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CHLORIDE NUTRITION: IMPACT IN PLANT DEVELOPMENT AND WATER RELATIONS

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ABSTRACT

Although Cl^- has been characterized as a micronutrient, we have observed that when available in the millimolar range (e.g. 1-5 mM), higher plants accumulate Cl^- to levels that are typical of the content of a macronutrient (Plant Cell Env. 2010, 33: 2012-27). Since this requires a considerable cost of energy, we speculate whether Cl^- might play a poorly understood function in plants when accumulated to macronutrient levels. Given that Cl^- is a major osmotically active solute in the plant vacuole, we propose that this element improves osmoregulatory and plant water relation mechanisms. To elucidate this hypothesis, tobacco plants were grown for 9 weeks with a basal nutrient solution (BS) and subjected to different treatments: 5 mM Cl^- solution (Cl); 5 mM NO_3^- (N); and 5 mM SO_4^{2-} and PO_3^{3-} (SP). Compared to BS and SP plants, chloride nutrition in the millimolar range promoted plant growth in terms of dry weight and total leaf area. This, together with the observation that Cl^- was preferentially included in growing and reproductive organs, suggested a role in plant development. In addition, compared to BS, SP and N plants, Cl plants exhibited the best water parameters.

INTRODUCTION

Chloride (Cl^-) is defined as a micronutrient since glycophyte plants can perform normally with low amounts of this element, having crop plants a critical requirement around 0.2 mg Cl^- g^{-1} leaf tissue dry weight (mg g^{-1}) (Marschner, 1995). As an essential micronutrient, Cl^- is involved in the regulation of important cellular functions like: the stabilization of the water splitting system of photosystem II; regulation of some enzymes activity; and electrical charge balance of essential cations like K^+ and H^+, playing crucial roles in the stabilization of the electric potential of cell membranes and the regulation of pH gradients (reviewed in Marschner, 1995; Xu et al, 2000; White and Broadley, 2001).

When Cl^- is available (e.g. 1-5 mM), despite being a micronutrient, it is actively taken and accumulated into leaf tissues to levels that exceed the critical requirement concentration by two orders of magnitude (Marschner, 1995; Brumós et al, 2010). Since this accumulation requires a very high cost of energy (Brumós et al, 2010), we propose that Cl^- fulfil a poorly understood role when accumulated to such high levels. It is known that Cl^- is a major osmotically active solute in the vacuole involved in both turgor and osmoregulation processes (Flowers, 1988). Chloride is particularly well suited to fulfil these roles given its relatively low biochemical activity and its high mobility in the short and long distance in plants (Maas, 1986; Xu et al, 2000; Hänsch and Mendel,

2009). We propose that, when accumulated to levels that are typical of the content of a macronutrient, Cl^- may have an impact in osmoregulation and water relations in higher plants.

MATERIALS AND METHODS

Plant material and growth conditions

Tobacco plants (*Nicotiana tabacum* L. var. Light Habana) were grown under greenhouse conditions at 24 ± 2 ºC / 17 ± 2 ºC (day/night), and a relative humidity of 60 ± 10% (EL-1-USB Data-logger, Lascar Electronics Inc., Erie, PA, USA), and a 16 h/8 h photoperiod with a photosynthetic photon-flux density of 300 µmol m^{-2} s^{-1} (quantum sensor, LI-6400; Li-COR, Lincoln, NE, USA). Tobacco seeds were sown in a mixture of peat and coarse sand in flat trays (cell size 4 cm × 4 cm × 10 cm). After 2 days vernalisation in a cold chamber (4ºC), seedlings were transferred to the greenhouse. After 3 weeks, seedlings were transplanted to 3.5 L pots containing a mix of perlite:coarse sand:vermiculite (2:3:5). Potted plants were then treated with four alternative nutritional solutions: 1) a basal nutrient solution **(BS)** containing 1.25 mM KNO_3, 0.625 mM KH_2PO_4, 0.053 mM K_2HPO_4, 1 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 0.1 mM FeNa-EDTA, 0.1 mM H_3BO_3, 0.1 mM $MnSO_4$, 29 µM $ZnSO_4$, 0.11 µM $CoCl_2$, 0.1 µM $CuSO_4$, 1 µM Na_2MoO_4, and 5 µM KI, and a final pH of 5.7; 2) BS supplemented with a 5 mM Cl^- salt mixture (**Cl**; with 2.5 mM KCl, 0.625 mM $MgCl_2$, and 0.625 mM $CaCl_2$); 3) BS supplemented with a 5 mM NO_3^- salt mixture (**N**; with 2.5 mM KNO_3, 0.625 mM $Mg(NO_3)_2$, and 0.625 mM $Ca(NO_3)_2$); 4) BS supplemented with a 3.125 mM SO_4^{2-} and PO_3^{3-} solution (**SP**; with 1.25 mM KH_2PO_4, 0.625 mM K_2SO_4, 0.625 mM $MgSO_4$, and 0.625 mM $CaSO_4$). Both N and SP controls contained the same cations balance of the Cl^- treatment. Plants treated with BS, N and SP solutions contained sufficient residual Cl^- (50-70 µM) to fulfill essential requirements. Pots were irrigated up to field capacity (2 mL g^{-1} substrate) throughout the experiment. Pots were weighted each week at field capacity to estimate the evolution of plants fresh weight over time. After 6 weeks (72 Days after seeding, DAS), plants were harvested, fresh weight (FW) measurements were obtained, and then samples were dried in a forced-air oven at 75ºC for 48 h, and the dry weight (DW) was recorded as grams per plant. Dry tissues were ground for subsequently analyses. Harvest index was obtained as follows: g Seeds DW g^{-1} Total Biomass DW (Cuellar-Ortiz et al 2008).

Chloride and nutrient content determination

Dry tissues were ground to powder and incubated overnight in a 0.1N HNO_3 and 10% glacial acetic acid solution. After centrifuging, 0.5 ml of the solution was used for the determination of Cl^- content in a Corning 926 chloridemeter (Sherwood Scientific Ltd. Cambridge, U.K.) by silver ion-titration according to the manufacturer instructions.

Total nitrogen was measured by the Kjeldahl method for organic nitrogen (RB Bradstreet, 1965). Soluble nitrate content was measured through a multiparameter autoanalyzer 'Bran+Luebbe' (Bran+Luebbe Analytics, Norderstedt, Germany); and the phosphorous (P), potassium (K), sodium (Na), calcium (Ca), magnesium (Mg), sulphur (S), iron (Fe), copper (Cu), manganese (Mn), zinc (Zn), nickel (Ni) and boron (B) content was determined through ICP-OES Varian ICP 720-ES spectrometers' (Varian Inc., Palo Alto, CA, USA).

Relative growth rate (RGR)

To determine the relative growth rate (RGR), tissues of 3 plants per treatment (n = 3) were sampled at 39 and 50 DAS. The plant tissues were dried in a forced-air oven at 70ºC for 48 h, and the dry weight (DW) was recorded as grams per plant. The remaining plants (n = 6) were sampled at 72 DAS (Tf). RGR was calculated from the increase in total, foliar, stem and root DW at the beginning and at each treatment, using the equation RGR = (ln DW_f – ln DW_i) / (Tf – Ti), where T is the time and the subscripts denote the final and initial sampling time (Rosales et al, 2012).

Leaf Area

Detached leaves were scanned in Epson Stylus DX4000 multifunction printer (SEIKO EPSON CORP., Owa, Suwa, Nagano, JAPAN). Scanning settings were defined as: colourless b/w and a very low resolution (72 ppp.). Leaf area was measured with the program 'Medición de Hojas v1.0' (Property of the Department of Ecology, University of Seville, SPAIN). Data were obtained in cm^2.

Water Parameters

Relative water content (RWC) and succulence were determined from ten discs (1 cm diameter) per plant as follows: RWC = (FW – DW) (fully hydrated weight – DW)$^{-1}$; Succulence (g cm^{-1}) = (FW – DQ) (area)$^{-1}$ (Barrs and Weatherley, 1962). Leaf osmolarity was obtained from dry ground tissue. In order to re-establish the average water content of mature leaves (previously calculated), a constant volume of ultrapure Milli-Q water was added to 0.1 g of the dry ground tissue obtained from different treatments. All samples were incubated for 2 hours in an orbital shaker at 36ºC. Samples were centrifuged at 12000g for 10 minutes, and the supernatant were transferred to 0.5 mL centrifuge tubes. Osmolarity was measured using the dew-point method with a dew-point microvoltimeter (model HR-33T, Wescor, UT, USA) and the C-52 sample chamber, as described in the instructions manual of the manufacturer.

Statistical analysis

The data compiled were submitted to an analysis of variance (ANOVA) and the differences between the means were compared by Tukey range test ($P < 0.05$). Values represent mean of six tobacco plants in each treatment (n = 6).

RESULTS AND DISCUSSION

Chloride content

Chloride content of Cl plants was higher in leaves (Table 1), exceeding by 150 times the critical requirement content (approximately 0.3 mg g^{-1} DW; Marschner et al, 1995). This accumulation corresponds to 100 mM Cl^-, which was similar to the content of the macronutrient K^+ (115 mM). In plants treated with low Cl^- (in the micromolar range: BS, N and SP plants) the highest accumulation occured in flowers (Table 1) and younger leaves (Fig.1). Contrary to general belief (reviewed by White and Broadley, 2001) this observation indicate that this nutrient is preferentially included in young and reproductive organs, indicating its potential involvement in developmental processes.

Table 1. Cl^- content in tobacco plant organs (mg g^{-1} DW)

ID	BS	SP	N	Cl
ROOTS	0.275 ± 0.001 b	0.256 ± 0.002 b	0.312 ± 0.004 b	10.843 ± 0.042 bc
STEM	0.318 ± 0.001 b	0.331 ± 0.003 b	0.337 ± 0.003 b	14.081 ± 0.053 b
LEAVES	0.506 ± 0.004 b	0.456 ± 0.004 b	0.381 ± 0.002 b	45.637 ± 0.223 a
INFLORESCENCE	1.368 ± 0.018 a	1.512 ± 0.023 a	0.848 ± 0.011 a	8.403 ± 0.035 c
P-Value[1]	***	***	***	***
$HSD_{0.05}$	0.46	0.35	0.19	4.56

[1]Level of significance is represented by *** P<0.001.

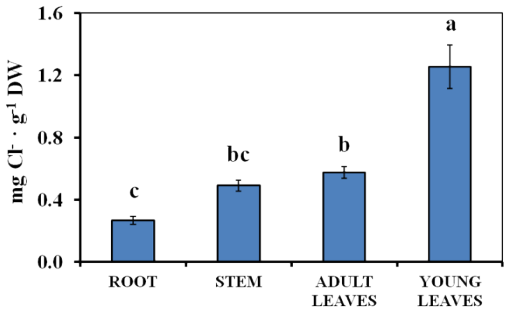

Figure 1. Cl^- content in vegetative organs of BS plants (treated with low Cl^-). Data obtained from a different experiment to that shown above *P*<0.001.

Growth

Chloride in the millimolar range stimulated plant growth (Figure 2). Fresh weight was higher in Cl plants compared to BS and SP plants, and was similar to that of N plants. RGR of Cl plants was significantly higher than that of SP plants and closer to RGR of N plants (Fig. 3). Since N plants, which exhibited the highest growth and biomass values (Fig. 2-4), presented the lowest Cl^- content, we can conclude that N plants, and therefore BS and SP plants, were not experiencing nutritional deficiency of Cl^-. Dry weight obtained at the end of the experiment also showed significantly higher biomass of Cl plants compared to BS and SP plants, but lower compared to N plants (Fig. 4 A). All organs analysed (Fig. 4B-E) exhibited this tendency (biomass N>Cl>SP=BS). Interestingly, Cl plants showed the highest harvest index (Fig. 4F).

Altogether, these results show that when available in the millimolar range, tobacco plants accumulated Cl^- to levels that are typical of the content of a macronutrient, stimulating plant development. The possibility that this effect was due to the accompanying cations was ruled out since SP plants, containing the same cations balance, did not improve growth compared to BS plants. The possibility that reduced growth of BS and SP plants could be due to Cl^- deficiency was also ruled out since N plants, with the lowest Cl^- content values, exhibited the highest growth. When accumulated to high levels, Cl^- may play functions that impact growth, possibly related with osmoregulatory and water relation processes.

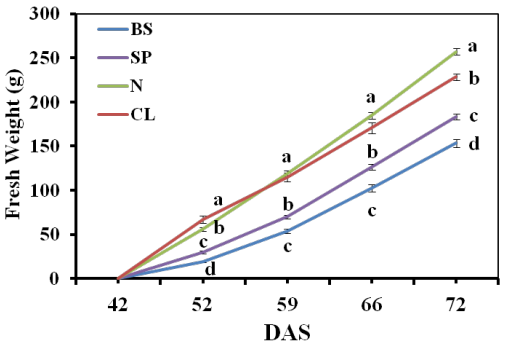

Figure 2. Evolution of plant **fresh weight** throughout the experiment. *P*<0.001.

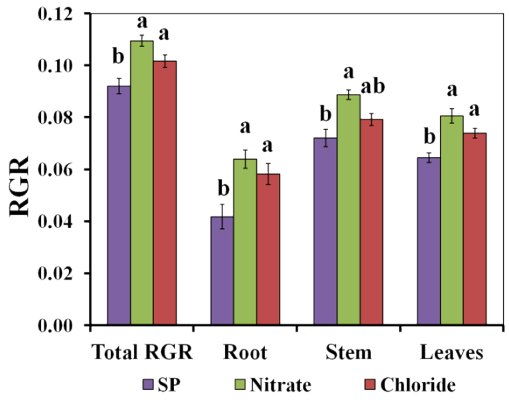

Figure 3. Relative growth Rate (RGR). In whole plants and leaves, *P*<0.001; in root and stem, *P*<0.01

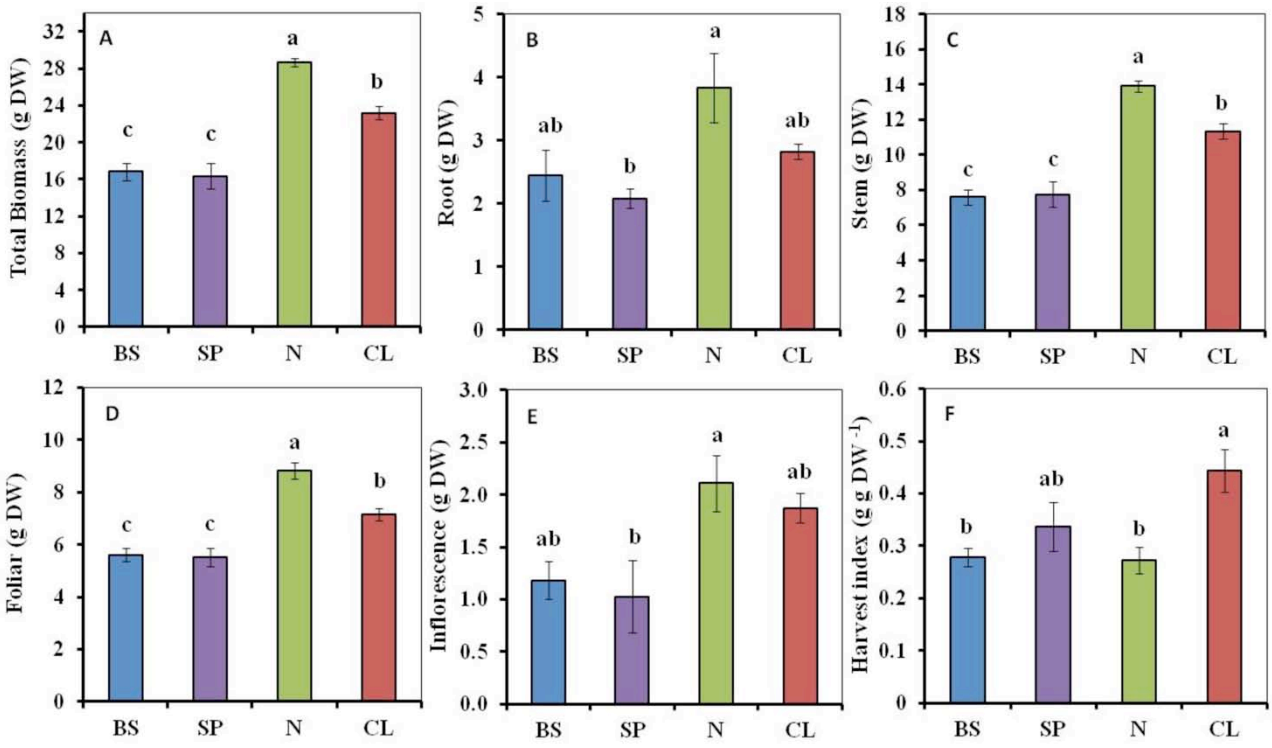

Figure 4. Biomass. Average DW per treatment and plant organ (**A** to **E**), and harvest index (**F**). $P<0.001$ (**A**, **B**, **D**). $P<0.05$ (**C**, **E**, **F**).

Water relations

The plant water status is linked to several leaf physiological variables, such as water and osmotic potential, relative water content and turgor (Kramer & Boyer, 1995). Since we hypothesized an osmotic effect of Cl^- nutrition on plants, we measured the leaf tissue osmolarity (Fig. 5A).

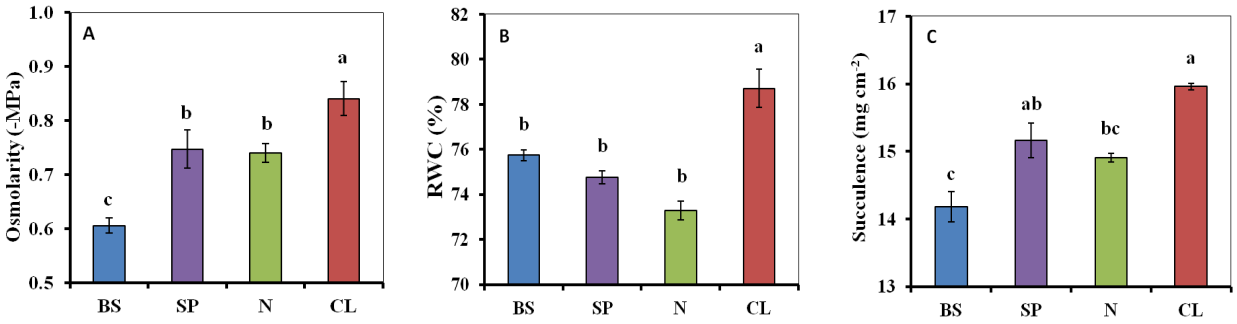

Figure 5. Leaf water balance parameters: osmolarity (**A**); RWC (**B**); and Succulence (**C**). $P<0.001$ (**A**, **C**). $P<0.01$ (**B**).

Cl plants exhibited the highest osmolarity, whereas BS plants exhibited the lowest one, having SP and N plants intermediate values. Accordingly, Cl plants also showed the highest relative water content (Fig. 5B) and succulence values (water content per unit area; Fig. 5C). When using a turgor pressure probe, no clear differences of leaf turgor among treatments during day/night oscillations were observed in well-irrigated plants (not shown). Correlation analyses of nutrient content *vs* water content showed optimal correlations (R^2 close to 1) with Cl^- and

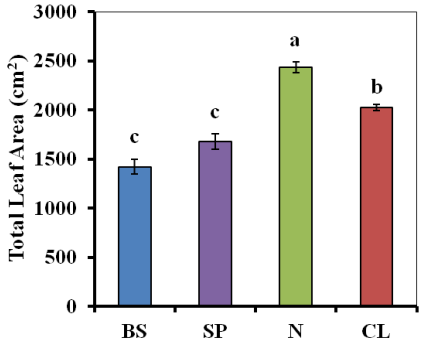

Figure 6. Total leaf area. $P<0.001$.

K^+ ions (Fig. 7).Therefore, leaves from Cl plants contained more water compared to the other treatments. Cl leaves also presented significantly higher dry biomass (Fig. 4D) and higher leaf area compared to BS and SP plants (Fig. 6), indicating that besides the osmoregulatory effect, Cl^- is playing a developmental role in plants.

Figure 7. **Ion content *vs.* water content correlationships:** Using Cl and N treatments, the leaf content of different macronutrients was correlated with the water content of the corresponding leaf. The Pearson correlation coefficient (R^2) was obtained and represented. **Cl^- (Cl)**, chloride content in Cl-treated plants; **K^+ (Cl)**, potassium content in Cl-treated plants; **NO_3^- (NO_3^-)**, nitrate content in Cl-treated plants; etc. **NT**, total nitrogen.

CONCLUSIONS

When Cl^- is available (5 mM), tobacco plants take and accumulate this nutrient to values that are typical of the content of a macronutrient, stimulating both vegetative and reproductive growth. This effect is not due either to the effect of accompanying cations or to a nutritional deficiency of non-Cl^- treated plants. Chloride increases the osmolarity and water content of leaves, probably stimulating its growth. Two observations, namely the inclusion of Cl^- into growing and reproductive organs, and the increased biomass and total area of leaves in Cl plants, indicates that Cl^- might be playing specific roles in plant development, a possibility that we are currently investigating in our laboratory.

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