</td>
<td>25.6 ± 0.38a</td>
</tr>
<tr>
<td>Exp. 2 (2010)</td>
<td>23.5 ± 0.38b</td>
</tr>
</tbody>
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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; ascendent and dotted arrows for Exp. 2.
3.5. Fluorescence and gas exchange relationships

Statistical analysis of the relationship between $A_{\text{max}}$ 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 $\mu$mol 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 $A_{\text{max}}$ 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 $A_{\text{max}}$ under a wide range of $C_i$ concentrations, was found for the overall $T_{-2}$ plants and the...
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).

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 $Fv/Fm$, 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.
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 $A_{\text{max}}$ 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 $A_{\text{max}}$ 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 (Gimeno et al., 2012) 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-stomatal 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 ($q_P$) 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 photochemical 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 $A_{\text{max}}$-ETR relationship (Fig. 6) provides evidence that, when exposed to cold stress, jatropha plants could increase electron transport to alternative electron sinks, such as photosynthesis, 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; Lichtenhalter 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; Ruellan 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 gs observed in Tc and Tg 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 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 gs with Ci 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 germoplasms 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 CO2 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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