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Chloride reduces plant nitrate requirement and alleviates low nitrogen stress symptoms

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ABSTRACT

Chloride (Cl^-) is traditionally categorized as an antagonist of nitrate (NO_3^-) because Cl^- hinders plant NO_3^- transport and accumulation. However, we have recently defined Cl^- as a beneficial macronutrient for higher plants, due to specific functions that lead to more efficient use of water, nitrogen (N) and CO_2 under optimal N and water supply. When accumulated in leaves at macronutrient levels, Cl^- promotes growth through osmotic, physiological, metabolic, anatomical and cellular changes that improve plant performance under optimal NO_3^- nutrition. Nitrate over-fertilization in agriculture can adversely affect crop yield and nature, while its deficiency limits plant growth. To study the relationship between Cl^- nutrition and NO_3^- availability, we have characterized different physiological responses such as growth and yield, N-use efficiency, water status, photosynthesis, leaf anatomy, pigments and antioxidants in tomato plants treated with or without 5 mM Cl^- salts and increasing NO_3^- treatments (3–15 mM). First, we have demonstrated that 5 mM Cl^- application can reduce the use of NO_3^- in the nutrient solution by up to half without detriment to plant growth and yield in tomato and other horticultural plants. Second, Cl^- application reduced stress symptoms and improved plant growth under low-NO_3^- conditions. The Cl^--dependent resistance to low-N stress resulted from: more efficient use of the available NO_3^-; improved plant osmotic and water status regulation; improved stomatal conductance and photosynthetic rate; and better antioxidant response. We proposed that beneficial Cl^- levels increase the crop ability to grow better with lower NO_3^- requirements and withstand N deficiency, promoting a more sustainable and resilient agriculture.

1. Introduction

Nowadays, the use of nitrogen (N) fertilizers to improve crop production and yield is an ordinary practice in agriculture (Ju et al., 2007; Coskun et al., 2017). However, their over-use cause profound environmental concerns and can also restrict plant growth and crop yield (Lupini et al., 2017; Cheng et al., 2021). Nitrogen application in agriculture is highly inefficient, since only about 30–50% of the N applied in soils is absorbed by crops (Coskun et al., 2017; Chen et al., 2020). The remaining N results in the emission of nitrogenous pollutants into the atmosphere (i.e. N_2O, NO, NH_3), contributing to the greenhouse effect and global warming. In addition, leaching and runoff cause a relevant part of the N to reach aquifers and surface waters, giving rise to eutrophication phenomena with severe environmental consequences (Motavalli et al., 2008; Martinez-Espinosa et al., 2011; Sutton et al., 2013). Nitrate (NO_3^-) is the most common source of N in nature, but excessive NO_3^- levels can negatively reduce crop yield and nutritional quality, becoming harmful to human health when excessively accumulated in leaves (Prasad and Chetty, 2008; Zhong et al., 2017; Xing et al., 2019). Fertilizations with NO_3^- exceeding 0.3 g N per kg of soil reduce the

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growth of crops like cabbage, rape or spinach (Chen et al., 2004). Additionally, NO_3^- disrupt plant development when applied at concentrations greater than 5 mM in maize (Saiz-Fernández et al., 2015) or 10 mM in arabidopsis (López-Bucio et al., 2003). However, N is the nutrient that most importantly limits the growth of terrestrial plants, playing a central role in plant metabolism, growth and development. N deficiency results in smaller leaves and roots (Rahayu et al., 2005; Gruber et al., 2013; de Bang et al., 2021), impairs water relations and photosynthesis and accelerates plant senescence, thereby decreasing crop productivity (Mu and Chen, 2021; Sakamoto et al., 2021). In addition, N deficiency also induces the appearance of stress symptoms caused by the accumulation of reactive oxygen species, generating a series of antioxidant responses in plants (Rubio-Wilhelmi et al., 2011a). Therefore, achieving higher crop yields under N-limited soil conditions or reducing N fertilization, by improving N use efficiency (NUE), is a fundamental requirement for sustainability in current agricultural systems (EU Nitrogen Expert Panel, 2015; Neocleous et al., 2021; Carillo and Rouphael, 2022).

NO_3^-, the major N source in plants, shares similar physical properties with chloride (Cl^-), playing both anions important roles in charge balance and cell osmoregulation and showing strong dynamic interactions in plant cells (Wege et al., 2017; Colmenero-Flores et al., 2019). These effects, together with the fact that both anions share membrane transport mechanisms, have been interpreted as an antagonistic interaction negatively affecting NUE, particularly under saline conditions (Xu et al., 2012; Wang et al., 2020; Carillo and Rouphael, 2022). Traditionally, the accumulation of Cl^- in high amounts in sensitive crops has been considered harmful to plants, affecting NO_3^- uptake by roots and its accumulation in the plant (Geilfus, 2018; Corrado et al., 2020). However, we recently demonstrated that the presence of Cl^- at levels typical of a macronutrient acts as a beneficial element for plant growth and development (Franco-Navarro et al., 2016; Colmenero-Flores et al., 2019; Cakmak et al., 2023). When available in the nutrient solution at concentrations ranging from 1 to 5 mM, plants actively take up Cl^- and accumulate it in leaves at levels significantly exceeding those required as a micronutrient (i.e. 10-50 mg g^{-1} DW depending on the plant species), thereby exerting a profound impact on the plant's osmoregulatory capacity and other cellular and physiological processes (Franco-Navarro et al., 2016; Colmenero-Flores et al., 2019; Cakmak et al., 2023). In consequence, it stimulates the size of leaf cells, leading to a reduction in stomatal density and stomatal conductance (g_s), but not affecting the net photosynthesis rate (A_N), dealing to increased efficiency in the use of water (WUE_i; Franco-Navarro et al., 2016). Furthermore, Cl^- has been demonstrated to improve mesophyll diffusion conductance to CO_2 (g_m) and, therefore, the photosynthetic performance (Franco-Navarro et al., 2019). Due to their roles in the regulation of water balance and whole-plant water relations, beneficial Cl^- nutrition has also been shown to improve plant resistance to water deficit (Franco-Navarro, et al., 2021). In addition, Cl^- allows for a more efficient assimilation of NO_3^- into organic N in plants grown under optimal NO_3^- concentrations (8 mM NO_3^- in the nutrient solution), resulting in a higher NUE and plant biomass, possibly as a result of the substitution of NO_3^- for Cl^- in the vacuole and in the osmoregulatory functions of plant cells (Rosales et al., 2020; Peinado-Torrubia et al., 2023). Hence, Cl^- nutrition emerges as a promising approach to enhance WUE and NUE in crops, particularly relevant in situations where NO_3^- availability becomes limited in the soil, a scenario where Cl^- nutrition remains largely unexplored.

We hypothesize that as a beneficial macronutrient, Cl^- can simultaneously replace partially NO_3^- while making a more efficient use of the less available NO_3^-, serving as a potential tool to reduce NO_3^- application in agriculture. We show in this study that 5 mM Cl^- application can: reduce the use of NO_3^- by up to half (for example from 12 to 6 mM NO_3^-) without detriment to the growth of tomato and other vegetables; reduce plant stress symptoms derived from low N treatments; and maintain a better growth, water status, photosynthesis and antioxidant capacity in the plants. These findings point to the possibility of designing novel formulations of fertilizers that reduce the amount of NO_3^- according to the Cl^- concentrations available in irrigation water and crop soil to promote a more sustainable and resilient agriculture.

2. Material and methods

2.1. Experimental design, plant material and nutritional treatments

Five independent experiments were carried out from April 2021 to May 2022. First, three experiments were performed with tomato plants and harvested at two different stages: i) vegetative, at 34 days after sowing (DAS), for the analysis of non-destructive (gas-exchange) and destructive (biomass, nutrients, potentials, microscopy and biochemical) parameters, and ii) ripening (90 DAS), for the determination of shoot biomass and tomato fruit production. Later, another set of two experiments was performed with lettuce, red chard and spinach plants for fresh biomass production at 30 DAS.

Tomato seeds (*Solanum lycopersicum* L. 'Ailsa Craig') were vernalized for 2 days in a cold chamber (4–7 °C) and sown in flat trays (cell size 4 x 4 × 10 cm) containing a mix of sand:vermiculite (2:8), previously washed with distilled water and the corresponding nutrient solutions. Tomato seedlings grown under semi-hydroponics conditions, i.e. semi-flooded in trays containing a constant 2–5 cm layer of nutrient solution, as described by Franco-Navarro et al. (2016). The greenhouse conditions were as follows: a temperature between 25 ± 3 °C/17 ± 2 °C (day/night), a relative humidity of 60 ± 10% (EL-1-USB Data-logger, Lascar Electronics Inc., Erie, PA, USA), a 16/8 h photoperiod with a photosynthetic proton flux density (average photosynthetically active radiation; PAR) of 300–350 μmol m^{-2} s^{-1} (quantum sensor, LI-6400; Li-COR, Lincoln, NE, USA), and a luminous emittance of 9000–10,000 lx (Digital Lux Meter, LX1010B; Carson Electronics, Valemount, Canada). At 14 DAS, seedlings were transferred to pots (15 cm × 15 cm × 20 cm), with the same sand/vermiculite substrate and semi-hydroponics conditions, and harvested at 34 DAS. For the ripening tomato experiment, plants were subsequently transferred to larger pots (20 cm × 20 cm × 25 cm) and harvested at 90 DAS. Throughout the entire growth cycle, tomato plants were treated with increasing NO_3^- concentrations (from 3 to 15 mM NO_3^-) in combination with two Cl^- treatments: 5 mM Cl^- salts (CL) and a mix of sulphate (SO_4^{2-}) and phosphate (PO_4^{3-}) salts as a control (SP; 0.075 mM Cl^-) that contained the same cation balance (as described in Franco-Navarro et al., 2016). Cations added with NO_3^- treatments were also equilibrated with SO_4^{2-}+PO_4^{3-} salts as described in Tables S1 and S2. A basal concentration about 0.075 mM Cl^- was monitored in all treatments coming from salts and water traces, which was sufficient to fulfil the micronutrient Cl^- functions in low Cl^- treatments (Franco-Navarro et al., 2016, 2019). All nutritional solutions were adjusted with KOH until a pH of 5.7 was reached. A half of the harvested plants were separated into organs and roots were rinsed with tap water and distilled water, removing the excess water with filter paper. Subsequently, tissues were dried at 75 °C for 48 h and weighted to obtain the dry weight (DW) biomass. The other half of the plants were conserved at −80 °C and subsequently used for the analysis of biochemical parameters.

For the horticultural species experiment, seeds of lettuce *(Lactuca Sativa* L. 'Batavia Solara amarilla'), red chard (*Beta vulgaris* subsp. cicla 'Rhubarb chard'), kale (*Brassica oleracea* L. 'Nero di Toscana') and spinach (*Spinacia oleracea* L. 'Butterflay') were vernalized and sown in flat trays containing a perlite:vermiculite mixture (7:3) under similar controlled and semi-hydroponics conditions as explained above for tomato plants. At 15 DAS, seedlings were transplanted to pots (15 cm × 15 cm x 20 cm) with the same inert substrate. Only shoots were harvested at 30 DAS and weighed on a precision scale to obtain the fresh weight. In these two experiments, only three treatments were used: the control SP treatment combined with 9 mM NO_3^- (9N:SP) and 15 mM NO_3^- (15N:SP), and 5 mM Cl^- treatment combined with 9 mM NO_3^- (9N:CL), as described in Tables S1 and S2.

2.2. Ion determination and NUE parameters

For the determination of the amount of nutrients, fully photosynthetic and expanded mature leaves from plants of 34 and 90 DAS were used. The dried leaf tissue was ground to powder to prepare the aqueous extracts. The concentration of Cl^-, NO_3^- and NH_4^+ were determined according to colorimetric assays using a microplate spectrophotometer (OMEGA) as previously reported in Franco-Navarro et al. (2016). Organic N was determined by the Kjeldahl method (Bradstreet, 1954). The amount of total N (TN) represents the sum of organic N and the N that forms part of both NH_4^+ and NO_3^- molecules, expressed as mg N g^{-1} DW. NUE is defined as total biomass per unit of N applied in the nutrient solution (g DW g^{-1} N; Moll et al., 1982). N utilization efficiency (NU_TE) was calculated as total DW divided by TN (g^2 DW mg^{-1} N; Siddiqi and Glass, 1981).

2.3. Leaf water and gas-exchange parameters

Leaf osmotic potential (Ψ_π) was calculated from the leaf sap obtained from leaf discs: 2 leaf discs per sample × three samples per plant × six plants per treatment. Leaf sap was extracted from leaf discs by transferring the samples, placed in 0.5 mL microcentrifuge tubes, from a block heated to 90 °C to liquid nitrogen. Tube caps were sealed with parafilm to avoid water evaporation. This thermal shock was repeated five times and leaf sap was collected in 1.5 mL tubes by centrifugation and filtration of tissue debris. For leaf water potential (Ψ_w) measurements, leaves were bagged with a sealed plastic bag during 20–30 min before collection (Begg and Turner, 1970). Samples were all double bagged in a plastic bag saturated with water vapour and carried to the laboratory in an insulated box. Ψ_w were measured with a Scholander pressure chamber (Soil Moisture Equipment Corp 3005; Santa Barbara Corp, Santa Barbara, CA, USA). Leaf turgor (or pressure) potential (Ψ_p) was calculated from the Ψ_w and Ψ_π values (experimentally obtained) according to the following equation:

$$\Psi_p \text{ (MPa)} = |\Psi_\pi| - |\Psi_w|$$

The gas-exchange parameters were measured between 11:00 and 14:00 h using an open gas-exchange system (LI-6400, LI-COR, Lincoln, NE, USA) at ambient temperature, saturating photosynthetic photon flux density (1600 μmol m^{-2} s^{-1}), an air temperature of 25 °C and an ambient CO_2 concentration (C_a) of between 50 and 1500 μmol mol^{-1}, as described in Franco-Navarro et al. (2016). The WUE_i was calculated as the ratio between the rate of photosynthesis and stomatal conductance (A_N/g_s).

2.4. Quantum yield

The PII quantum yield (Qy) is considered as a marker of plant stress that quantifies the efficiency of Photosystem II (Franco-Navarro et al., 2021). Chlorophyll fluorescence in light-adapted plants was measured using a portable fluorometer (FluorPen FP-100; Photon System Instruments, Brno, Czech Republic). The Qy was calculated according to Maxwell and Johnson (2000):

$$Qy = \Phi_{PSII} = F_m'(F_v')^{-1}$$

through the measurement of the following variable parameters:

$$F_v' = (F_m' - F_t)$$

where F_v' is the difference between F_m' (the maximum fluorescence in the light-adapted state) and F_t (the basal fluorescence in the light-adapted state).

For each treatment, three measurements per leaf were recorded, and three leaves per plant were used to obtain the mean value. The results correspond to the mean values collected during the 2–4 days prior to harvest.

2.5. Anatomical parameters

For specific leaf area (SLA), tomato leaves were placed on a white filter paper with a ruler, images were recorded and subsequently analysed using ImageJ software. Data were obtained in cm^2. SLA was calculated as described in Marcelis et al. (1998):

$$SLA = (\text{Total leaf area}) / (\text{Total leaf DW})^{-1}$$

Epidermal peelings and epidermal impressions of abaxial leaf cells were made following the method described by Allen et al. (1999) and He et al. (2013) with slight modifications. The peels were obtained from the abaxial surface by scraping the epidermis with a scalpel and removing it with tweezers (Franco-Navarro et al., 2019). Images of the epidermal impressions were taken with the Leica DM2000 optical microscope. Both the size of leaf cells and the absolute stomatal aperture area were measured using ImageJ Software. Data were obtained in μm^2.

2.6. Measurement of photosynthetic pigments and antioxidant capacity

Tomato leaf extracts were prepared with pure methanol (0.2 g in 10 mL) and centrifuged at 12,000 rpm for 15 min at room temperature. The optical density of the supernatants was used to spectrophotometrically measure chlorophylls and carotenoids at 665.2 nm, 652.4 nm, and 470 nm, using pure methanol as a blank. The respective concentrations of these photosynthetic pigments were calculated based on the formulas established by Lichtenthaler and Buschmann (2001):

$$C_a = 16.72\ A_{665.2} - 9.16\ A_{652.4}\ (\mu g\ mL^{-1})$$

$$C_b = 34.09\ A_{652.4} - 15.28\ A_{665.2} (\mu g\ mL^{-1})$$

$$\text{Carotenoids} = (x + c) = (1000\ A_{470} - 1.63\ C_a - 104.96\ C_b) / 225$$

The concentration of anthocyanins was calculated according to the method of Mancinelli et al. (1974) modified by Rosales et al. (2011) by measuring at 530 and 653 nm using a spectrophotometer after acidifying the extracts with 1% HCl. Total phenolic compounds (phenols and flavonoids) were also measured by colorimetric methods using the Folin-Ciocalteu reagent and gallic acid as a reference standard described by Rosales et al. (2011). Their absorbance was measured at 415 nm.

The amount of H_2O_2 of leaf samples was determined as described by Mukherjee and Choudhuri (1985) and modified by Rubio-Wilhelmi et al. (2011a). The determination of malondialdehyde (MDA) was carried out according to Taulavuori et al. (2001), using the same extracts as those for photosynthetic pigments in the spectrophotometer at 440, 532, and 600 nm. The concentration of MDA was calculated following the formula:

$$A = Abs_{532} - Abs_{600} - (Abs_{532\ TCA} - Abs_{600\ TCA})$$

$$B = (Abs_{440} - Abs_{600})\ 0.0571$$

$$MDA\ (mM) = (A - B / 157000)\ 10^6$$

To analyse the antioxidant activity, various tests were conducted following the methodology described by Rosales et al. (2006): The Ferric Reducing Ability of Plasma (FRAP) assay, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging effect and the reducing power.

2.7. Statistical analysis

For the statistical analysis, the software STATGRAPHICS Centurion XVI (StatPoint Technologies, Warrenton, VA, United States) was used. Shapiro–Wilk (W) test was used to verify the normality of the data sets. One-way ANOVA and multivariate analysis of variance (MANOVA) were performed to determine significant differences between groups of samples, and levels of significance were described by asterisks: $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$. Non-significant (ns) differences were

indicated when $P > 0.05$. The means were compared using Tukey's post hoc HSD (honestly significant difference) test and MRT (multiple range test) statistical tests included in the above-mentioned software. Principal component analysis (PCA) was also performed. Values represent the mean of at least six plants per treatment, which were reproduced in at least two independent experiments.

3. Results

3.1. Plant biomass, anions and fruit production

To study whether Cl^- nutrition can be used as a potential strategy to reduce the NO_3^- supply in fertilizers, we conducted several greenhouse experiments with tomato plants grown under a basal nutrient solution alternatively supplemented with 0 and 5 mM Cl^- salts in combination with increasing NO_3^- treatments, ranging from deficiency (3 mM NO_3^-; 3N) to excess (15 mM NO_3^-; 15N). To determine the effect of these Cl^-/NO_3^- treatments on plant growth and fruit production, tomato plants were collected at 34 and 90 DAS, corresponding to vegetative and ripening stages, respectively. During the vegetative stage, the results showed that Cl^--free plants (control SP) positively responded to increasing NO_3^- treatments, reaching the maximum values of total, leaf and root biomass at 12 mM NO_3^- application (Fig. 1A–C). Interestingly, the Cl^--treated plants significantly surpassed the SP plants in total, leaf and root biomass in the 3–12 mM NO_3^- treatments, showing a higher increase of 93% in total biomass values under the 3N treatment in CL plants compared to SP plants (Fig. 1A–C). The 15N treatment produced reduction in total biomass compared to the 12N treatment in both SP plants (22% reduction) and CL plants (35% reduction), pointing to a toxic effect on plant growth due to excessive NO_3^- supply (Fig. 1A).

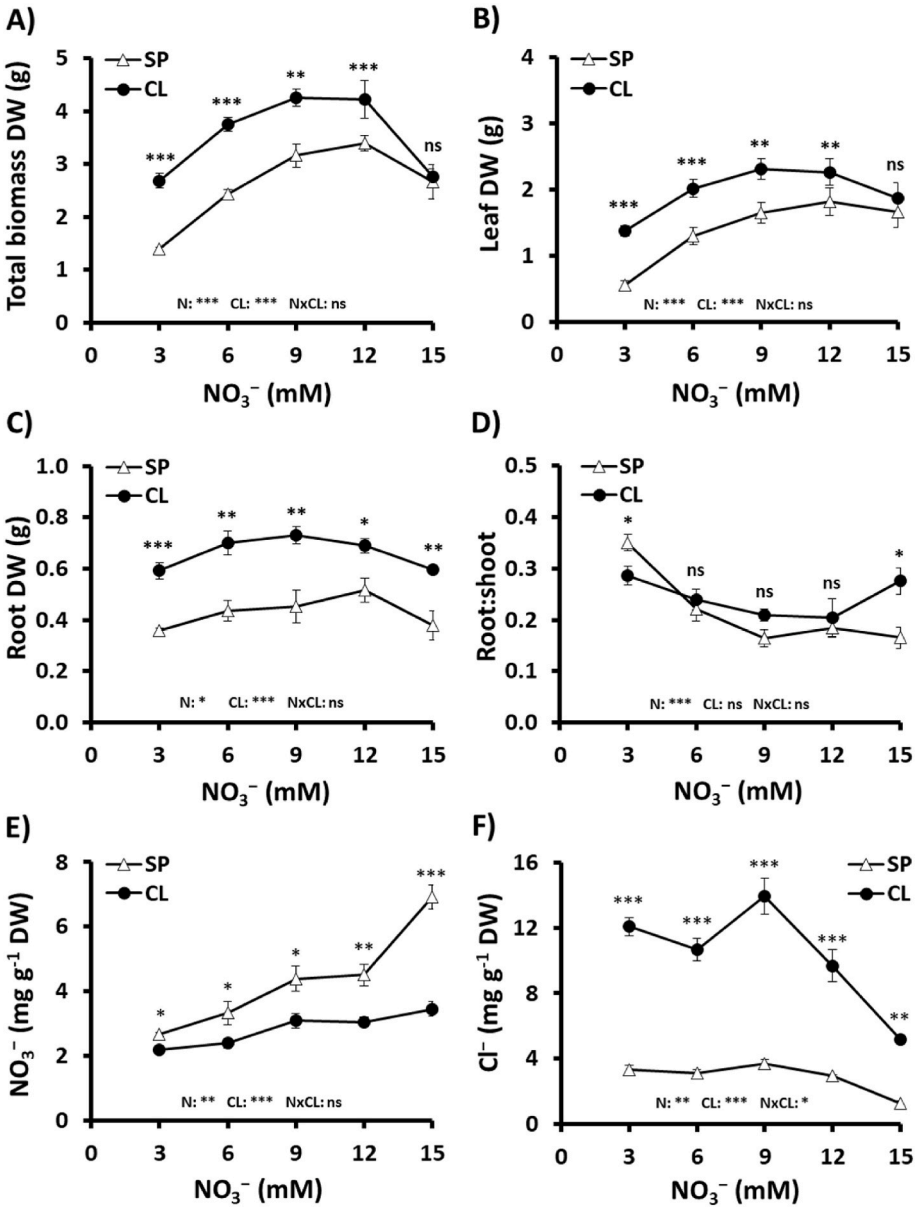

Fig. 1. Effect of Cl^- and NO_3^- treatments on plant growth and anion amount in 34 days-old tomato plants. Tomato plants were treated with increasing NO_3^- concentrations (N; 3, 6, 9, 12, and 15 mM), and alternatively with two different Cl^- treatments: 0.075 mM Cl^- with a mix of SO_4^{2-} and PO_4^{3-} salts as a control (SP), and 5 mM Cl^- salts (CL), maintaining the same balance of cations. (A) Total dry weight (DW); (B) Total leaves DW; (C) Total roots DW; (D) Root:shoot ratio; (E) The amount of NO_3^- per g of leaf DW; and (F) The amount of Cl^- per g of leaf DW. Mean values ± SE; $n = 6$–12. 'Homologous group' statistics was calculated through ANOVA and MANOVA, where mean values of SP and CL treatments are compared in each N treatment. Levels of significance: $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; and 'ns' $P > 0.05$.

These results confirmed the stimulatory effect of Cl^- on plant growth parameters and evidenced the occurrence of nutritional stress symptoms in SP plants under low N conditions. Regarding the root-to-shoot ratio, significant differences between SP and CL plants were found in both 3 mM and 15 mM NO_3^- treatments (Fig. 1D).

Consistently with the NO_3^- application, its concentration in leaves also gradually increased in both SP and CL plants, reaching the highest amount at 15 mM NO_3^- treatment as expected (Fig. 1E). However, the amount of NO_3^- in Cl^--treated plants remained below SP plants, with stronger differences at higher NO_3^- treatments. It is important to note that, as previously demonstrated (Franco-Navarro et al., 2016, 2021; Rosales et al., 2020), SP plants are not in conditions of Cl^- deficiency as an essential micronutrient, showing plants no symptoms of wilting, bronzing or chlorosis (Fig. S1). Furthermore, the amount of Cl^- in leaves of SP-treated plants ranges around 1.0–3.5 mg g^{-1} DW (Fig. 1F), which is an order of magnitude above the deficiency threshold of 0.1–0.2 mg g^{-1} DW defined in glycophyte plants (Xu et al., 2000; White and Broadley, 2001). The results also showed a negative effect of NO_3^- supply on plant Cl^- transport/accumulation in the range of 9–15 mM NO_3^- supply, with 15 mM NO_3^- strongly reducing Cl^- concentration in CL plants (Fig. 1F). This reduction could be related to the sharp decrease in total biomass observed (Fig. 1A).

Considering that the maximum growth of SP plants (i.e. 9-12 mM NO_3^-) was exceeded by Cl^--treated plants under 6 mM NO_3^- (Fig. 1A), a complementary experiment with 90 days-old tomato plants was performed to determine the role of Cl^- as a potential substitute of NO_3^- to improve plant growth and tomato yield. To this end, SP tomato plants were grown in pots up to the ripening stage (90 DAS) under 5 and 9 mM NO_3^- conditions and compared to CL plants treated with 5 mM NO_3^-. Results showed that the 5N:CL treatment again surpassed the 5N:SP in both shoot and fruit DW and the fruit number, exhibiting similar yield values than 9N:SP plants (Fig. 2A–B). To demonstrate whether the Cl^- application also reduces the NO_3^- requirements in crop plants other than tomato, and can be proposed as a potential tool to reduce NO_3^- fertilization in agriculture, plant species such as lettuce, red chard, kale and spinach were grown under 9 and 15 mM NO_3^- (9N:SP and 15N:SP, respectively) and compared to 9 mM NO_3^- + 5 mM Cl^- (15N:CL). In these species, the 9N:CL treatment showed a significantly increase in FW biomass than the 9N:SP treatment, which resulted in similar biomass values exhibited by the 15N:SP treatment. Therefore, these results confirm the positive effect of Cl^- nutrition in reducing NO_3^- requirements in plants.

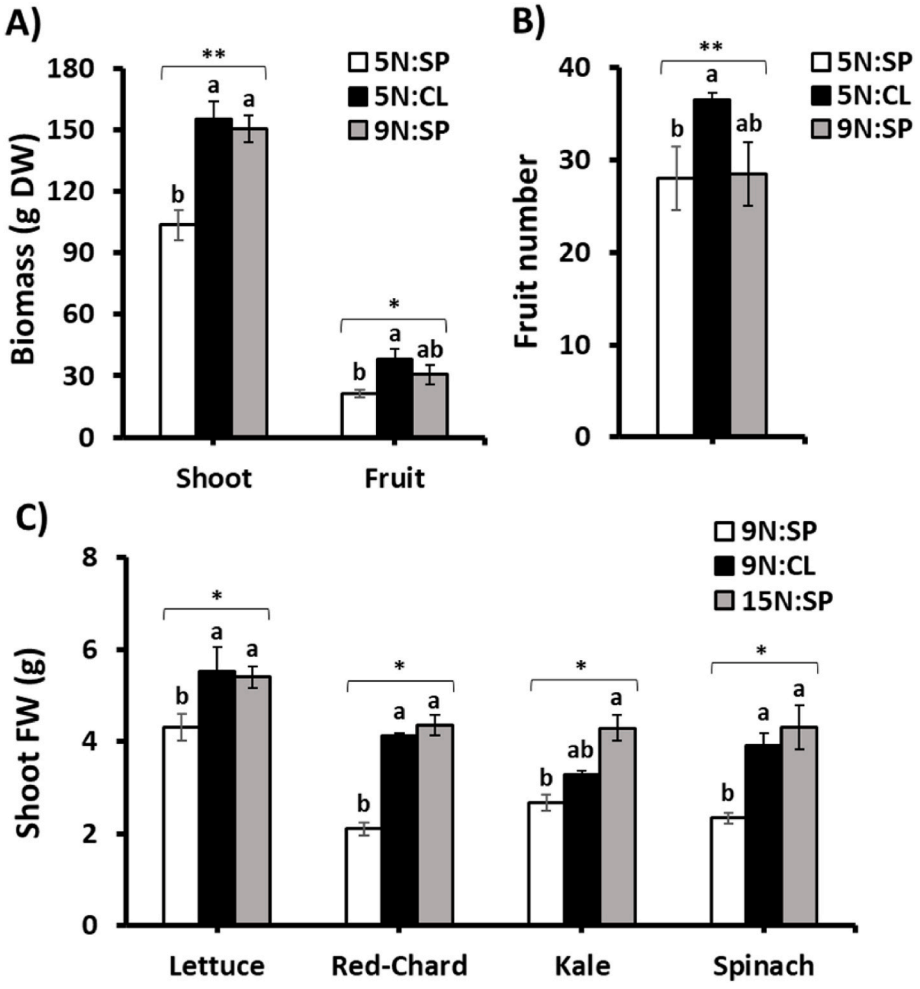

Fig. 2. Effect of Cl^- and NO_3^- treatments on plant growth and fruit production in tomato (90 DAS) and other horticultural plants (30 DAS). Tomato plants were treated with 5 and 9 mM of NO_3^- concentrations and alternatively with two different Cl^- treatments: 0.075 mM Cl^- with a mix of SO_4^{2-} and PO_4^{3-} salts as a control (SP), and 5 mM Cl^- salts (CL), maintaining the same balance of cations. Lettuce, red-chard, and kale plants were treated with 9 and 15 mM of NO_3^- concentrations and with SP and CL salts. (A) Shoot and fruit DW of 90 days-old tomato plants; (B) Fruit number of 90 days-old tomato plants; and (C) Shoot FW of 30 days-old lettuce, red-chard, kale and spinach. Mean values ± SE; $n = 5$ (tomato) and $n = 6$–12 (leafy vegetables). 'Homologous group' statistics was calculated through ANOVA, where mean values with different letters are significantly different for each plant species, according to Tukey's test at $P \leq 0.05$. Levels of significance: $^*P \leq 0.05$; and $^{**}P \leq 0.01$.

3.2. Nitrogen use efficiency

To better understand the physiological responses of Cl^--treated plants that result in higher growth with lower NO_3^- availability, we first analysed the impact of Cl^- on the amount of total N and NUE. The results showed no significant differences in the amount of TN per biomass unit between SP and CL plants independently of the NO_3^- supply (Fig. 3A). Besides, although NUE values increased in both SP and CL plants with lower NO_3^- treatments, we observed that NUE values of CL plants significantly surpassed those of SP plants (Fig. 3B). To verify that Cl^- promotes a more efficient use of N, we determined another NUE component, the NO_3^- utilization efficiency (NU_TE), which estimates how efficiently the transported N is used by the plant (Moll et al., 1982). Accordingly, NU_TE values significantly increased in Cl^--treated plants under all NO_3^- treatments studied (Fig. 3C). The fact that CL plants exhibited significantly less amount of NO_3^- (Fig. 1E) and higher NUE than SP plants clearly pointed to a more efficient assimilation of NO_3^- in CL-treated plants.

3.3. Leaf water status parameters

Considering the osmoregulatory properties of Cl^- under optimal NO_3^- availability (Franco-Navarro et al., 2016), we hypothesized in this work whether Cl^- participates in water-status responses under low NO_3^- conditions, by measuring Ψ_π, Ψ_w and calculating Ψ_p in leaves. The results of Ψ_π showed that Cl^--treated plants exhibited lower (more negative) values in both optimal and low NO_3^- treatments (9 and 3 mM, respectively) compared to SP plants (Fig. 4A). Interestingly, the low N availability generated a significant reduction of Ψ_w (more negative) in SP plants, meaning less tissue water available under low N conditions. On the contrary, CL plants showed the opposite effect, reaching the highest Ψ_w values (more tissue water available) under low NO_3^- conditions, which was progressively reduced under growing NO_3^- availability (Fig. 4B). Interestingly, turgor (Ψ_p) was significantly higher in CL-treated plants relative to SP-treated plants under all the NO_3^- treatments assayed, with the greatest differences observed under low NO_3^- conditions (Fig. 4C). Specifically, 3N:CL plants showed about 13 times greater turgor than 3N:SP plants, suggesting that the severity of NO_3^- deficiency symptoms on water-balance parameters are exacerbated in the absence of Cl^-. Interestingly, in Cl^--treated plants, turgor decreased as the availability of NO_3^- increased (Fig. 4C), as a result of the reduction in the amount of water in leaves (Ψ_w; Fig. 4B), rather than of the osmotic adjustment capacity (Fig. 4A).

3.4. Gas exchange and non-invasive stress marker parameters

Plant growth depends on both plant turgor and photosynthesis. For that reason, it is crucial to study how NO_3^- availability and Cl^- nutrition affect photosynthetic parameters and WUE_i. In this study, the A_N values of both SP and CL plants grown under 6 and 9 mM NO_3^- remained unaffected (Fig. 5A). However, under 6N and 9N treatments Cl^--treated plants exhibited significantly lower g_s and higher WUE_i than SP plants, respectively (Fig. 5B–C). Interestingly, under low NO_3^- levels, SP plants suffered a significant decrease of photosynthetic capacity (A_N) not observed in CL plants (Fig. 5A), which correlated with a reduction of the gas-exchange capacity (Fig. 5B), not affecting WUE_i (Fig. 5C). On the contrary, CL plants maintained photosynthetic parameters similar to optimal NO_3^- conditions (Fig. 5A–C). To compare the degree of stress symptoms between SP and CL plants resulting from N starvation, the photosynthetic efficiency of PII (Qy) was measured with a chlorophyll fluorometer in dark-adapted state. The Cl^- treatment exhibited higher Qy values than the SP treatment under low N conditions (Fig. 5D) and, therefore, greater protection of the photosynthetic machinery in line with the higher A_N values observed (Fig. 5A). These results show that, as a result of the low N levels imposed by the 3 mM NO_3^- treatment, SP plants exhibited stress symptoms as a result of a N deficiency, which were not observed in CL plants. On the other hand, Cl^--treated plants were unaffected by this reduction in N, as they maintained A_N, g_s, and WUE_i values very similar to those of plants treated with higher N concentrations.

3.5. Leaf anatomical parameters

To deepen into the Cl^--dependent physiological responses that reduced the stress symptoms of tomato plants under low N conditions, anatomical changes were analysed in leaf epidermal cells. The results showed an increase in total leaf area proportional to the increase in NO_3^- supply in both SP and CL plants (Fig. 6A). However, the Cl^- application promoted higher expansion of total leaf area compared to SP plants in all NO_3^- treatments, which correlated with the leaf biomass results (Fig. 1B). In addition, SLA values were also higher in Cl^--treated plants (Fig. 6B). Under low N, 3N:SP plants exhibited a strong reduction in the size of both epidermal cells and stomata with respect to 9N:SP plants, whereas CL plants maintained similar sizes under the three treatments (Fig. 6C–D). The stomatal density was higher in SP plants under low N (Fig. 6E) as a consequence the small size of the leaf epidermal cells observed in N-starved plants (Fig. 6C). The Cl^- treatment reversed this phenotype in low-NO_3^- plants, which showed similar levels of stomatal density under the different N treatments. Finally, we quantified the absolute stomatal aperture by measuring the stomatal pore area. The results showed that SP plants exhibited significantly higher stomatal aperture area than CL plants in both 6N and 9N treatments in line with higher g_s and values obtained under these treatments. However, under low N conditions the aperture area was severely impaired in SP plants, whereas this value was maximal in 3N:CL plants (Fig. 6F). The highest stomatal pore opening values observed in 3N:CL plants, are in line with their high Ψ_p and g_s and A_N values.

3.6. Photosynthetic pigments, oxidative stress markers and antioxidant capacity

To further investigate the better performance of Cl^--treated tomato plants under low NO_3^- conditions, we analysed metabolic responses such as the accumulation of photosynthetic pigments, indicator parameters of lipid peroxidation and oxidative stress (i.e. MDA and H_2O_2), total phenolic compounds and flavonoids, and various biochemical tests that determine their antioxidant capacity (i.e. FRAP, DPPH and reducing power). The results showed that under low N conditions, a significant reduction in the amount of photosynthetic pigments such as anthocyanins, chlorophyll *b*, and carotenoids was observed in both SP and CL treatments (Table 1). However, the Cl^- treatment stimulated the synthesis, phenolic compounds and flavonoids, as well as the plant antioxidant capacity (FRAP, DPPH, and reducing power) under low N conditions, which is in line with a reduction in the amount of H_2O_2 (Table 1). Although no differences were observed in the amount of pigments, a higher chlorophylls/carotenoids ratio was found in Cl^--treated plants under both 3N and 9N treatments.

3.7. Principal component analysis (PCA)

A PCA was performed to identify potential physiological parameters that participate in the Cl^--dependent responses to low N stress conditions in tomato plants. The PCA showed two axes explaining the 55.67% (PC1: 34.75%; PC2: 20.92%) of the total variance (Fig. 7). In this study, the loading matrix indicates that variation in stomatal density, Ψ_w and the amount of H_2O_2 are more closely aligned with the 3N:SP treatment, whereas the 3N:CL treatment excels in Ψ_p, aperture area and epidermal cell size. Interestingly, the 9N-CL treatment containing the highest amount of Cl^- showed higher values of total biomass and leaf area, SLA and NU_TE (Fig. 7).

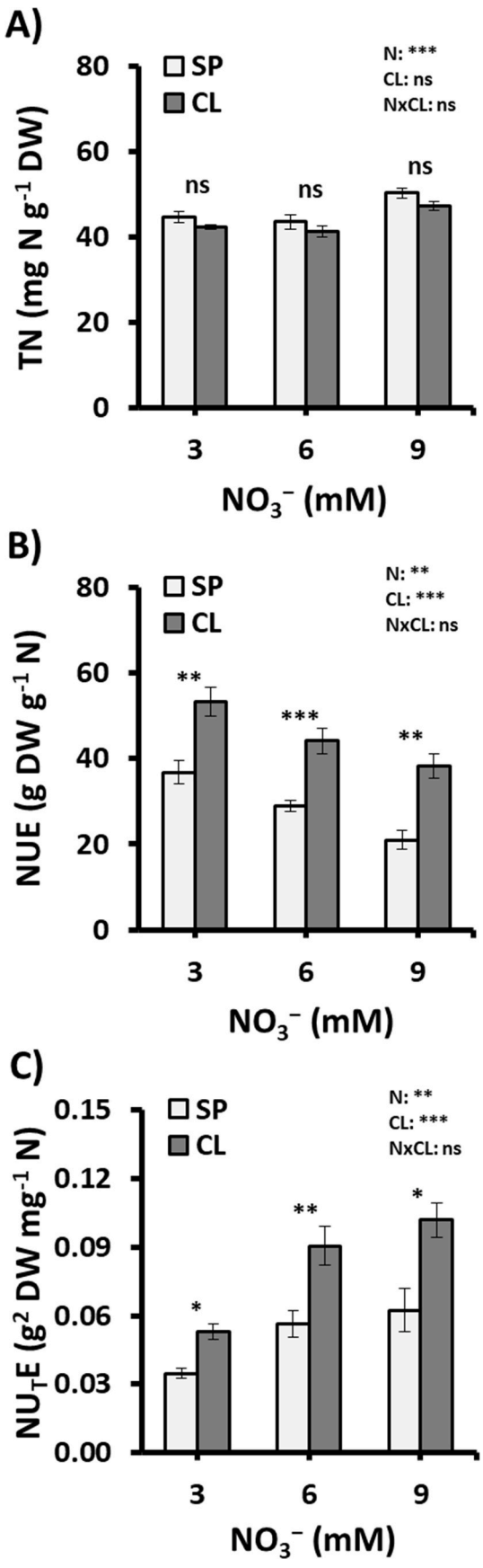

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Fig. 3. Effect of Cl^- and NO_3^- treatments on the amount of total N (TN) and Nitrogen Use Efficiency (NUE) parameters in tomato leaves at 34 DAS. Tomato plants were treated with increasing NO_3^- concentrations (N; 3, 6, and 9 mM), and alternatively with two different Cl^- treatments: 0.075 mM Cl^- with a mix of SO_4^{2-} and PO_4^{3-} salts as a control (SP), and 5 mM Cl^- salts (CL), maintaining the same balance of cations. (A) Total nitrogen (TN) represents the sum of organic N and N–NH_4^+ and N–NO_3^- molecules; (B) Nitrogen use efficiency (NUE) as total DW per unit of N applied in the nutrient solution; and (C) N utilization efficiency (NU_TE) as total DW divided by TN. Mean values ± SE; n = 6. 'Homologous group' statistics was calculated through ANOVA and MANOVA, where mean values of SP and CL treatments were compared in each N treatment. Levels of significance: $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; and 'ns' $P > 0.05$.

3.8. *Correlations between A_N, Ψ_p and biomass under different NO_3^- treatments*

Total biomass increased in both SP and CL treatments as the NO_3^- supply increased up to 12 mM (Fig. 1A). However, when two main physiological factors related to growth, photosynthesis and leaf turgor, were correlated between them and with total biomass, we found relevant correlations (Fig. 8A–C), which were unique for each treatment. The correlations were mostly positive for SP plants, but negative for Cl^- ones. The supply of N always increased the total biomass of SP plants; however, our results point out that turgor (Fig. 8C) might be more relevant than photosynthesis (Fig. 8B) for inducing growth. In fact, discarding the lower NO_3^- treatment in SP plants, all the other treatments, including those of the Cl^- treatment, showed a very similar photosynthetic rate. When A_N is plotted against Ψ_p, it can be clearly seen that even at higher Ψ_p it is not demanded more photosynthesis (Fig. 8A). Therefore, our results suggest that the N demand for growth is not driven by the need to synthesise more photoassimilates, but for structural or N-metabolism processes other than photosynthesis. Although the presence of NO_3^- when applying Cl^- as a macronutrient would not be a limiting factor for photosynthesis, it could indeed limit plant growth.

Taken together, under low NO_3^- supply, this being the only source of N, Cl^- is capable of eliminating the nutritional deficiency symptoms and recovering normal plant growth by simultaneous improvement of: NUE, tissue water content, turgor, cell growth, stomatal functioning, g_s, Qy, A_N and antioxidant capacity.

4. Discussion

Although it is difficult to infer from our results the main mechanism that drove the enhanced growth in Cl^--treated plants, both the higher turgor (Franco-Navarro et al., 2016) and N-use efficiency (NUE; Rosales et al., 2020) are expected to be relevant processes involved. NO_3^-, the major N source in plants, shares similar physical properties with Cl^- and plays important roles in charge balance and cellular osmoregulation (Wege et al., 2017; Colmenero-Flores et al., 2019). This effect and the strong dynamic interactions between both anions, particularly under saline conditions, have been interpreted as an antagonistic interaction negatively affecting NUE (Carillo and Rouphael, 2022). Recently, we discovered that Cl^- nutrition improves NUE in various agronomical relevant plants under optimal N fertilization levels (8 mM NO_3^-; Rosales et al., 2020). This was the case in this study under optimal N application, but interestingly also under low NO_3^- levels (Fig. 3B). Additionally, we found that the accumulation of Cl^- at macronutrient levels in tobacco leaves not only favoured growth and water relations (Franco-Navarro et al., 2019) but also NO_3^- availability in the cytosol, stimulating its assimilation through NO_3^- reductase and photorespiration pathways (Peinado-Torrubia et al., 2023). These findings position Cl^- as a molecule with great potential to substitute NO_3^- in plant osmoregulation functions, promoting N metabolic assimilation and its incorporation into plant biomass.

The application of 5 mM Cl^- reduced the demand for NO_3^- in the

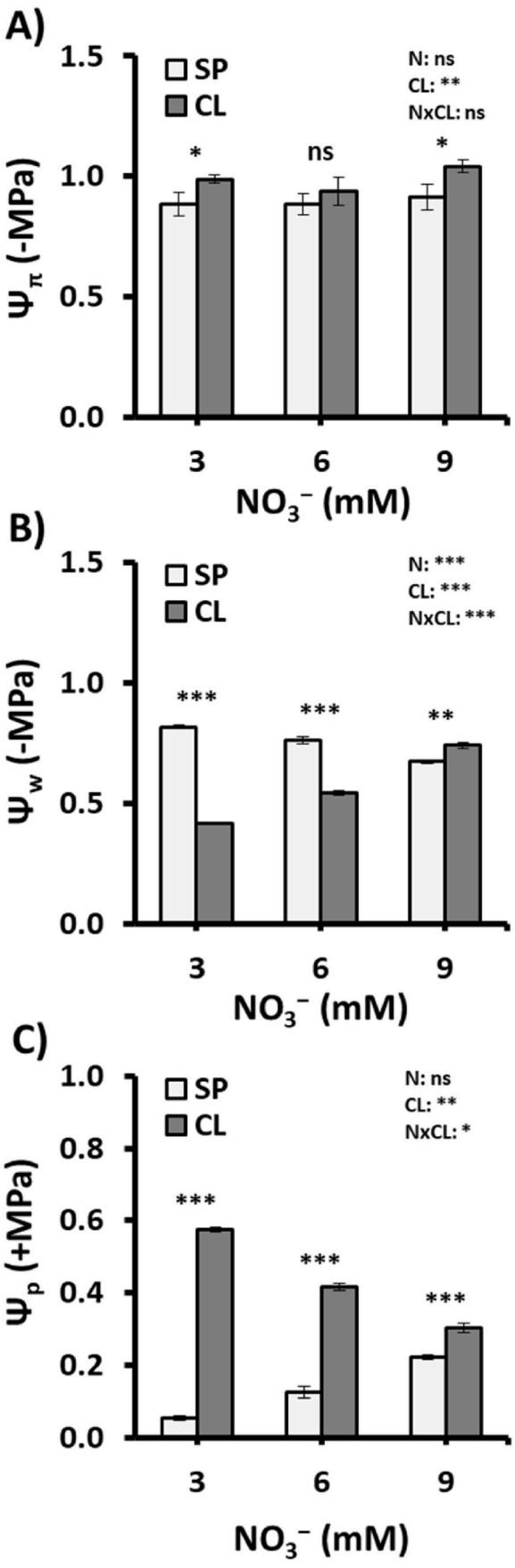

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Fig. 4. Effect of Cl^- and NO_3^- treatments on leaf water status of 34 days-old tomato plants. Tomato plants were treated with increasing NO_3^- concentrations (N; 3, 6, and 9 mM), and alternatively with two different Cl^- treatments: 0.075 mM Cl^- with a mix of SO_4^{2-} and PO_4^{3-} salts as a control (SP), and 5 mM Cl^- salts (CL), maintaining the same balance of cations. (A) Leaf osmotic potential (Ψ_π); (B) Leaf water potential (Ψ_w); and (C) Leaf turgor (or pressure) potential (Ψ_p). Mean values ± SE; $n = 6$–8. 'Homologous group' statistics was calculated through ANOVA and MANOVA, where mean values of SP and CL treatments were compared in each N treatment. Levels of significance: $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; and 'ns' $P > 0.05$.

plant. The increased concentration of NO_3^- applied was not reflected in a higher concentration of NO_3^- in the Cl^--treated plants, contrary to what was observed in SP plants (Fig. 1E and F). Consequently, the NUE of CL plants was higher than that of SP. These results are consistent with those reported by Neocleous et al. (2021), who found that replacing one-third of NO_3^- with Cl^- in greenhouse hydroponic culture solution doubled NUE without affecting tomato fruit production. Several studies also demonstrated that Cl^- salt application, at levels known as eustress, promotes growth and reduces NO_3^- accumulation in edible leafy vegetables and thus their nutritional quality (Carillo and Rouphael, 2022). Therefore, our results reveal the great potential of Cl^- nutrition to reduce NO_3^- requirements and improve NUE in agriculture, through the design of N fertilization programs appropriate to the Cl^- concentrations available in irrigation water and crop soil.

Other hypothesis tested in this work was that Cl^- plays an essential role as osmoregulatory driver in plant cells, replacing NO_3^- in the osmotic function and leaving more N available in assimilation processes, therefore alleviating its deficiency under conditions of low N availability. This was the case under optimal N nutrition, but it is unknown when the supply of NO_3^- is limiting. Thus, our results show that, as osmoregulatory driver, Cl^- is capable of improving the plant's water status, favouring plant growth in tomato plants in both the vegetative and ripening stages (Figs. 1 and 2A), as well as in other species like lettuce, red-chard, kale and spinach (Fig. 2B). Furthermore, 5 mM Cl^- application could reduce up to 7 mM NO_3^- with similar vegetative growth in tomato plants (Fig. 1) and up to 6 mM NO_3^- in other horticultural species (Fig. 2C). Specially under the low NO_3^- treatment, strong turgor build-up induced by the Cl^- treatment (Fig. 4C) suggests that Cl^- has a relevant role in improving plant water status and growth under NO_3^- treatment limitation. Photosynthesis, the other key component of growth, which provides photoassimilates to biomass accumulation, cannot explain the differences found in total biomass among treatments (Fig. 8B), suggesting that the process of growth is mainly limited by sink strength (Körner, 2003; Fatichi et al., 2014).

Despite the difference in total biomass found among Cl^-/NO_3^- treatments, Fig. 8A shows that A_N did not vary significantly among them all, except in SP with a 3 mM NO_3^- treatment, which was significantly lower. On the other hand, the range of turgor varied significantly as a function of the NO_3^- applied and whether Cl^- was supplemented or not. The increment of NO_3^- increased the total biomass in both Cl^- treatments, but meanwhile in the case of SP treatment this occurred as A_N and turgor increased, and in the case of Cl^- treatment A_N and turgor decreased. This suggests that: i) under micronutrient Cl^- levels (SP), plants used NO_3^- not only as a N source for growing processes, but also as an alternative osmoregulatory driver to achieve the necessary turgor to ensure cell survival and growth; ii) when Cl^- availability allowed macronutrient Cl^- levels and optimal turgor, since photosynthetic capacity was not limiting growth (Fig. 8A), higher NUE and N availability released other N-limited processes, further improving plant growth. Such processes might include the synthesis of amino acids, secondary metabolites, compatible osmolytes, etc.

The SP treatment constitutes a complete nutrient solution that slightly increases the SO_4^{2-} (1.875 mM) and PO_4^{3-} (1.25 mM) levels compared to the Cl^- treatment, far from levels that could cause nutritional stress in plants. Consistently, tobacco plants treated with a basal

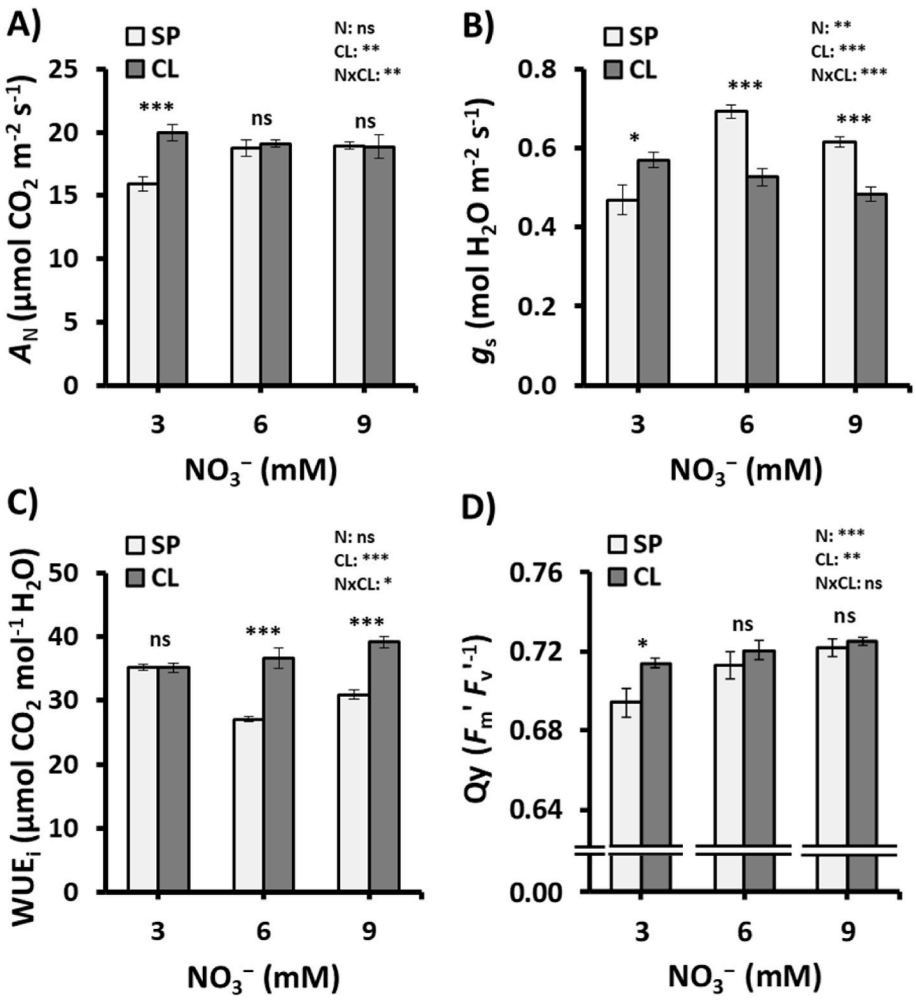

Fig. 5. Effect of Cl^- and NO_3^- treatments on gas exchange parameters, water-use efficiency and stability of PII of 34 days-old tomato plants. Tomato plants were treated with increasing NO_3^- concentrations (N; 3, 6, and 9 mM), and alternatively with two different Cl^- treatments: 0.075 mM Cl^- with a mix of SO_4^{2-} and PO_4^{3-} salts as a control (SP), and 5 mM Cl^- salts (CL), maintaining the same balance of cations. (A) Net photosynthetic rate (A_N); (B) Stomatal conductance (g_s); and (C) Photosynthetic or instantaneous water-use efficiency (WUE_i); and the highly sensitive physiological stress marker quantum yield (Qy; stability of PII). Mean values ± SE, $n = 6$–8. 'Homologous group' statistics was calculated through ANOVA and MANOVA, where mean values of SP and CL treatments were compared in each N treatment. Levels of significance: $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; and 'ns' $P > 0.05$.

solution (without the addition of SP or CL salts) have shown similar growth values to those treated with SP, thus ruling out the existence of nutritional stress in SP compared to CL plants (Franco-Navarro et al., 2016). Low NO_3^- treatments (i.e. 3 mM) reduced the growth of tomato plants in both SP and CL treatments, which is consistent with results reported in many plant species such as tobacco or wheat (Rubio--Wilhelmi et al., 2011b; Huang et al., 2022). However, Cl^- exhibited a lower decrease in growth than SP plants (35% and 59%, respectively) under low NO_3^- supply compared to the optimal 12N treatment (Fig. 1A–C), suggesting lower appearance of low NO_3^- stress symptoms than SP plants. This is supported by the higher root:shoot ratio in SP plants under low N conditions (Fig. 1C), which is considered an important parameter to study the coordination and balance in growth between roots and shoots (Lloret et al., 1999). Under N deficiency, it is known that plants induce changes in root architecture by increasing root length, density and branching as a response to enhance the nutrient acquisition and improve NUE, in detriment of shoot growth (Nacry et al., 2013). In SP plants, the higher growth reduction is in line with the reduced Qy values observed under low N conditions (Fig. 5D), supporting the appearance of some symptoms of stress as a result of N deficiency. The N deficiency has been shown to lead to elevated levels of reactive oxygen species that are detrimental to the plant (Rubio-Wilhelmi et al., 2011a). Our results revealed that Cl^--treated plants are capable of developing efficient antioxidant mechanisms as part of their adaptive response to N deficiency. Although the low-N treatment reduced the amount of photosynthetic pigments, no differences were observed between SP and CL plants (Table 1). However, the chlorophyll/carotenoid ratio was higher in Cl^--treated plants, correlating with the enhanced photosynthetic performance in these plants. The higher antioxidant activity (measured as FRAP, DPPH and reducing power tests) of Cl^--treated plants under low NO_3^- conditions, along with increased levels of phenols and flavonoids (Table 1), also support that Cl^- nutrition could be beneficial to withstand low N stress conditions by enhancing the antioxidant response of tomato plants.

The higher turgor level in the Cl^- treatment was also reflected in other aspects related to growth. For instance, we observed a progressive increase in SLA along with NO_3^- concentrations in both Cl^- and SP plants (Fig. 6B) as both biomass (Fig. 1) and leaf area increased (Fig. 6A). The Cl^- treatment consistently exhibited higher values than SP, indicating that Cl^--treated plants, even under low NO_3^- conditions, had a larger light capture surface. N deficiency, in turns, leads to a reduction in leaf area, decreased photosynthesis and a lower plant yield (Bojović and Marković, 2009; Mofokeng et al., 2015), as we observed in SP plants with 3 mM of NO_3^- (Fig. 6A, 5A and 5D). Additionally, in Cl^--treated

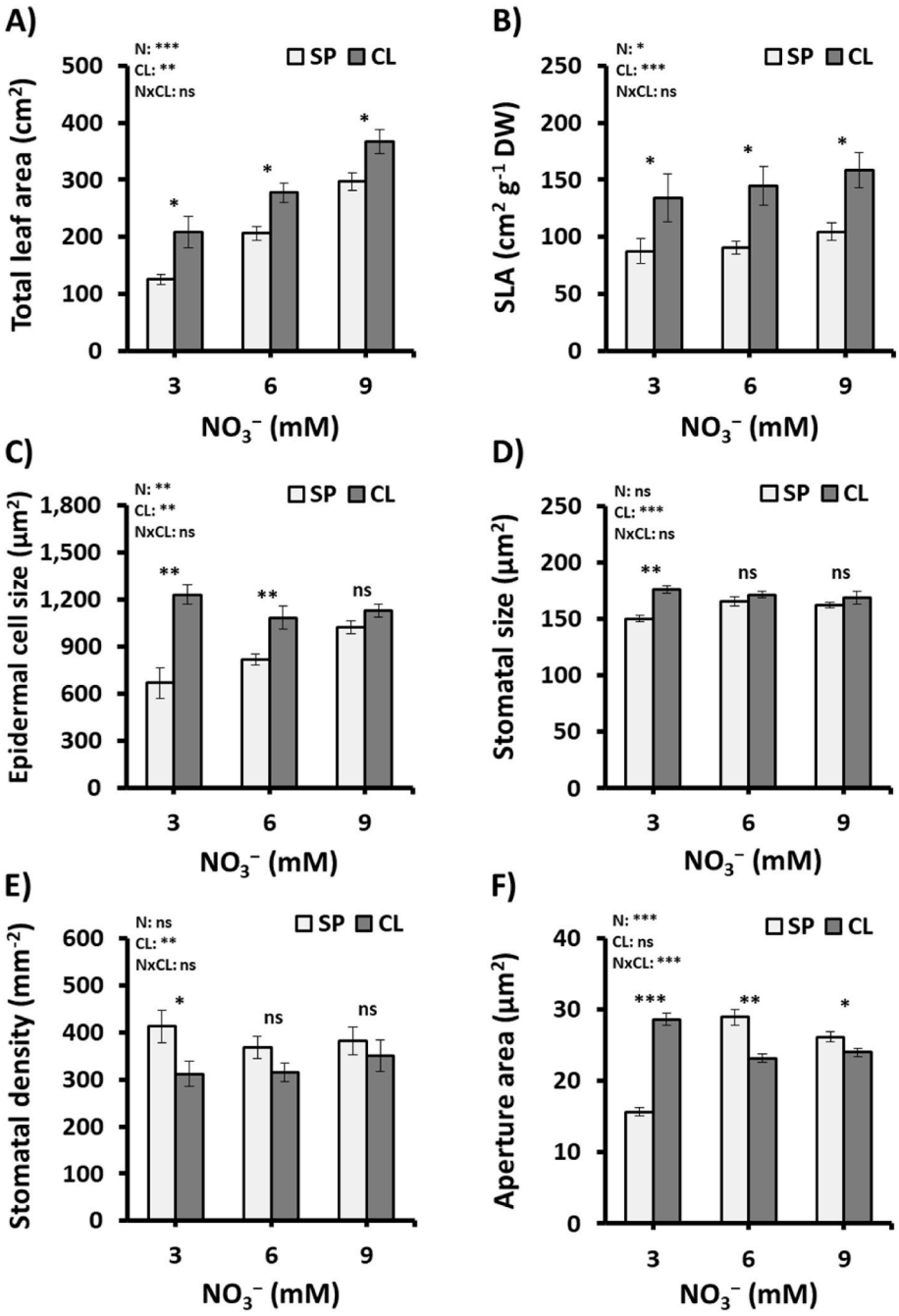

Fig. 6. Effect of Cl^- and NO_3^- treatments on leaf anatomy parameters of 34 days-old tomato plants. Tomato plants were treated with increasing NO_3^- concentrations (N; 3, 6, and 9 mM), and alternatively with two different Cl^- treatments: 0.075 mM Cl^- with a mix of SO_4^{2-} and PO_4^{3-} salts as a control (SP), and 5 mM Cl^- salts (CL), maintaining the same balance of cations. (A) Total leaf area; (B) Specific leaf area (SLA); (C) Epidermal cell size; (D) Stomatal size, (E) Stomatal cell density; and (F) Aperture pore area of the stomata. Mean values ± SE, $n = 6$–9. 'Homologous group' statistics was calculated through ANOVA and MANOVA, where mean values of SP and CL treatments were compared in each N treatment. Levels of significance: $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; and 'ns' $P > 0.05$.

plants with low NO_3^- levels, the growth stimulation primarily occurred in the epidermal cells (Fig. 5C), while the cells of SP plants appeared to be affected by the decrease in NO_3^- levels. Additionally, the increase in SLA was associated to an increase in the size of epidermal cells in 3N:Cl treatment, which could be related to the rise in Ψ_p (Fig. 4C). It suggests that under low NO_3^- conditions, Cl^-'s osmoregulatory function is essential for maintaining optimal turgor, resulting in larger cells and enhanced vegetative growth. Our results showed that 3N:CL tomato plants, as previously mentioned, were larger compared to 3N:SP plants but smaller than 9N:CL plants. The difference in epidermal size between treatments was also mirrored by the larger size of stomata and the lower stomatal density (Fig. 6D and E). Plant growth, in addition to being limited by the availability of C, can be affected by cell development. Thus, turgor bellow a minimum threshold could limit cell elongation (Hernandez-Santana et al., 2021). This would help explain why SP plants under low NO_3^- conditions had lower growth since they had lower turgor, leading to a lower rate of cell division and elongation, i.e., smaller leaf cells and, consequently, lower vegetative growth, which appears to be compensated in the presence of Cl^-.

On the other hand, in response to stress situations, plants tend to increase stomatal density, resulting in a higher number of stomata but of smaller size (Zhao et al., 2015). Additionally, stomatal closure is a

Table 1
Effect of Cl^- and NO_3^- treatments on different physiological parameters related to photosynthetic pigments, lipid peroxidation and antioxidants in 34 days-old tomato plants.

	Treatments	3 mM NO_3^-	9 mM NO_3^-	*P* value
Anthocyanins (μg g^{-1} FW)	SP	7.433 ± 0.307	9.623 ± 0.511	**
	CL	7.288 ± 0.037	10.068 ± 0.102	*
	P value	ns	ns	
Chlorophyll *a* (mg g^{-1} FW)	SP	0.841 ± 0.073	1.133 ± 0.142	ns
	CL	0.991 ± 0.027	1.108 ± 0.064	ns
	P value	ns	ns	
Chlorophyll *b* (mg g^{-1} FW)	SP	0.528 ± 0.039	0.713 ± 0.086	*
	CL	0.574 ± 0.032	0.669 ± 0.025	*
	P value	ns	ns	
Chlorophyll *a*+b (mg g^{-1} FW)	SP	1.369 ± 0.098	1.935 ± 0.210	*
	CL	1.446 ± 0.038	1.78 ± 0.084	**
	P value	ns	ns	
Carotenoids (mg g^{-1} FW)	SP	20.512 ± 1.265	28.905 ± 3.827	*
	CL	20.284 ± 0.693	23.892 ± 1.086	*
	P value	ns	ns	
Chlorophylls/carotenoids	SP	0.067 ± 1.528 × 10^{-3}	0.068 ± 1.214 × 10^{-3}	ns
	CL	0.071 ± 8.435 × 10^{-4}	0.074 ± 6.494 × 10^{-4}	ns
	P value	*	*	
MDA (nmol g^{-1} FW)	SP	3.253 ± 0.413	4.292 ± 0.629	ns
	CL	4.016 ± 0.445	4.391 ± 0.252	ns
	P value	ns	ns	
H_2O_2 (μg g^{-1} FW)	SP	1.734 ± 0.314	0.700 ± 0.183	ns
	CL	0.894 ± 0.179	0.319 ± 0.035	ns
	P value	*	ns	
Phenols (mg/100 g FW)	SP	0.135 ± 0.005	0.155 ± 0.014	ns
	CL	0.161 ± 0.010	0.134 ± 0.007	ns
	P value	*	ns	
Flavonoids (mg/100 g FW)	SP	0.159 ± 0.006	0.181 ± 0.013	ns
	CL	0.206 ± 0.016	0.133 ± 0.014	ns
	P value	*	*	
FRAP (mM g^{-1} FW)	SP	13.102 ± 0.592	13.865 ± 1.432	ns
	CL	15.043 ± 0.531	10.160 ± 0.510	ns
	P value	*	ns	
DPPH (%)	SP	89.388 ± 1.861	95.205 ± 0.413	*
	CL	94.248 ± 0.759	86.159 ± 2.791	*
	P value	*	ns	
Reducing power (% activity equivalent to ascorbic acid)	SP	2020.33 ± 8.359	2250.15 ± 109.205	ns
	CL	2371.32 ± 71.637	1778.33 ± 70.664	*
	P value	*	ns	

Tomato plants were treated with increasing NO_3^- concentrations (N; 3, 6, and 9 mM), and alternatively with two different Cl^- treatments: 0.075 mM Cl^- with a mix of SO_4^{2-} and PO_4^{3-} salts as a control (SP), and 5 mM Cl^- salts (CL), maintaining the same balance of cations. Mean values ± SE, $n = 4–6$. 'Homologous group' statistics was calculated through ANOVA, where mean values of SP and CL treatments are compared in each N treatment. Levels of significance: $*P \leq 0.05$; $**P \leq 0.01$; and 'ns' $P > 0.05$. MDA, Malondialdehyde.

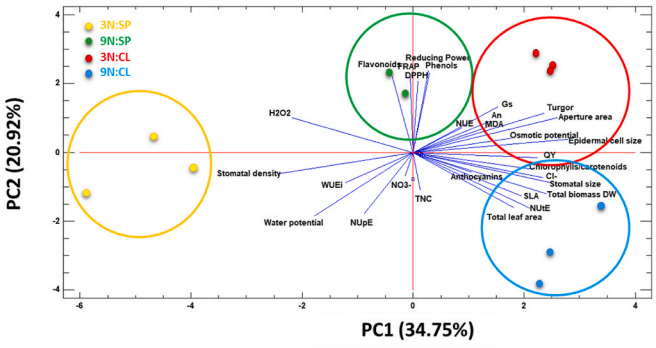

Fig. 7. PCA biplot with the first two PCA axes, with projected centroids of different Cl^- and NO_3^- treatments in tomato leaves (3N:SP-yellow, 9N:SP-green, 3N-CL-red, 9N-CL-blue).

physiological mechanism employed by plants to survive. However, this natural closure response may have a negative impact on photosynthetic processes, subsequently affecting overall plant development and growth. This is due to the important role that stomata play in gas exchange, regulating the opening and closing of their pores to make a balance between water availability and the plant's need for CO_2 (Tricker et al., 2005; Song et al., 2023). Under low NO_3^- conditions, SP plants exhibited adverse effects on their water status and A_N, subsequently impacting their development and growth. The decrease in g_s in these SP plants could be attributed to an enhanced stomatal pore closure mechanism, in order to reduce water loss through transpiration. In contrast, Cl^--treated plants exhibited lower g_s and higher WUE_i under 6N and 9N treatments (Fig. 5B–C). These results are in line with those observed in tobacco plants under optimal N conditions, which demonstrated that Cl^- as a macronutrient reduces stomatal density, decreasing g_s and preventing excessive water loss through transpiration, improving WUE_i and photosynthetic performance (Franco-Navarro et al., 2019).

5. Conclusions

Cl^- nutrition could be an important tool to enhance current agricultural sustainability by improving plant water status and optimizing N utilization. In this study, we demonstrated that its application as a macronutrient in tomato plants was capable of reducing NO_3^- levels and alleviating low N stress symptoms. By applying Cl^- as a beneficial macronutrient, we successfully enhanced the plant's water status under low N levels while maintaining its photosynthetic capacity and increasing the plant's antioxidant capacity. As a result, we observed greater vegetative growth and improvements in fruit yield. Therefore, Cl^- operates as an osmoregulator in leaf cells, effectively achieving this objective by utilizing N more efficiently and reducing its levels, which is crucial for improving crop yield.

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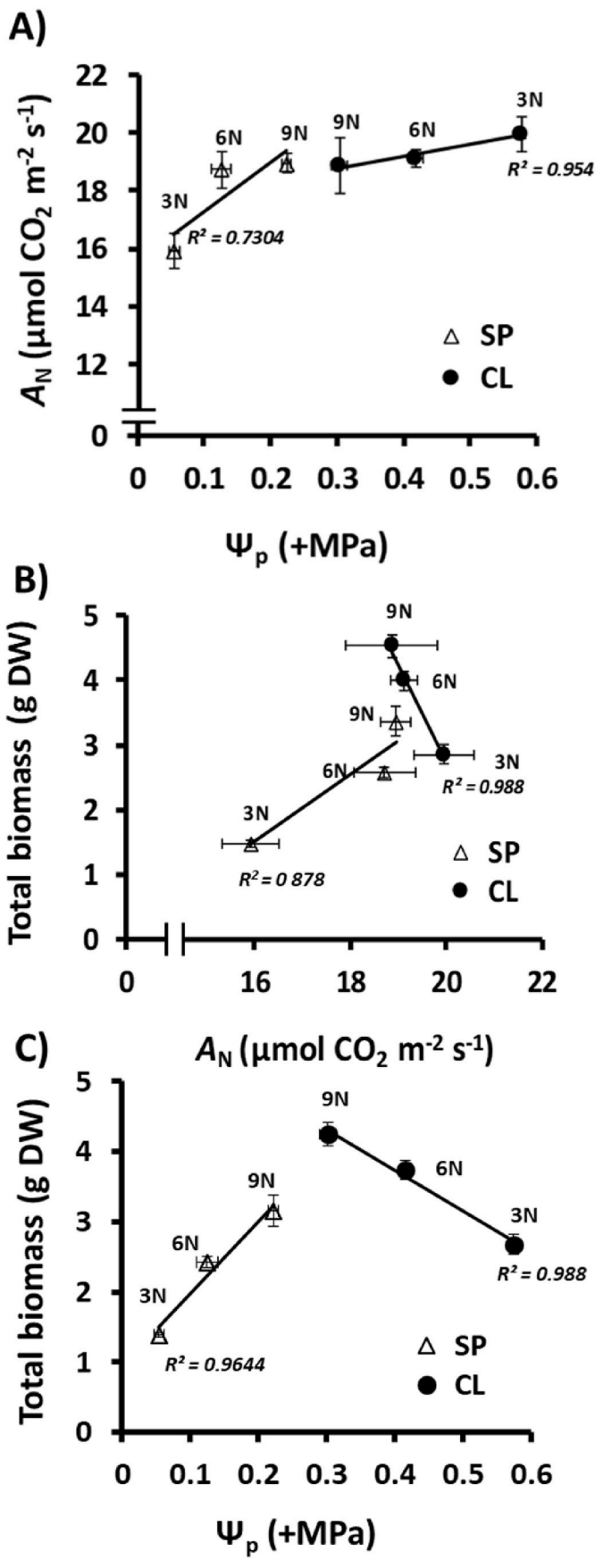

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Fig. 8. Correlations between net photosynthesis rate (A_N), total biomass and turgor (Ψ_p) of tomato leaves under different of Cl^- and NO_3^- treatments. Tomato plants were treated with increasing NO_3^- concentrations (N; 3, 6, and 9 mM), and alternatively with two different Cl^- treatments: 0.075 mM Cl^- with a mix of SO_4^{2-} and PO_4^{3-} salts as a control (SP), and 5 mM Cl^- salts (CL), maintaining the same balance of cations. (A) Correlation between A_N and Ψ_p; (B) Correlation between total biomass and A_N; and (C) Correlation between total biomass and Ψ_p. Mean values ± SE, $n = 6$. The R^2 value represents the Pearson's R-squared linear correlation test.

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CRediT authorship contribution statement

Marta Lucas: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Antonio Diaz-Espejo:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization. **David Romero-Jimenez:** Methodology, Investigation, Formal analysis. **Procopio Peinado-Torrubia:** Methodology, Investigation. **Alba Delgado-Vaquero:** Methodology, Investigation. **Rosario Álvarez:** Supervision, Methodology, Investigation. **José M. Colmenero-Flores:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Miguel A. Rosales:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.108717.

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