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# Leaf nitrogen supply improves sugarcane photosynthesis under low temperature

G. CERQUEIRA*, M.C. SANTOS*, P.E.R. MARCHIORI**, N.M. SILVEIRA*, E.C. MACHADO*, and R.V. RIBEIRO***,+

*Laboratory of Plant Physiology 'Coaracy M. Franco', Center for Research and Development in Ecophysiology and Biophysics, Agronomic Institute, P.O. Box 28, 13020-902, Campinas SP, Brazil**

*Department of Biology, Federal University of Lavras, P.O. Box 3037, 37200-000, Lavras MG, Brazil***

*Laboratory of Crop Physiology (LCroP), Institute of Biology, University of Campinas, P.O. Box 6109, 13083-970, Campinas SP, Brazil****

## Abstract

This study aimed to test the hypothesis that increases in leaf nitrogen concentration would reduce the sensitivity of sugarcane photosynthesis to low temperature. IACSP95-5000 plants were grown inside a growth chamber at 30/20°C (day/night) and we evaluated the effects of leaf nitrogen spraying (2.5% urea) on plants facing low temperature (22/12°C) for eight days. The leaf nitrogen supply increased leaf nitrogen concentration and plants exhibited higher leaf gas exchange as compared to nonsprayed ones. We also found higher activity of the carboxylation enzymes, Rubisco and phospho*enol*pyruvate carboxylase, as well as a higher chlorophyll content in plants sprayed with nitrogen. Such enhancement of photosynthetic performance was associated with an increase in number of leaves and in total leaf area. Our results suggest that the effects of low temperature on photosynthesis of field-grown sugarcane plants could be alleviated by leaf nitrogen supply, with likely consequences for biomass production and crop yield.

*Additional key words*: *Saccharum* spp.; carboxylation; leaf gas exchange; urea; winter.

## Introduction

$C_4$ plants are rare in environments where the average air temperature is below 16°C during the growing season (Sage *et al.* 1999), possibly due to the high sensitivity of the photosynthetic metabolism to low temperatures. Although sugarcane plants exhibit high photosynthetic rates in subtropical regions, plants are also subjected to seasonal limitations such as low air temperature during the winter period. In fact, sugarcane photosynthesis increases with the temperature up to 35°C (Sage *et al*. 2013), with both photosynthesis and vegetative growth being inhibited below 15°C (Sales *et al.* 2013).

Dysfunction in photochemical and biochemical reactions are the main causes of declines in photosynthesis at low temperatures, with low phospho*enol*pyruvate carboxylase (PEPC) activity being motivated by increases in its activation energy and also by its liability (Sage and Kubien 2007). Under low temperature, $C_4$ photosynthesis is also limited by the activity of Rubisco and by the regeneration of ribulose-1,5-bisphosphate (RuBP), phospho*enol*pyruvate (PEP), and inorganic phosphorus (Sage and Kubien 2007). In general, $C_4$ species are intolerant to cold stress and their low Rubisco content – as compared to $C_3$ plants – makes the adaptation and acclimation to cold difficult (Sage *et al*. 2008). In addition to the metabolic aspects, low photosynthetic activity at low temperature is also a consequence of stomatal closure due to changes in guard cell sensitivity to $CO_2$ (Allen and Ort 2001).

In field-grown plants under subtropical conditions, significant reduction of light-conversion efficiency into biomass ($\varepsilon_C$) associated with decreases in leaf N concen-

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+Corresponding author; phone: +55 19 35216214, e-mail: rvr@unicamp.br

*Abbreviations*: Chl – chlorophyll; $C_i$ – intercellular $CO_2$ concentration; CNC – culm nitrogen concentration; DM – dry mass; $E$ – transpiration rate; FM – fresh mass; $F_v/F_m$ – maximum quantum yield of PSII photochemistry; $g_s$ – stomatal conductance; LNC – leaf nitrogen concentration; NPQ – nonphotochemical quenching of variable fluorescence; PEPC – phospho*enol*pyruvate carboxylase; $P_N$ – net photosynthetic rate; $P_{NI}$ – integrated net photosynthetic rate; $Q$ – photosynthetically active radiation; $q_P$ – photochemical quenching of variable fluorescence; RNC – root nitrogen concentration; $\Delta F/F_m'$ – effective quantum yield of photochemical energy conversion in PSII.

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tration (LNC) was reported in sugarcane during the winter season (Magalhães Filho 2014). Decreases in LNC are due to crop aging and low LNC may explain the reduced growth phenomenon found in sugarcane (Park *et al*. 2005, van Heerden *et al.* 2010). Accordingly, leaf $CO_2$ assimilation increases with increasing LNC of sugarcane plants (Allison *et al.* 1997, Santos *et al.* 2017) as a significant fraction of leaf N is invested into the photosynthetic machinery. Sugarcane plants have 0.3–2.0% of N in dry biomass (Robinson *et al*. 2014), with most of N allocated into amino acids, proteins, amides, nucleic acids, nucleotides, and enzymes (Crawford *et al*. 2000, Robinson *et al*. 2014). A considerable fraction of leaf N is driven to the synthesis of the light-harvesting complexes and of carboxylation enzymes, such as Rubisco and PEPC (Crawford *et al*. 2000, Maranville and Madhavan 2002).

As the photosynthetic capacity and crop yield are closely related to N availability throughout a crop cycle (Saliendra *et al*. 1996, Marchiori *et al*. 2014), we could suggest that low LNC is one of the factors associated with low photosynthesis during sugarcane ripening, which occurs during the cold and dry winter season under subtropical conditions. Then, our aim was to test the hypothesis that increases in LNC through leaf spraying would reduce the sensitivity of sugarcane photosynthesis to low temperature, with plants showing higher $CO_2$ assimilation and increased activities of carboxylation enzymes.

## Materials and methods

**Plant material and experimental strategy**: Two-month old plants of sugarcane (*Saccharum* spp., cv. IACSP95-5000, supplied by the Advanced Centre for Technological Research of Sugarcane, Agronomic Institute, Ribeirão Preto SP, Brazil) were transplanted into 10-L pots filled with soil fertilized with superphosphate (375 mg $kg^{-1}$), potassium chloride (125 mg $kg^{-1}$), magnesium sulphate (100 mg $kg^{-1}$), boric acid (0.5 mg $kg^{-1}$), Fe-EDTA (5 mg $kg^{-1}$), manganese sulphate (5 mg $kg^{-1}$), copper sulphate (1.5 mg $kg^{-1}$), and zinc sulphate (5 mg $kg^{-1}$). After transplanting, plants received two fertilizations with urea [125 mg(N) $kg^{-1}$, each] in intervals of 15 d (Santos *et al.* 2017). Sugarcane plants were irrigated daily up to the maximum water holding capacity of soil.

Plants were grown under greenhouse conditions for 83 d, where average air temperature was 27.2 ± 5.5°C during the day and 18.2 ± 2.5°C at night, relative air humidity (RH) was 82.5 ± 15.3%, and the maximum photo-synthetically active radiation (*Q*) was 830 µmol(photon) $m^{-2}$ $s^{-1}$. When LNC reached 20 g $kg^{-1}$ in leaves +1 [following the Kuijper numbering system (Bonnett 2014), leaf +1 is the first fully expanded leaf with visible dewlap, while leaf +3 is the third one and older than leaf +1], indicating N sufficiency for sugarcane (Santos *et al*. 2017), plants were placed inside a growth chamber for nine days under a thermal regime of 30/20°C (day/night). During the acclimation, a group of plants received two leaf N sprays (urea at 2.5% diluted in water and *Triton*® at 0.1%) in two consecutive days, defining the treatment +N. The other group of plants was sprayed two times with a solution composed by water and *Triton*®, being called as treatment N. At each spraying, plants received 180 mL of solution, corresponding to 4.5 g(urea) per plant.

Two days after the second N spray, plants were subjected to a thermal regime of 22/12°C (day/night) for eight days – time for plants reaching null values of photosynthesis. The other environmental conditions inside the growth chamber were constant, with *Q* of 500 µmol(photon) $m^{-2}$ $s^{-1}$, RH of 70/80% (day/night), and 12-h photoperiod. The temperature regimes were based on the temperatures that usually occur during the summer (warm and humid) and winter (cold and dry) seasons in São Paulo, Brazil. As the effects of increasing nitrogen on photosynthesis of plants under nonlimiting conditions are already known (Feng *et al.* 2012), we designed our experiment to test the working hypothesis about nitrogen effects under low temperature. Based on this, we needed plants under low temperature and supplied or not with leaf nitrogen. As this study was done inside a growth chamber (with constant environmental conditions) and the time under low temperature was relatively short (8 d), measurements taken after nitrogen spraying and before reducing temperature were used as references.

**Gas exchange, photochemical activity and chlorophyll (Chl) relative content**: Leaf gas exchange was initially assessed (eight days before the cooling) to characterize the physiological state of sugarcane plants. After beginning the experiment, measurements were performed every two days in leaves +1 between 10:00 and 14:00 h, with an infrared gas analyzer (*LI-6400*, *LI-COR*, Lincoln NE, USA), under air $CO_2$ concentration of 380 µmol($CO_2$) $mol^{-1}$ and *Q* of 2,000 µmol(photon) $m^{-2}$ $s^{-1}$. The $CO_2$ assimilation ($P_N$), stomatal conductance ($g_s$), and intercellular $CO_2$ concentration ($C_i$) were evaluated and the measurements registered under temporal stability and low coefficient of variation. The photochemical activity was evaluated with a modulated fluorometer (*6400-40 LCF*, *LI-COR*, Lincoln NE, USA) attached to the *LI-6400*. The measurements of Chl fluorescence were taken simultaneously to the leaf gas exchange in light-adapted tissues, according to the technique of light saturation pulse [630 nm, 6,000 µmol(photon) $m^{-2}$ $s^{-1}$, 0.8 s]. Such technique was also applied to dark-adapted tissues (30 min), with the minimal ($F_0$) and maximal ($F_m$) fluorescence in dark-adapted tissues and the minimal [$F_0$’, 740 nm, <5 µmol(photon) $m^{-2}$ $s^{-1}$, 4 s], maximal ($F_m$’) and steady-state ($F_s$) fluorescence in light-adapted tissues being used to estimate the following indices: variable fluorescence in the dark- ($F_v = F_m - F_o$) and light-adapted ($\Delta F = F_m' - F_s$) leaves; the maximum quantum yield of PSII photochemistry ($F_v/F_m$); and effective quantum yield of photochemical energy conversion in PSII ($\Delta F/F_m'$); the photochemical [$q_P = (F_m' - F_s)/(F_m' - F_0')$] and nonphotochemical [$NPQ = (F_m - F_m')/F_m'$] quenching of ΔF (Roháček 2002). By using a Chl meter (*CFL1030*, *Falker*, Porto Alegre RS, Brazil), we measured the relative content of Chl *a* and *b* in the same leaves used for photosynthesis measurements (leaves +1) and also in leaves +3.

**Nitrogen concentration in plant organs**: LNC was estimated every two days in the leaves +1 and +3 through the equation: LNC = 1.488 + 2.133*(Chl *b*) – 0.049*(Chl *b*)$^2$, in which Chl *b* is an indirect measure of Chl *b* content taken with the Chl meter model *CFL1030* (Cerqueira *et al*. 2017). LNC measurements were taken during the cooling and also three days before leaf sprayings. Culm (CNC) and root (RNC) N concentrations were determined according to the Kjeldahl method (Bremner 1965), before cooling (at 30/20°C) and eight days after cooling (22/12°C).

**Nitrate, ammonium and amino acids**: Fractions of leaf +1 were collected prior (30/20°C) and after cooling (22/12°C), immediately frozen in liquid N, and stored at –80°C. About 500 mg of leaves were macerated in liquid N and then 2.5 mL of methanol:chloroform:water (MCW, 12:5:3) were added and mixed at room temperature for 2 h. After this period, samples were centrifuged at 2,000 × *g* for 5 min. The extraction was performed once more with 2.5 mL of MCW, joining the supernatants. Finally, 4 mL of the leaf extract were taken (the remaining volume was measured and discarded) and mixed with 1 mL of chloroform and 1.5 mL of water. After phase separation (12 h), about 4 mL were collected from the superior aqueous phase and the extract was kept in water bath at 37°C. The extracts were then concentrated by evaporation with a $N_2$ jet in a concentrator (*TE-019-E3*, *Tecnal*, Piracicaba SP, Brazil) and stored at −20°C before evaluating nitrate, ammonium and amino acids.

Nitrate concentration ($NO_3^-$) was determined in accordance to Cataldo *et al*. (1975). 0.2 mL of 5% salicylic acid (salicylic acid in concentrated $H_2PO_4$) was added to 0.05 mL of the MCW extract. After mixing, the solution was left to stand for 20 min at room temperature. Then, 4.75 mL of NaOH (2 N) was added, and the mixture was agitated again. After cooling at room temperature, absorbance was read at 410 nm with a spectrophotometer (*80-2109-10*, *Pharmacia Biotech*, Cambridge MA, UK). The standard curve of potassium nitrate was performed from 1 to 10 µmol mL$^{-1}$ and the nitrate concentrations were expressed as µmol g$^{-1}$.

The determination of ammonium ($NH_4^+$) followed the protocol proposed by McCullough (1967) and Weatherburn (1967). In test tubes, we added 0.1 mL of MCW extract, 0.5 mL of the reagent #1 [0.1 M phenol (2.5 g) and 170 µM sodium nitroprusside (12.5 mg) in 250 mL of distilled water] and 0.5 mL of reagent #2 [0.125 M NaOH (1.25 g), 0.15 M $Na_2HPO_4$.12 $H_2O$ (13.4 g) and NaOCl (3% $Cl_2$, 10 mL) in a final 250 mL]. Then, tubes were agitated, sealed and incubated in water bath at 37°C for 35 min. After cooling at room temperature, the absorbance was read at 625 nm (*80-2109-10*, *Pharmacia Biotech.*, Cam-bridge, UK). The ammonium concentrations were calculated from the standard curve of ammonium sulphate between 100 and 1,000 nmol mL$^{-1}$ and expressed as µmol g$^{-1}$.

The content of amino acids was determined following the Cocking and Yemm (1954) protocol. Previously, MCW extracts were diluted in water to a final volume of 0.5 mL in test tubes, with the addition of 0.25 mL of 0.2 M citrate buffer (pH 5.0), 0.1 mL of 5% ninhydrin [ninhydrin (*Merck*®) in ethylene glycol monomethyl ether (methyl cellosolve, *Merck*®)] and 0.5 mL of 0.01 M KCN in methyl cellosolve. The tubes were agitated, sealed, and incubated in water bath at 100°C for 20 min, being cooled at room temperature. The sample was then transferred to a cuvette, adding 0.5 mL of 60% ethanol, and the absorbance read at 570 nm with a spectrophotometer (*80-2109-10*, *Pharmacia Biotech.*, Cambridge, UK). The standard curve was performed with leucine 20 to 100 nmol mL$^{-1}$, and the total soluble amino acid values were expressed as µmol g$^{-1}$.

**Total soluble protein**: The analysis of total soluble protein was done according to Bradford (1976) in leaf tissues sampled at 30/20°C (prior to cooling) and 22/12°C (after eight days of cooling) and stored at −80°C. Leaf extract was obtained with 100 g of fresh leaf macerated in liquid N with 2% polyvinylpyrrolidone (PVPP). After crushing the tissues, 2.0 mL of extraction buffer [0.1 mol L$^{-1}$ potassium phosphate (pH 6.8), 0.1 mmol L$^{-1}$ ethylenediaminetetraacetic acid (EDTA), and 1.0 mmol L$^{-1}$ phenylmethylsulfonyl fluoride (PMSF)] was added to the leaf extract and mixed for 2 min. The solution was then transferred to a tube and centrifuged at 15,000 × *g* at 4°C for 15 min. The supernatant was collected and kept on ice. In a cuvette, we added 30 µL of leaf extract, 80 µL of water and 5.0 mL of the Bradford reagent [*Comassie Blue G-250* (100 mg), 95% ethanol (50 mL), 85% orthophosphoric acid (100 mL) and distilled water (850 mL)]. The sample absorbance was read with a spectrophotometer (*Genesys 10S UV Scanning*, *Thermo Fisher Scientific*, Madison WI, USA) at 595 nm. Based on a standard curve with concentrations between 0 and 100 mg mL$^{-1}$ of bovine serum albumin (BSA), the content of total soluble proteins was estimated and expressed as mg g$^{-1}$.

**Carboxylation enzymes**: Leaf samples stored at −80°C were used for assessing PEPC (EC 4.1.1.31) activity according to Ashton *et al*. (1990) and Degl'Innocenti *et al*. (2002) and Rubisco (EC 4.1.1.39) activity according to Sharkey *et al*. (1986), Sage *et al*. (1988), and Reid *et al*. (1997). The activity of both PEPC and Rubisco were evaluated at 30/20°C (prior to cooling) and under 22/12°C (eight days after cooling).

**PEPC**: In a mortar, about 100 mg of leaf +1 were macerated with liquid N and 2% PVPP. After grinding, 0.5 mL of extraction buffer [100 mM potassium phosphate (pH 7.5) and 1 mM EDTA] were added and macerated for 2 min. The leaf extract was then transferred to a tube and centrifuged at 14,000 × *g* at 4°C for 25 min. The supernatant was removed and kept at 4°C until the analysis. The reaction buffer [50 mM Tris-HCl (pH 7.8), 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 33 nKat malic dehydrogenase (MDH), and 0.3 mM NADH] was kept at 30°C during the analyses. In a cuvette, 800 µL of reaction buffer was added to 100 µL of leaf extract and 100 µL of 4.0 mM PEP. By using a spectrophotometer (*Genesys 10S*), the decrease in absorbance at 340 nm was followed for 4 min, which reflects PEP carboxylation and

subsequent NADH oxidation.

**Rubisco**: About 100 mg of leaf +1 was also used as described for PEPC. After grinding, 0.5 mL of extraction buffer [100 mM Bicine-NaOH (pH 7.8), 5 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol (DTT), 1 mM PMSF, and 10 µM leupeptin] was added to the leaf extract and mixed. Such solution was transferred to a tube and centrifuged at 14,000 × *g* at 4°C for 5 min. The supernatant was removed and kept at 4°C. The reaction buffer [100 mM Bicine-NaOH (pH 8.0), 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 3.4 mM ATP, 5 mM phosphocreatine, 0.25 mM NADH, 80 nkat (4,799 U.A.) glyceraldehyde-3-phosphate dehydrogenase, 80 nkat (4.799 U.A.) creatine phosphofructokinase, and 80 nkat (4.799 U.A.) 3-phosphoglycerate phosphokinase] was kept at 25°C during the analyses. The initial activity of Rubisco was measured by using 900 µL of the reaction buffer, 70 µL of leaf extract and 30 µL of 0.5 mM RuBP added to the cuvette. Decreases in absorbance induced by NADP oxidation were followed for 3 min at 340 nm, using the *Genesys 10S* spectrophotometer. Both Rubisco and PEPC activities were expressed as µmol g(protein)$^{-1}$ min$^{-1}$.

**Biometry**: Leaves and culms (main culm and tillers) were counted and plant height measured with a measuring tape. The total leaf area was determined with an electronic planimeter (*LI-3000C* and *LI-3050C*, *LI-COR*, Lincoln NE, USA). Leaves, culms, and roots were separated, dried in an oven with forced air circulation at 60°C until they reached constant mass, and then the dry matter was determined. Those evaluations were performed at the end of the experiment, *i.e.* after eight days under 22/12°C.

**Statistical analyses**: The experimental design was in randomized blocks, with two causes of variation: leaf N supply (with and without spraying); and evaluation times (0, 2, 4, 6, and 8 d), with four replications. The data were submitted to the analysis of variance (*ANOVA*) and the mean values compared through the *Scott Knott´*s test at 1%, 5%, and 10% of significance, using the statistical software *Sisvar® v.5.4* (Ferreira 2011).

## Results

**Gas exchange and photochemistry**: $P_N$ and $g_s$ increased after leaf N spraying under adequate thermal conditions, with both variables being reduced during cooling (Fig. 1). However, decreases of $P_N$ and $g_s$ due to cold occurred less intensely and plants supplied with N exhibited higher $P_N$ and $g_s$ than ones nonsupplied (Fig. 1). By integrating the $CO_2$ assimilation throughout the experimental period ($P_{NI}$), our data revealed that leaf N spraying reduced the sensitivity of photosynthesis to low temperature, with plants supplied with N presenting an increase of 27% in photosynthesis (Fig. 1*A*).

The photochemical activity was also markedly affected by low temperature, but leaf N supply did not alleviate the cooling effects (Fig. 2*B*,*C*). While $F_v/F_m$ was higher in +N plants only on the second day under low temperature (Fig. 2*A*), NPQ was higher in N plants on the eighth day of cooling (Fig. 2*D*).

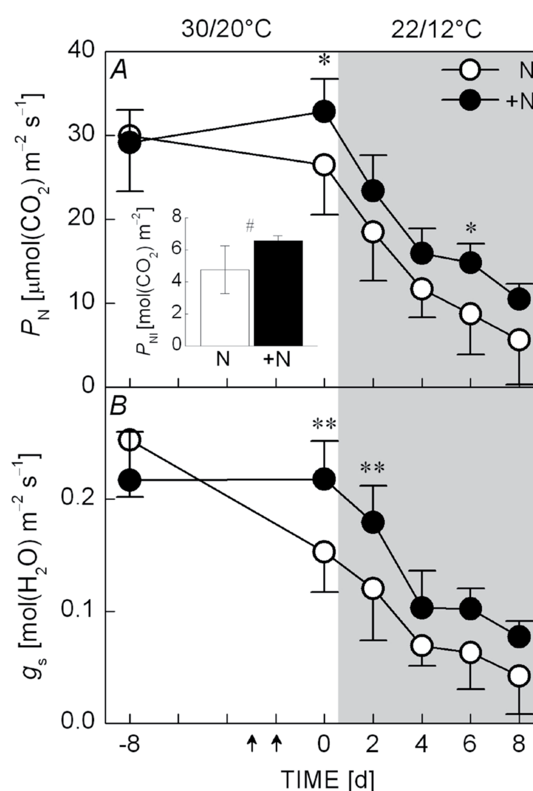


Fig. 1. Effects of leaf N spraying on $CO_2$ assimilation ($P_N$, *A*) and the stomatal conductance ($g_s$, *B*) of sugarcane plants subjected to changes in growth temperature from 30/20°C to 22/12°C (day/night). $CO_2$ assimilation was integrated over the experimental period ($P_{NI}$) and shown in *A*. Plants were sprayed with water (N) or 2.5% urea (+N) in two consecutive days, indicated by arrows. Mean values (*n* = 4, ± SD) were compared by the *Scott Knott´*s test. **, *, and # – statistical differences between the treatments in each evaluation time at $p<0.01$, $0.01<p<0.05$ and $0.05<p<0.10$, respectively.

The contents of Chl *a* and *b* in leaves +1 and +3 increased due to leaf N supply before reducing temperature (time 0), and were not reduced during exposure to low temperature (Fig. 3*A*,*C*). In N plants, Chl *a* and *b* contents were reduced by low temperature and leaves +3 were more sensitive than leaves +1.

**Nitrogen**: Low temperature reduced LNC in plants without extra N supply, and this effect was more evident in leaves +3 (Fig. 3*D*). Leaf N spray increased culm N concentration (CNC), but low temperature reduced CNC in both treatments (Fig. 3*E*). Interestingly, plants sprayed with N did not present reduction in root N concentration (RNC) under low temperature (Fig. 3*F*).

Leaf nitrate and ammonium concentrations did not vary significantly between the treatments in both temperature regimes (Table 1). On the other hand, leaf N supply stimulated the synthesis of total soluble amino acids before the cooling, and no significant changes were noticed for total soluble proteins (Table 1).

**Carboxylation enzymes**: Leaf N supply increased PEPC

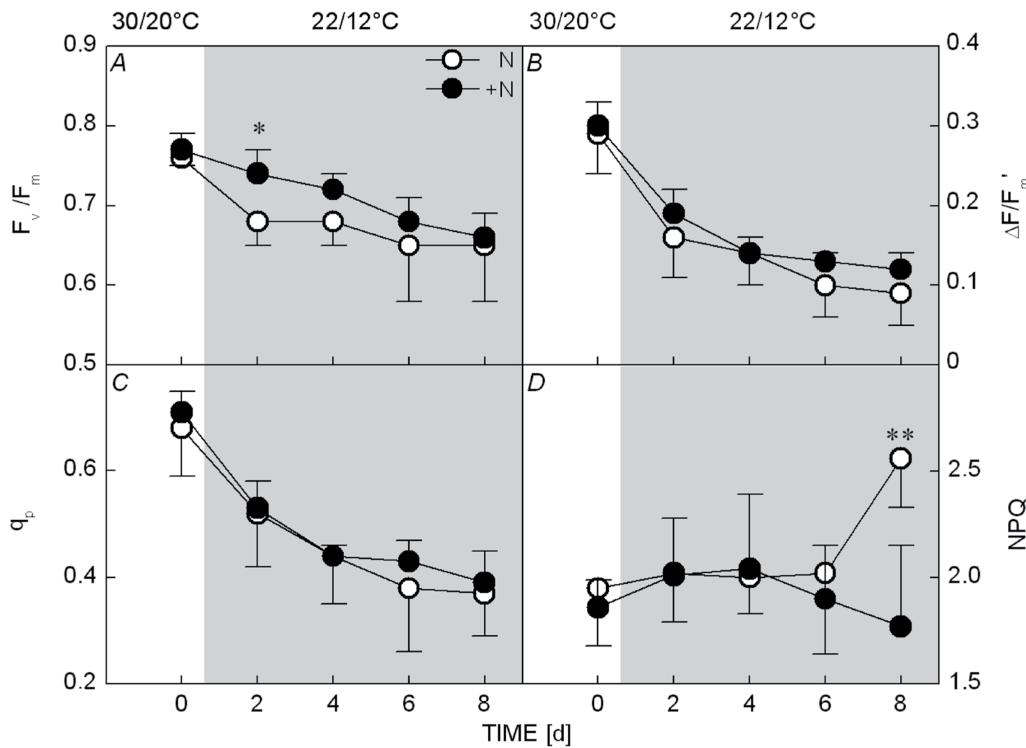


Fig. 2. Effects of leaf N spraying on the maximum quantum yield of PSII photochemistry ($F_v/F_m$), the effective quantum yield of photochemical energy conversion in PSII ($\Delta F/F_m$'); and photochemical ($q_P$, *C*) and nonphotochemical (NPQ, *D*) quenching of variable fluorescence by sugarcane plants subjected to changes in growth temperature from 30/20°C to 22/12°C (day/night). Plants were sprayed with water (N) or 2.5% urea (+N) in two consecutive days, indicated by arrows. Mean values ($n$ = 4, ± SD) were compared by the *Scott Knott´*s test. ** and * – statistical differences between the treatments in each evaluation time at $p<0.01$ and $0.01<p<0.05$, respectively.

and Rubisco activities under both temperature regimes (Fig. 4). In addition, leaf N spray prevented the reduction in Rubisco activity due to low temperature (Fig. 4*B*).

**Biometry**: At the end of the experiment, leaf N supply caused an increase in the number of leaves, the total leaf area and plant height (Table 2). However, the number of culms and dry matter of leaves, culm and roots were not affected by leaf N spraying (Table 2).

## Discussion

The initial hypothesis of this research was that increases in LNC could alleviate the negative impact of low temperature on sugarcane photosynthesis. Through leaf N spraying, we increased LNC and found that plants supplied with N presented higher photosynthesis during cold exposure when compared to plants without leaf N spraying (Fig. 1*A*). Such treatment allowed the maintenance of LNC during cooling, which was not observed in the plants without extra N supply (Fig. 3*B*,*D*). In fact, low temperature caused large reductions in LNC in plants that did not receive extra N through leaf spraying, with values lower than 15 g kg$^{-1}$ suggesting N deficiency (Santos *et al*. 2017). Such decrease in LNC due to cooling was even higher in leaves +3 of plants without leaf N supply, which would indicate the translocation of this nutrient to the younger leaves (Masclaux-Daubresse *et al*. 2010). Leaf N supply also increased the culm N concentration and maintained root N concentration during exposure to low temperature (Fig. 3*E*,*F*), if the overall plant N status changed by leaf spraying.

Leaf nitrate and ammonium concentrations were not changed by leaf N supply, but enhancements in concentrations of amino acids indicated that N supplied through leaf spraying was metabolized by sugarcane plants (Table 1). Increases in contents of amino acids were also reported in grapevine leaves sprayed with urea (Pérez-Álvarez *et al*. 2017). Interestingly, the concentrations of amino acids were similar at the end of exposure to low temperature (Table 1), suggesting higher amino acids consumption and N partitioning for synthesis and maintenance of Chl pigments (Fig. 3*A,C*) and photosynthetic components (Figs. 1*A*, 4) in plants sprayed with N. Accordingly, our results clearly revealed a gradual reduction in Chl *a* and *b* contents along the exposure to low temperature only in the plants that did not receive extra N (Fig. 3*A*,*C*). Based on this finding, we may argue that leaf N supply was able to prevent Chl degradation under low temperature, improving plant ability to absorb light energy. Even though proteins are composed by amino acids, significant changes were not found in the total soluble protein contents between treatments (Table 1).

Our data also revealed the sugarcane sensitivity to low temperature and a possibility to reduce such sensitivity through leaf N supplying (Fig. 1*A*). While it

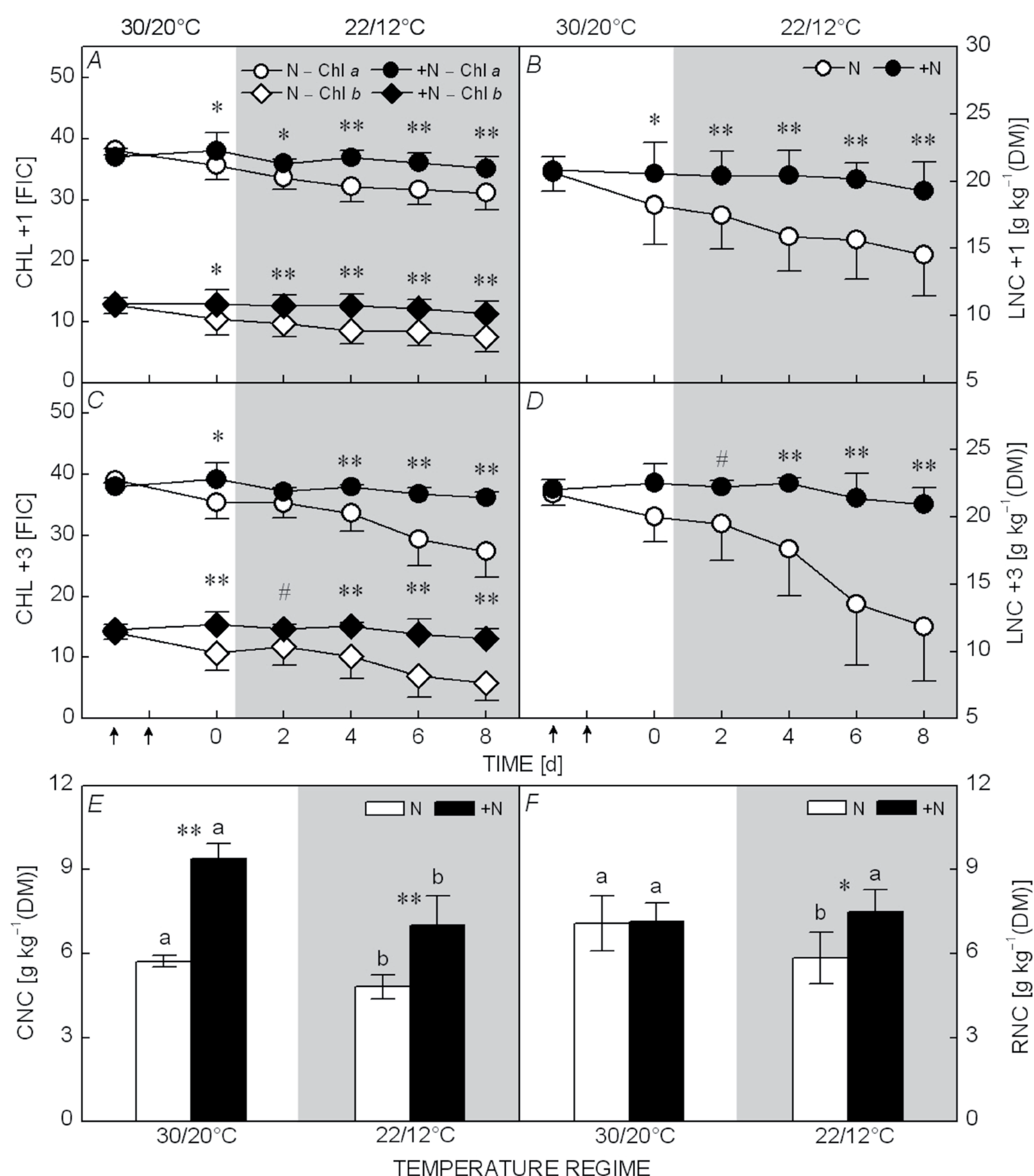


Fig. 3. Effects of leaf N spraying on the chlorophyll *a* and *b* of leaves +1 (CHL +1, *A*) and +3 (CHL +3, *C*), on the nitrogen concentration of leaves +1 (LNC +1, *B*) and +3 (LNC +3, *D*) and on the nitrogen concentration in culms (CNC, *E*) and roots (RNC, *F*) of sugarcane plants subjected to changes in growth temperature from 30/20°C to 22/12°C (day/night). Plants were sprayed with water (N) or 2.5% urea (+N) in two consecutive days, indicated by *arrows*. CNC and RNC were evaluated before cooling (30/20°C) and after eight days at low temperature (22/12°C). Mean values ($n = 4$, ±SD) were compared by the *Scott Knott´s* test. In *A* to *D*, **, *, and # – statistical differences between the treatments in each evaluation time at $p<0.01$, $0.01<p<0.05$, and $0.05<p<0.10$, respectively. In *E* and *F*, ** and * – statistical differences between the treatments at $p<0.01$ and $0.01<p<0.05$, whereas *different letters* show statistical difference ($p<0.05$) between the temperature regimes. FIC – Falker chlorophyll index.

is known that increases in LNC improve photosynthetic rates in $C_4$ species (Tazoe *et al.* 2006, Uribelarrea *et al.* 2009), we provided evidence that such benefit was also found for sugarcane plants under unfavourable thermal conditions. Stomatal responses were similar to those found for photosynthesis under low temperature (Fig. 1*B*), with stomatal closure being a likely consequence of cold-induced synthesis of abscisic acid (Ashraf and Harris 2013). Plants supplied with N presented higher $g_s$ than that of the nonsupplied ones (Fig. 1*B*) and this would be a reason for the higher photosynthetic activity of +N plants, as found in *Miscanthus* (Feng *et al.* 2012). However, we did not find any increase in intercellular $CO_2$ availability due to higher stomatal aperture, which would justify high photosynthetic rates. $C_i$ varied between 129 and 195 μmol($CO_2$) $mol^{-1}$ in N plants and between 131 and 172 μmol($CO_2$) $mol^{-1}$ in +N plants. In general, exposure to low temperature increased $C_i$ – as found in *Flaveria bidentis* L. (Kubien *et al.* 2003) – and this response accompanied by reductions in $P_N$ suggests nonstomatal limitation of

Table 1. Leaf concentrations of $NO_3^-$, $NH_4^+$, amino acids, and total soluble protein as affected by leaf N spraying on sugarcane plants under 30/20°C to 22/12°C (day/night). Plants were sprayed with water (treatment N) or 2.5% urea (treatment +N) in two consecutive days before cooling. Evaluations were performed before (30/20°C) and 8 d after reducing temperature (22/12°C). The average values ($n$ = 4, ± SD) were compared by the *Scott Knott´s* test ($p$<0.05). Different *capital letters* show statistical difference between the treatments in each thermal regime, and different *lowercase letters* show difference between the temperature regimes in each treatment.

| Variable | Temperature (day/night) [°C] | Treatment N | Treatment +N |
|---|---|---|---|
| $NO_3^-$ [µmol $g^{-1}$(FM)] | 30/20 | $9.7 \pm 2.0^{Ab}$ | $11.1 \pm 1.8^{Aa}$ |
| | 22/12 | $14.6 \pm 2.4^{Aa}$ | $14.4 \pm 3.9^{Aa}$ |
| $NH_4^+$ [µmol $g^{-1}$(FM)] | 30/20 | $0.2 \pm 0.1^{Aa}$ | $0.3 \pm 0.1^{Aa}$ |
| | 22/12 | $0.2 \pm 0.0^{Aa}$ | $0.3 \pm 0.1^{Aa}$ |
| Amino acids [µmol $g^{-1}$(FM)] | 30/20 | $4.6 \pm 0.5^{Ba}$ | $18.8 \pm 4.8^{Aa}$ |
| | 22/12 | $3.4 \pm 0.4^{Aa}$ | $4.6 \pm 2.0^{Ab}$ |
| Protein [mg $g^{-1}$(FM)] | 30/20 | $7.4 \pm 3.1^{Aa}$ | $7.9 \pm 2.0^{Aa}$ |
| | 22/12 | $6.4 \pm 1.0^{Aa}$ | $8.3 \pm 0.6^{Aa}$ |

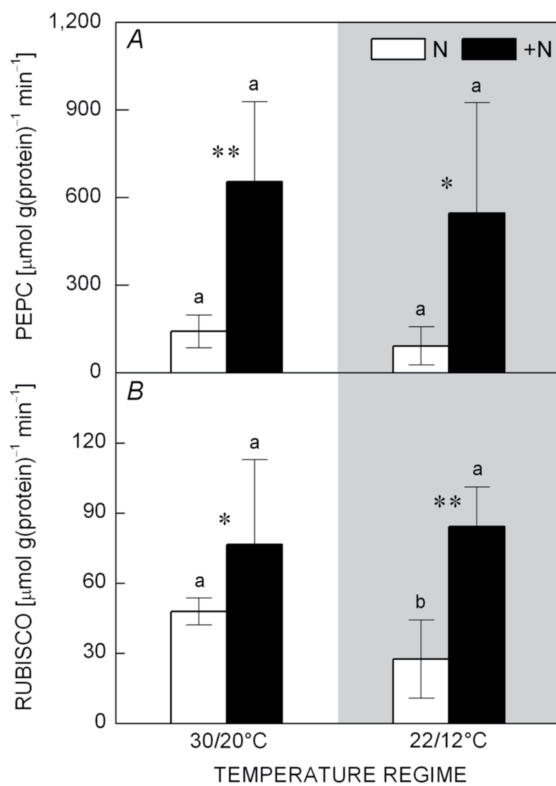


Fig. 4. Effects of leaf N spraying on the activities of PEPC (*A*) and Rubisco (*B*) of sugarcane plants subjected to changes in growth temperature from 30/20°C to 22/12°C (day/night). Plants were sprayed with water (N) or 2.5% urea (+N) in two consecutive days, before cooling. Samples were taken before cooling (30/20°C) and after eight days at low temperature (22/12°C). Mean values ($n$ = 4, ± SD) were compared by the *Scott Knott´s* test. ** and * – statistical differences between the treatments in each temperature regime at $p$<0.01 and 0.01<$p$<0.05, respectively. *Different letters* show statistical difference ($p$<0.10) between the temperature regimes.

photosynthesis.

Regarding the primary photochemistry, decreases in $F_v/F_m$ and $\Delta F/F_m$' with cooling (Fig. 2*A,B*) indicate thermoinhibition of PSII activity and likely reductions in ATP synthesis and RuBP regeneration (Allen and Ort 2001). Only marginal effects of leaf N supply were found on photochemical activity, with N-supplied plants showing higher $F_v/F_m$ only at the beginning of cold exposure (Fig. 2*A*). On the other hand, leaf N supply had significant effects on biochemistry of photosynthesis. Due to higher leaf N status during cooling, plants supplied with N showed maintenance of PEPC activity and higher Rubisco activity under low temperature (Fig. 4).

Table 2. Number of active leaves of the main culm, number of culms, main culm height, total leaf area and leaf, culm and root dry matter (DM) of sugarcane plants sprayed with N and subjected to cooling for eight days. Plants were sprayed with water (treatment N) or 2.5% urea (treatment +N) in two consecutive days before cooling. The average values ($n$ = 4, ± SD) were compared by the *Scott Knott´s* test (*0.05<$p$<0.10 and **$p$<0.05). Different *capital letters* show statistical difference between the treatments.

| Variable | Treatment N | Treatment +N |
|---|---|---|
| Leaf [units]* | $4.3 \pm 0.5^{B}$ | $5.3 \pm 0.5^{A}$ |
| Culm [units] | $15.5 \pm 5.4^{A}$ | $11.5 \pm 3.1^{A}$ |
| Plant height [cm]* | $41.0 \pm 2.4^{B}$ | $45.8 \pm 2.8^{A}$ |
| Leaf area [$m^2$]** | $0.52 \pm 0.06^{B}$ | $0.63 \pm 0.08^{A}$ |
| Leaf DM [g] | $94.3 \pm 13.9^{A}$ | $103.3 \pm 16.0^{A}$ |
| Stem DM [g] | $57.8 \pm 7.8^{A}$ | $66.2 \pm 7.8^{A}$ |
| Root DM [g] | $31.1 \pm 7.4^{A}$ | $31.5 \pm 7.4^{A}$ |

$C_4$ plants have a mechanism of $CO_2$ concentration that increases the catalytic capacity of Rubisco, minimizing its oxygenase activity (photorespiration) and maximizing the carboxylation during the photosynthetic process. Such physiological advantage is possible due to PEPC, which catalyses the initial carboxylation of $CO_2$ after the action of a carbonic anhydrase in mesophyll cells (Sage *et al.* 2012). As Rubisco is less abundant in $C_4$ species as compared to $C_3$ species and both PEPC and Rubisco lose activity under low temperature (Sage and Pearcy 1987, Sage 2002, Sage

and Kubien 2007), those two enzymes have an important role in $C_4$ photosynthesis under stressful conditions. Taken together, our results indicate that the photosynthetic reactions in both mesophyll and bundle sheath cells of sugarcane were improved by leaf N supply, with increases in carbon pumping from mesophyll to bundle sheath cells by PEPC and consequent maintenance of Rubisco activity under low temperature (Fig. 4). Large differences found in PEPC and Rubisco activity due to nitrogen supply would be checked *in vivo* through $P_N$-$C_i$ response curves, when photosynthetic reactions are not necessarily occurring under optimum conditions, as in our biochemical assays. In addition, the causes of increased carboxylation activity under +N conditions would be further investigated, considering upregulation of gene expression, increases in amount of those specific enzymes and/or their higher activity.

Leaf N supply did not change biomass accumulation in sugarcane under low temperature (Table 2), even with plants showing increases in photosynthetic activity (Fig. 1*A*). In fact, leaf N spraying enhanced the number of leaves and leaf area without significantly changing biomass. Such absence of positive effects of leaf N supply on plant growth could be justified by the short period of experimentation, *i.e.* eight days. Thus, further research is needed to reveal how the nutritional management with leaf N supply would affect biomass production and yield of field-grown sugarcane plants.

In conclusion, stomatal, photochemical, and biochemical limitations were responsible for low photosynthesis of sugarcane under low temperature. However, leaf N supply was able to decrease the sensitivity of photosynthesis to cooling, impeding reductions in leaf N and chlorophyll concentrations and increasing the activity of Rubisco and PEPC. Those results suggest that leaf N supply could increase the light-conversion efficiency into biomass – in which photosynthesis is the main component – in sugarcane fields, where low temperature occurs during the winter season and photoassimilate supplying is critical for sucrose yield.

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