



Physiological and nutritional mechanisms underlying chloride-induced drought resistance in tomato

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ABSTRACT

Chloride (Cl^-), long regarded as a micronutrient or salinity-associated ion, is now recognized as a beneficial macronutrient in higher plants. This study evaluated whether Cl^- supplied at macronutrient concentrations enhances drought resistance in tomato (*Solanum lycopersicum* L.) through improved physiological performance and nutrient efficiency. Tomato plants were grown under greenhouse conditions under well-watered (CTR) or water-deficit (WD) regimes, receiving either micronutrient (SP) or macronutrient (CL) Cl^- supply. At macronutrient levels, Cl^- alleviated drought-induced growth inhibition, maintaining both vegetative and reproductive biomass and promoting adaptive root growth. CL-treated plants exhibited enhanced leaf water status, reduced stomatal conductance (g_s) without compromising net photosynthesis rate (A_N), and increased intrinsic water use efficiency (WUEi) during WD. Cl^- also preserved PSII efficiency, maintained chlorophyll levels, reduced lipid peroxidation (MDA), and enhanced total antioxidant capacity. Nutritionally, Cl^- improved nitrogen and cation (K^+ , Ca^{2+} , and Mg^{2+}) utilization efficiencies under drought conditions. Principal component analysis integrating physiological and nutritional traits under WD revealed that CL plants maintained a coordinated and functionally integrated response associated with improved water status, photosynthetic efficiency, and nutrient use. Overall, these findings indicate that Cl^- acts as a true macronutrient enhancing drought resilience in tomato through coordinated effects on osmotic adjustment, nutrient optimization, and oxidative stress mitigation. Incorporating Cl^- into fertilization programs may therefore represent a cost-effective and sustainable strategy to improve crop WUE and productivity under water-limited conditions.

1. Introduction

In the current context of climate change, drought represents one of the main abiotic threats to agricultural productivity worldwide due to its direct effects on plant growth and yield (Comas et al., 2013). Water deficit (WD) reduces tissue water potential and alters key physiological processes, leading to oxidative stress and growth inhibition (Chaves et al., 2009; Rosales et al., 2019). The decrease in leaf water content and water potential leads to stomatal closure, limiting CO_2 uptake and reducing photosynthetic rate (A_N) and enhancing water use efficiency (WUE; Rosales et al., 2012). Improving crop resilience to WD requires strategies that enhance both water and nutrient use efficiency. For

example, acclimatization responses include changes in gene expression, hormone accumulation and antioxidant mechanisms (Shao et al., 2009). However, there is little information regarding responses associated with changes in mineral nutrition patterns (Nieves-Cordones et al., 2019). Identifying physiological drought-adaptation traits linked to mineral nutrition is particularly valuable, as they can be targeted through agronomic management approaches (Tuberosa, 2012). In this context, nutrients that contribute not only to metabolism but also to osmotic adjustment and tissue water retention emerge as potential tools (Nieves-Cordones et al., 2019; Franco-Navarro et al., 2021; Cakmak et al., 2023). One promising approach is the fertilization with such nutrients to strengthen plant performance under drought conditions.

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Chloride (Cl^-) has traditionally been classified as a micronutrient due to its essential roles in photosystem II, charge balance, and membrane potential regulation (White and Broadley, 2001). Additionally, it acts as an osmotically active vacuolar solute and modulates enzymatic activity (Cakmak et al., 2023). However, its agricultural use has been limited by toxicity in sensitive crops under salinity conditions (Geilfus, 2018) and by presumed antagonism with nitrate (NO_3^-), which affects Cl^- transport and accumulation (Xu et al., 1999; Wege et al., 2017). More recently, this view has shifted as studies have demonstrated that, when supplied at concentrations above the micronutrient range but below toxicity (1–5 mM) under well-watered conditions, Cl^- can provide additional benefits. These include regulation of turgor and osmolarity, increased leaf cell size, reduced stomatal density and stomatal conductance (g_s), and lower water consumption, all without compromising photosynthesis due to increased mesophyll conductance to CO_2 (g_m ; Franco-Navarro et al., 2016, 2019). These responses contribute to greater WUE and nitrogen use efficiency (NUE), positioning Cl^- as a beneficial macronutrient for higher plants (Raven, 2017; Colmenero-Flores et al., 2019; Rosales et al., 2020; Cakmak et al., 2023; Peinado-Torrubia et al., 2023). In addition, Cl^- promotes the early development of the photosynthetic apparatus, which, together with the benefits mentioned, induces a strong stimulation of growth during early vegetative development (Peinado-Torrubia et al., 2025).

Although the physiological role of Cl^- has gained attention in recent years, the impact of drought on physiological traits and its interaction with other essential nutrients in crop species remains poorly understood. Drought is known to negatively affect mineral uptake in crops such as rice, maize, soybean, and wheat although the magnitude of this effect depends on the species, genotype, and environmental conditions (Tanguilig et al., 1987; Nieves-Cordones et al., 2019). In tomato, additional factors, including salinity and light intensity, also modulate nutrient absorption (Gent and Ma, 1998; Fernández-García et al., 2004). Understanding the role of mineral nutrients, particularly Cl^- or $\text{NO}_3^-/\text{Cl}^-$ ratios, in drought-resistance mechanisms, can contribute to optimizing fertilization management in arid and semi-arid regions (Hu and Schmidhalter, 2005; Sánchez-Rodríguez et al., 2010a; Franco-Navarro et al., 2021). Balanced mineral nutrition not only mitigates drought effects but also promotes plant water transport and overall physiological performance (Marschner, 1995). However, under low nutrient availability, plants require greater water uptake to sustain metabolism, presenting an additional challenge during WD. Thus, improving both nutrient uptake capacity and use efficiency becomes a priority to ensure crop productivity and conserve natural resources (Sharma and Bali, 2017). High nutrient use efficiency has been associated with increased growth, photosynthesis, leaf area, and yield (Barraclough et al., 2010; Haile et al., 2012). In addition to improving nutrient dynamics, Cl^- might also play a role in modulating oxidative stress responses under drought, a critical but less understood aspect of its physiological function.

Under drought conditions, plants develop strategies including: (i) water loss prevention (osmotic adjustment); (ii) cellular protection (pigments, solutes, hydrophilins, and heat-shock proteins); and (iii) oxidative damage repair via antioxidants (Garay-Arroyo et al., 2000; Reyes et al., 2005; Sánchez-Rodríguez et al., 2010b; Rosales et al., 2012; Franco-Navarro et al., 2025). Cl^- may contribute to drought resilience through its roles in cellular water balance (Shabala and Lew, 2002) and stomatal regulation (Nieves-Cordones et al., 2019). At macronutrient levels, Cl^- improves plant growth and alleviates drought effects in tobacco by promoting vacuolar Cl^- accumulation, which enhances osmotic adjustment, tissue succulence, and water retention under both well-watered and drought conditions (Franco-Navarro et al., 2016, 2021). This response appears to represent an active component of drought adaption, and has been observed in several species, including papaya, broad bean, the xerophyte *Pugionium cornutum*, and drought-tolerant tomato cultivars (Shabala et al., 2000; Mahouachi et al., 2006; Sánchez-Rodríguez et al., 2010b; Cui et al., 2020). In

tobacco, it was further demonstrated that Cl^- provides more effective osmotic adjustment than NO_3^- (Franco-Navarro et al., 2021), supporting its role as a preferential osmoticum under water-limiting conditions. This highlights the need to deepen our understanding of the physiological mechanisms by which Cl^- contributes to drought responses in agronomically important species such as tomato.

The redox balance is also disrupted by WD in plants, promoting the accumulation of reactive oxygen species (ROS) that damage cellular components such as proteins, lipids, and DNA (Rosales et al., 2012; Kabiri et al., 2014). To mitigate this damage, plants activate both enzymatic and non-enzymatic antioxidant systems, including flavonoids, carotenoids, and phenolic compounds, which protect cells and help maintain turgor pressure for gas exchange (Mittler, 2002; Rosales et al., 2009; Faize et al., 2011; Suzuki et al., 2014). Interestingly, Cl^- has been shown to enhance antioxidant capacity under low NO_3^- conditions, increasing total antioxidant activity and the accumulation of flavonoids, along with improved photosynthetic traits (Lucas et al., 2024). However, it remains unknown whether these Cl^- -mediated effects are also effective under water-limited conditions, opening new questions about its role in oxidative stress tolerance during drought conditions.

In this context, the objective of this work was to evaluate whether Cl^- nutrition at macronutrient levels can activate additional physiological responses in a crop of high agronomic interest, such as tomato, to enhance water status, nutrient use efficiency, and redox balance under WD conditions. We hypothesize that Cl^- accumulation in plant tissues at macronutrient levels enhances the uptake and use efficiency of essential nutrients such as nitrogen (N), potassium (K), magnesium (Mg), and calcium (Ca), in addition to enhancing antioxidant systems, thereby contributing to a more effective response to adverse conditions and helping to identify physiological traits associated with drought resilience. These results would provide new evidence on the functional role of Cl^- as a macronutrient in plant nutrition and reinforce its potential as an agronomic management tool to improve drought tolerance in crops of interest.

2. Materials and methods

2.1. Plant material and growth conditions

Tomato seeds (*Solanum lycopersicum* L. 'Ailsa Craig') were vernalized for 48 h at temperatures between 4 and 7 °C. They were then sown in trays measuring 4 × 4 × 10 cm containing a mixture of sand and vermiculite in a 2:8 ratio, previously washed with distilled water and treated with the corresponding nutrient solutions described below. An inert sand-vermiculite substrate was purposely selected to provide strict control of water availability and ionic composition, minimizing soil-derived variability and enabling a mechanistic assessment of Cl^- -dependent physiological responses.

Seedlings were grown with a basal nutrient solution composed of: 4.5 mM KNO_3 , 1.125 mM $\text{Mg}(\text{NO}_3)_2$, 1.125 mM $\text{Ca}(\text{NO}_3)_2$, 1.75 mM KH_2PO_4 , 0.875 mM K_2SO_4 , 0.875 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.875 mM $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 0.1 mM FeNa-EDTA , 0.1 mM H_3BO_3 , 0.1 mM $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.1 μM CuSO_4 , 30 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.1 μM $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, and 5 μM KI. From this base solution, two different treatments were prepared according to the Cl^- concentration: (i) a control treatment (SP), with Cl^- at micronutrient level (0.075 mM), where cations were balanced with SO_4^{2-} and PO_4^{3-} anions; and (ii) a treatment with Cl^- at macronutrient level (5 mM Cl^- ; CL). In both cases, an equivalent cationic balance was maintained, as described by Franco-Navarro et al. (2016). The salts used for the SP treatment were: 1.25 mM KH_2PO_4 , 0.625 mM K_2SO_4 , 0.625 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.625 mM $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, while for the CL treatment, 2.5 mM KCl, 0.625 mM MgCl_2 , and 0.625 mM CaCl_2 were employed. All solutions were adjusted to a pH of 5.7 with KOH. The SP concentration (0.075 mM Cl^-) was selected because it meets the minimal Cl^- requirement of plants while maintaining low tissue accumulation, thus avoiding artefacts associated with

Cl^- deficiency. In contrast, the CL treatment (5 mM Cl^-) represents a moderate macronutrient supply known to promote Cl^- accumulation without inducing salinity stress (Franco-Navarro et al., 2016). A zero- Cl^- treatment was not included because complete omission of Cl^- is known to induce deficiency symptoms and disrupt PSII function and ion homeostasis (Xu et al., 1999), which would confound the interpretation of Cl^- -specific physiological responses. The greenhouse environmental conditions were as follows: temperatures of $25 \pm 3^\circ\text{C}$ during the day and $17 \pm 2^\circ\text{C}$ at night, with a relative humidity of $60 \pm 10\%$ (recorded using a datalogger EL-1-USB, Lascar Electronics Inc., Erie, PA, USA). A photoperiod of 16 h of light and 8 h of darkness was maintained, with light intensity of $300\text{--}350 \mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation (measured with a LI-6400 quantum sensor; Li-COR, Lincoln, NE, USA) and a general illumination of 9000–10,000 lx (digital luxmeter LX1010B; Carson Electronics, Valemount, Canada).

During the first two weeks, seedlings were maintained under a semi-hydroponic system, i.e. partially submerged in trays with 2–5 cm of nutrient solution (Franco-Navarro et al., 2016). At 14 days after sowing (DAS), a subset of the plants was harvested to determine initial biomass values for subsequent calculation of the relative growth rate (RGR). The remaining seedlings were transplanted into pots measuring $15 \times 15 \times 20$ cm, maintaining the same substrate and growing conditions. From this point on, in addition to the two nutritional treatments (i.e. SP and CL), two different watering conditions were established: well-watered (CTR) and sustained water deficit (WD). Under WD conditions, irrigation was performed every 2–3 days to reach 60% of the field capacity of the substrate. To ensure accurate and homogeneous drought intensity, each pot was weighed individually and the exact water loss was replenished gravimetrically. As a result of this irrigation cycle, field capacity fluctuated between 26% (immediately before irrigation) and 60% (target FC after watering), corresponding to the controlled gravimetric management used throughout the experiment (Fig. S1). This drought level (60% FC) was selected because preliminary assays comparing 100%, 80%, and 60% FC showed that 60% FC induced a consistent moderate drought signal without causing severe damage, whereas responses at 80% FC were weaker (Fig. 1A). In CTR, plants were maintained constantly at 100% of field capacity (semi-hydroponic conditions) by irrigating on demand to ensure full substrate saturation. For each irrigation event under WD conditions, the exact volume of nutrient solution applied to each pot was recorded to monitor water supply and nutrient input, whereas CTR plants were irrigated freely without quantification.

In total, the experiment was structured with two nutritional treatments (SP and CL) and two watering regimes (CTR and WD), and it was maintained until harvest after 25 days of water restriction (i.e. 38 DAS). After harvesting, half of the plants were separated by organ. The roots were rinsed with tap water and distilled water, and excess moisture was removed with filter paper. The tissues were dried in an oven at 75°C for 48 h to determine biomass by dry weight (DW). Leaves of the other half of the plants were frozen at -80°C for subsequent biochemical analysis. Total biomass was calculated as the sum of leaves, roots, stems and flowers.

2.2. Determination of ion concentration

The Cl^- concentration in plant tissues was determined using a colorimetric assay based on the method described by Frankenberger et al. (1996), which relies on the displacement of mercury thiocyanate ($\text{Hg}(\text{SCN})_2$) by Cl^- , forming colorless mercury chloride (HgCl_2) and releasing ferric thiocyanate ($\text{Fe}(\text{SCN})_3$), a red-colored complex whose absorbance is measured at 480 nm. Fully expanded, oven-dried tissues were finely ground using a homogenizer (Taurus 25790, Barcelona, Spain). Aqueous extracts were prepared by incubating 18 mg of dry tissue in 1.8 mL of Milli-Q water at room temperature for 2 h under gentle agitation. After incubation, the extracts were centrifuged, and aliquots of the supernatant were used for colorimetric quantification.

For the assay, 20 μL of each diluted sample were pipetted into 96-well microplates, followed by the sequential addition of 20 μL of 20.2% ferric nitrate [$\text{Fe}(\text{NO}_3)_3$] and 20 μL of 0.417% mercury thiocyanate [$\text{Hg}(\text{SCN})_2$] in methanol. Finally, 190 μL of Milli-Q water were added to each well to reach a total volume of 250 μL . Each sample was measured in triplicate. A standard calibration curve was prepared using two linear ranges: a low-range curve (0–5 ppm Cl^-) and a high-range curve (4–16 ppm Cl^-), ensuring accurate quantification across the full concentration spectrum. Absorbance was measured at 480 nm using a microplate spectrophotometer (OMEGA, BMG Labtech, Ortenberg, Germany). The results, initially calculated as mg Cl^- per g dry weight (DW), were subsequently converted to mM by considering the leaf water content.

For the quantification of NO_3^- and ammonium (NH_4^+), aqueous extracts were prepared and analyzed using colorimetric assays with a microplate spectrophotometer (OMEGA), following the methods described in Peinado-Torrubia et al. (2023).

Total amount of calcium (Ca), potassium (K), and magnesium (Mg) was quantified from leaf and root extracts obtained after controlled acid digestion with ultrapure nitric and hydrochloric acid of the samples, using a DigiPREP block digestion system (SCP Science). Quantification was performed by inductively coupled plasma optical emission spectroscopy (ICP-OES) (Varian Inc., Palo Alto, California, USA). Additionally, the amount of cations, initially expressed in mg g^{-1} , was converted to mM to evaluate the total ionic availability under the treatment and irrigation conditions. These analyses were performed on both leaves and roots as described in Sánchez-Rodríguez et al. (2010b), providing a comprehensive view of nutritional availability under the experimental conditions.

2.3. Leaf water and gas-exchange parameters

Leaf water content (WC) and relative water content (RWC) were measured according to Rosales et al. (2013) and calculated using the formulas described by Barrs and Weatherley (1962):

$$\text{WC (\%)} = 100 \times (\text{FW} - \text{DW}) / \text{FW}$$

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

where FW is the fresh weight and DW is the dry weight of the tissue.

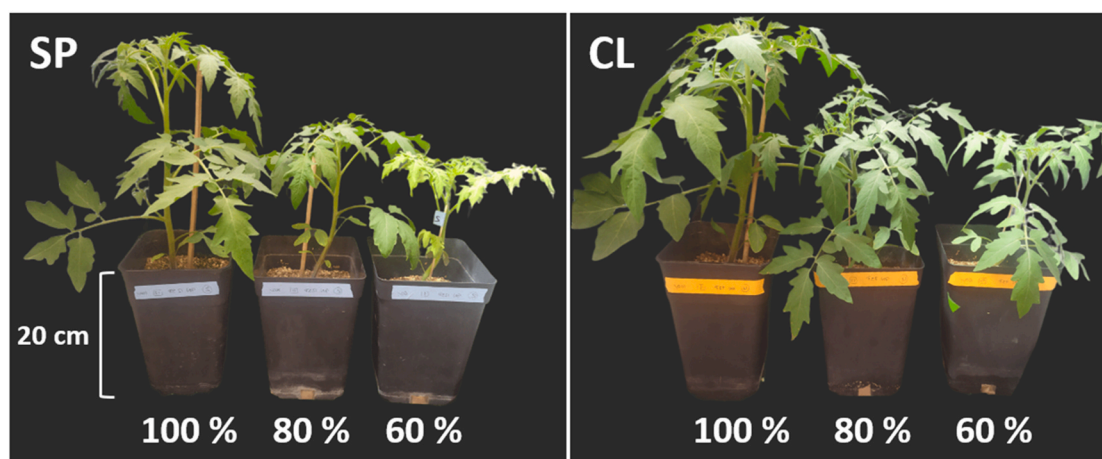
Leaf osmotic potential (Ψ_π) was determined from leaf sap obtained from leaf discs, following the procedure previously described in Franco-Navarro et al. (2016). Measurements were performed using a dew-point microvoltmeter and a C-52 sample chamber after sample equilibration.

Gas-exchange measurements were performed between 11:00 and 14:00 h, using an open-circuit measurement system (LI-6400; LI-COR, Lincoln, NE, USA). The measurement conditions were: ambient temperature, photon flux density of $1600 \mu\text{mol m}^{-2} \text{s}^{-1}$, air temperature of 25°C , and CO_2 concentration in the chamber (C_a) ranging from 50 to $1500 \mu\text{mol mol}^{-1}$, following the methodology of Franco-Navarro et al. (2016). Under these controlled conditions, the main physiological parameters were evaluated: net photosynthetic rate (A_N), stomatal conductance (g_s), electron transport rate (ETR); and intrinsic water use efficiency (WUEi). The latter was estimated as the ratio of net photosynthetic rate to stomatal conductance (A_N/g_s).

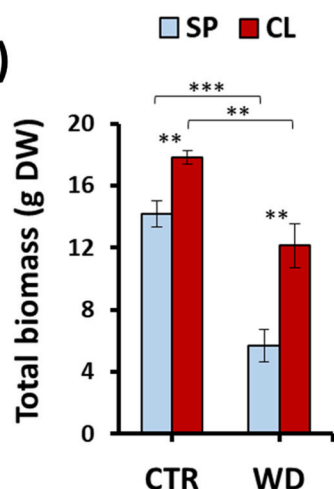
2.4. Plant stress symptoms

During the two weeks prior to the final harvest, two types of non-invasive plant stress markers were used. On the one hand, the efficiency of photosystem II was quantified through the quantum yield PII (Qy) (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). For each treatment, three measurements were recorded per leaf, using between three and four leaves per plant to obtain an average value. The Qy

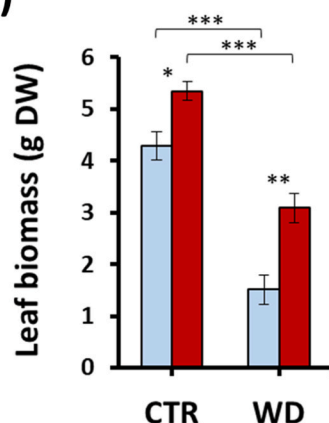
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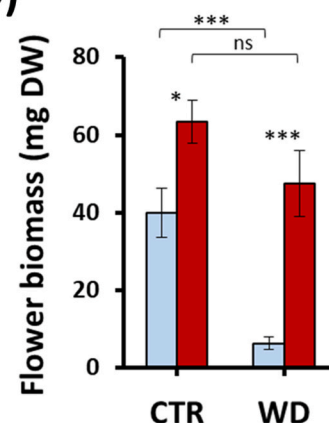
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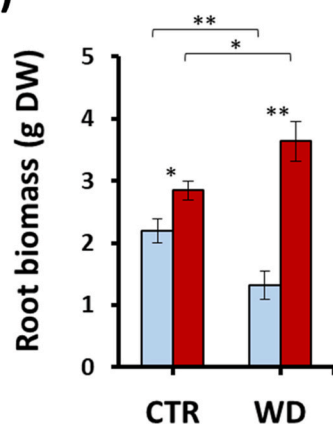
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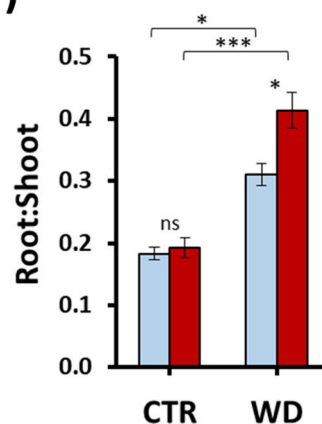


Fig. 1. Effect of Cl^- nutrition and sustained water deficit on plant growth. Tomato plants were grown under two nutritional treatments: SP, a control treatment with Cl^- at micronutrient levels (0.075 mM Cl^-); and CL, with Cl^- supplied at macronutrient levels (5 mM Cl^-), maintaining an equivalent cationic balance in both cases. At 13 days after sowing, two irrigation treatments were applied for 25 days: 100% field capacity (CTR, well-watered control) and 60% field capacity (WD, water deficit). (A) Representative images of SP and CL plants grown under 100%, 80% and 60% field capacity. Photographs were taken 21 days after the onset of the irrigation treatments. Scale bar = 20 cm. (B) Total dry weight (DW); (C) leaf DW; (D) flower DW; (E) root DW; and (F) root:shoot ratio. Total dry weight corresponds to the sum of leaf, root, stem and flower dry biomass. Data represent mean $\pm$ SE ($n = 12$). Statistical significance was assessed by ANOVA. Asterisks denote significance levels: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns, not significant ($P > 0.05$).

value was calculated according to the formula of [Maxwell and Johnson \(2000\)](#):

$$Q_y = \Phi_{PSII} = F_v'/F_m'$$

by measuring 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).

On the other hand, to estimate chlorophyll concentration, a portable chlorophyll meter SPAD 502 Plus (Spectrum Technologies, Inc., Plainfield, IL, USA) was used, which measures the SPAD index, proportional to the chlorophyll content based on light transmittance through the leaf tissue. Six plants per treatment were randomly selected to obtain SPAD readings, with three to four repetitions per plant. Both QY and SPAD were measured on randomly selected photosynthetically active leaves.

As for the malondialdehyde (MDA) concentration, an indicator of cellular damage due to oxidative stress, it was determined following the protocol described in [Rosales et al. \(2006\)](#). The same extracts used for measuring photosynthetic pigments were analyzed in a spectrophotometer at 440, 532, and 600 nm. The MDA concentration was calculated using the following formula:

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

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

$$MDA \text{ (mM)} = (A - B / 157000) 106$$

where A and B are the absorbance values at the specified wavelengths. Tomato leaf extracts were prepared with pure methanol (0.2 g in 10 mL), then centrifuged at 12,000 rpm for 15 min at room temperature.

2.5. Photosynthetic pigments and antioxidants

Leaf extracts were obtained from 0.2 g of tomato leaf tissue, which was homogenized in 10 mL of absolute methanol and centrifuged at 12,000 rpm for 15 min at room temperature. The absorbance of the supernatant was measured by spectrophotometry at 665.2, 652.4, and 470 nm, using methanol as a blank control. Concentrations of chlorophyll *a*, chlorophyll *b*, and total carotenoids were calculated using the equations described by [Lichtenthaler and Buschmann \(2001\)](#):

- Chlorophyll *a* (C_a ; $\mu\text{g mL}^{-1}$) = $16.72 A_{665.2} - 9.16 A_{652.4}$
- Chlorophyll *b* (C_b ; $\mu\text{g mL}^{-1}$) = $34.09 A_{652.4} - 15.28 \times A_{665.2}$
- Carotenoids ($\mu\text{g mL}^{-1}$) = $(1000 A_{470} - 1.63 \times C_a - 104.96 C_b) / 225$

For anthocyanin determination, the extracts were acidified with 1% HCl and their absorbance was measured at 530 and 653 nm, according to [Rosales et al. \(2011\)](#). Additionally, the total content of phenolic compounds (phenols and flavonoids) was quantified by a colorimetric method using the Folin-Ciocalteu reagent, employing gallic acid as a standard, with readings at 415 nm.

Total phenolic compounds and total flavonoids were extracted using a mixture of methanol, chloroform, and 1% (w/v) NaCl in a 2:2:1 ratio, as described by [Rosales et al. \(2011\)](#). The total phenolic content was quantified by spectrophotometry at 765 nm using the Folin-Ciocalteu reagent. For total flavonoid content, aliquots of the phenolic extract were mixed with 2 mL of Milli-Q water and 0.15 mL of 5% (w/v) NaNO_2 . After 5 min, 0.15 mL of 10% (w/v) $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ solution was added. Following an additional 5 min, 1 mL of 1 M NaOH was added, and the reaction mixture was kept at room temperature for 15 min. Absorbance was then measured at 415 nm. Results were expressed as mg of caffeic acid 100 g^{-1} FW for phenols and mg of rutin 100 g^{-1} FW for flavonoids.

Antioxidant capacity was evaluated using three complementary assays: FRAP (ferric reducing antioxidant power), DPPH (2,2-diphenyl-1-

picrylhydrazyl) free radical scavenging activity, and total reducing power. The FRAP assay was based on the reduction of Fe^{3+} to Fe^{2+} in the presence of antioxidants, with absorbance measured at 593 nm. The DPPH assay measured the ability of extracts to scavenge free radicals by monitoring the decrease in absorbance at 517 nm. The total reducing power assay assessed electron-donating activity through the reduction of ferricyanide, with absorbance measured at 700 nm. All protocols were performed following the procedures described in [Rosales et al. \(2009\)](#), and results were calculated based on appropriate standard curves.

2.6. Nutrient use efficiency parameters

For the determination of nutrient content, fully expanded and photosynthetically active leaves were collected from plants harvested at 38 DAS. Plant tissues were oven-dried, ground to a fine powder, and subjected to aqueous extraction as described above. The quantification of NO_3^- and NH_4^+ was performed as described above. Organic N content was quantified using the Kjeldahl method ([Bradstreet, 1954](#)), subtracting the mineral forms. Total N accumulation (TNA) was calculated as the sum of all N forms multiplied by total plant DW, as described by [Rubio-Wilhelmi et al. \(2011\)](#). The NUE was defined as the total dry biomass produced per unit of N supplied via the nutrient solution ($\text{g DW g}^{-1} \text{N}$), as described by [Moll et al. \(1982\)](#). The NUE was further divided into two components: (i) N uptake efficiency (NUpE), calculated as TNA divided by root DW ($\text{g N g}^{-1} \text{root DW}$); and (ii) N utilization efficiency (NuTE), calculated as the ratio between total biomass and total N content ($\text{g}^2 \text{DW mg}^{-1} \text{N}$; [Siddiqi and Glass, 1981](#)).

For macronutrient cations (K^+ , Ca^{2+} , and Mg^{2+}), efficiency parameters were determined using the same conceptual framework. Agro-nomic use efficiency (UE) for each cation was then estimated following [White \(2013\)](#). The KUE, MgUE, and CaUE were defined as the total plant DW produced per unit of nutrient supplied ($\text{g DW}_{\text{plant}} \text{g}^{-1} \text{Nutrient}_{\text{supplied}}$). According to [White \(2013\)](#), the uptake efficiency (UpE) was calculated by dividing the nutrient content in the plant by the total amount of nutrient supplied ($\text{g Nutrient}_{\text{plant}} \text{g Nutrient}_{\text{supplied}}^{-1}$), and the utilization efficiency (UtE) was determined as the plant yield per unit of nutrient content in the plant ($\text{g DW}_{\text{plant}} \text{g Nutrient}_{\text{plant}}^{-1}$).

Nutrient use efficiency parameters (NUE, KUE, MgUE, and CaUE) were determined and presented only for plants subjected to WD, since in this treatment the exact volume of nutrient solution applied to each pot was individually recorded throughout the 25-day restriction period (see [Fig. S1](#)). This allowed a precise estimation of the total amount of each nutrient supplied per plant, enabling accurate calculation of the corresponding efficiency parameters ([Fig. 5](#); [Table S1](#)). In contrast, plants grown under well-watered (CTR) conditions received abundant nutrient solution under semi-hydroponic conditions, and therefore the total volume of nutrient applied per plant could not be accurately quantified. For this reason, nutrient use efficiency parameters in CTR plants were approximated based on the nominal ion concentrations of each nutrient in the irrigation solution, assuming uniform nutrient availability among plants. These estimated values are provided as supplementary information ([Table S1](#)).

2.7. Statistical analysis

All statistical analyses were conducted using STATGRAPHICS Centurion XVI software (StatPoint Technologies, Warrenton, VA, USA). Data normality was assessed using the Shapiro-Wilk (W) test. One-way analysis of variance (ANOVA) was performed to identify statistically significant differences among treatments. Significance levels were indicated as follows: $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$, while non-significant differences were denoted as “ns” ($P > 0.05$). Additionally, principal component analysis (PCA) was performed to evaluate multivariate patterns among treatments. Data represent means of 5-12 biological replicates per treatment. Although data from a representative experiment are presented, the results were consistently reproduced

across three independent experiments.

3. Results

3.1. Chloride promotes plant growth and shifts biomass towards roots during water deficit

The impact of Cl^- nutrition on growth and biomass distribution of tomato plants was evaluated under well-watered (CTR) and sustained WD conditions. Both treatments reached 60% field capacity after 11 to 12 days of water restriction and were subsequently maintained under WD conditions ranging between 26 and 60% of field capacity (Fig. S1). Under well-watered conditions, Cl^- application resulted in a significant increase in total, leaf, flower, and root biomass compared with SP plants (Fig. 1B–E), consistent with the previously reported stimulation of shoot growth and leaf expansion induced by Cl^- in tobacco plants (Franco-Navarro et al., 2016). Under WD, although both nutritional treatments led to a reduction in total and leaf biomass, CL-treated plants exhibited significantly higher growth than SP plants, with reductions of 32% and 42% in total and leaf biomass in CL plants, compared with 60% and 65% in SP plants, respectively (Fig. 1B–C). Flower biomass was not significantly affected by WD in CL plants but was drastically reduced by 84% in SP plants (Fig. 1D). Interestingly, WD reduced root biomass by 40% in SP plants, while it was significantly increased by 28% in CL plants (Fig. 1E). Consequently, the root-to-shoot ratio was higher in CL plants under WD conditions (Fig. 1F), indicating a shift in biomass allocation towards roots under drought stress. A comparative overview of biomass differences between Cl^- treatments and irrigation regimes is provided in Fig. 1A.

3.2. Chloride modifies the leaf ionic profile and improves nutrient balance during water deficit

To verify whether the observed growth responses were associated with Cl^- accumulation, we first quantified Cl^- concentrations in leaves under both irrigation regimes. As expected, CL-treated plants showed a pronounced increase in Cl^- concentration compared with SP plants under both CTR and WD conditions (Table 1). Under CTR, CL plants accumulated about sevenfold more Cl^- than SP plants, while under WD, the difference decreased by approximately 50%, due to increased Cl^- concentration in SP plants. However, no significant irrigation effect was observed in CL plants (Table 1).

To further characterize the ionic adjustments associated with Cl^- nutrition, we analyzed the concentrations of major inorganic ions in leaves (Table 1). Under well-watered conditions, CL plants exhibited markedly lower levels of SO_4^{2-} and PO_4^{3-} than SP plants, reflecting the partial substitution of these anions by Cl^- in the nutrient solution. A reduction in NO_3^- concentration was also observed in CL plants, consistent with the enhanced NO_3^- assimilation previously reported under Cl^- supplied at macronutrient levels (Peinado-Torrubia et al., 2023). In contrast, NH_4^+ concentrations were slightly higher in CL plants, while K^+ , Ca^{2+} , and Mg^{2+} did not differ significantly between treatments (Table 1).

Drought induced distinct ion-specific responses. In SP plants, WD caused a significant rise in Cl^- and NH_4^+ concentrations, whereas NO_3^- , PO_4^{3-} , and SO_4^{2-} showed no significant changes (Table 1). In contrast, CL plants maintained high Cl^- concentrations and exhibited a significant reduction in SO_4^{2-} and PO_4^{3-} , consistent with the persistence of Cl^- -driven anionic substitution under drought. Notably, Ca^{2+} concentrations showed an opposite trend between treatments: while WD markedly reduced Ca^{2+} in SP plants, it significantly increased in CL plants, indicating a differential regulation of Ca^{2+} homeostasis associated with Cl^- nutrition. Moreover, WD promoted higher NH_4^+ , K^+ , and Mg^{2+} concentrations in CL plants compared with SP plants. Altogether, these results indicate that Cl^- nutrition substantially modifies the leaf ionic composition under drought, leading to a distinct and more stable ionic balance

Table 1

Effect of Cl^- nutrition and sustained water deficit on the concentration of major soluble inorganic ions in tomato leaves.

	Treatments	CTR	WD	P-value
Cl^- (mM)	SP	8.81 ± 0.81	21.22 ± 2.03	***
	CL	63.40 ± 2.74	67.76 ± 3.20	ns
	P-value	***	***	
SO_4^{2-} (mM)	SP	28.88 ± 2.16	30.73 ± 6.81	ns
	CL	16.73 ± 0.82	11.24 ± 1.28	*
	P-value	**	*	
PO_4^{3-} (mM)	SP	23.52 ± 1.35	25.09 ± 4.79	ns
	CL	10.88 ± 0.81	8.60 ± 0.22	*
	P-value	**	*	
NO_3^- (mM)	SP	37.39 ± 2.76	30.99 ± 3.14	ns
	CL	23.06 ± 2.13	27.98 ± 2.83	ns
	P-value	**	ns	
NH_4^+ (mM)	SP	1.33 ± 0.07	2.21 ± 0.11	***
	CL	1.66 ± 0.12	2.14 ± 0.07	**
	P-value	*	ns	
K^+ (mM)	SP	149.20 ± 7.74	132.85 ± 6.81	ns
	CL	130.73 ± 5.40	168.10 ± 5.60	**
	P-value	ns	*	
Ca^{2+} (mM)	SP	21.82 ± 0.83	12.00 ± 2.19	*
	CL	20.83 ± 1.36	27.44 ± 1.78	*
	P-value	ns	*	
Mg^{2+} (mM)	SP	45.25 ± 2.98	54.85 ± 4.48	ns
	CL	39.01 ± 0.99	52.20 ± 2.34	**
	P-value	ns	ns	

Tomato plants were grown under two nutritional treatments: SP, a control treatment with Cl^- at micronutrient levels (0.075 mM Cl^-); and CL, with Cl^- supplied at macronutrient levels (5 mM Cl^-), maintaining an equivalent cationic balance in both cases. At 13 days after sowing, two irrigation treatments were applied for 25 days: CTR, maintained at 100% field capacity (well-watered control), and WD, 60% field capacity (water deficit). The concentrations of chloride (Cl^-), sulfate (SO_4^{2-}), phosphate (PO_4^{3-}), nitrate (NO_3^-), ammonium (NH_4^+), potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}) were determined in the soluble fraction of ground dry leaf tissue. Data are expressed as concentrations (mM) and represent mean ± SE ($n = 3-6$). Statistical significance was assessed by ANOVA. Asterisks denote significance levels: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns, not significant ($P > 0.05$).

compared with control plants (Table 1).

3.3. Chloride mitigates drought-induced physiological stress symptoms

Given that Cl^- nutrition has been associated with improved water status regulation and enhanced WUE in tobacco plants under both well-watered and drought conditions (Franco-Navarro et al., 2016, 2021), we first assessed its effect on key leaf water-related parameters of tomato plants. Under well-watered conditions, CL-treated plants exhibited significantly higher leaf WC and RWC compared with SP plants (Fig. 2A–B), together with a more negative Ψ_π (Fig. 2C). This was accompanied by lower g_s , while net A_N and ETR remained unchanged, resulting in increased WUEi in CL plants (Fig. 3A–D), as previously observed in tobacco plants (Franco-Navarro et al., 2019). Under WD conditions, SP plants showed a stronger reduction in leaf WC, RWC (Fig. 2A–B) and g_s (Fig. 3A) than CL plants, which was associated with decreased A_N and ETR, and a lower WUEi response (Fig. 3B–D). In contrast, WD did not significantly affected leaf WC, Ψ_π , A_N , or ETR in CL-treated plants, while the reductions in g_s and RWC were smaller in CL (22% and 9%, respectively) than in SP plants (31% and 13%, respectively), and CL plants maintained higher WUEi under WD (Figs. 2 and 3).

Non-invasive indicators supported these physiological observations. SP plants exhibited a significant decline in both the maximum quantum yield of PSII (Qy) and SPAD values (i.e. relative chlorophyll index), whereas CL plants showed no significant changes under WD conditions (Fig. 4A–B). Similarly, leaf MDA content revealed higher lipid peroxidation levels in SP plants during WD, while no significant differences were observed in CL plants between irrigation regimes (Fig. 4C). Together, these results indicate that Cl^- nutrition could mitigate

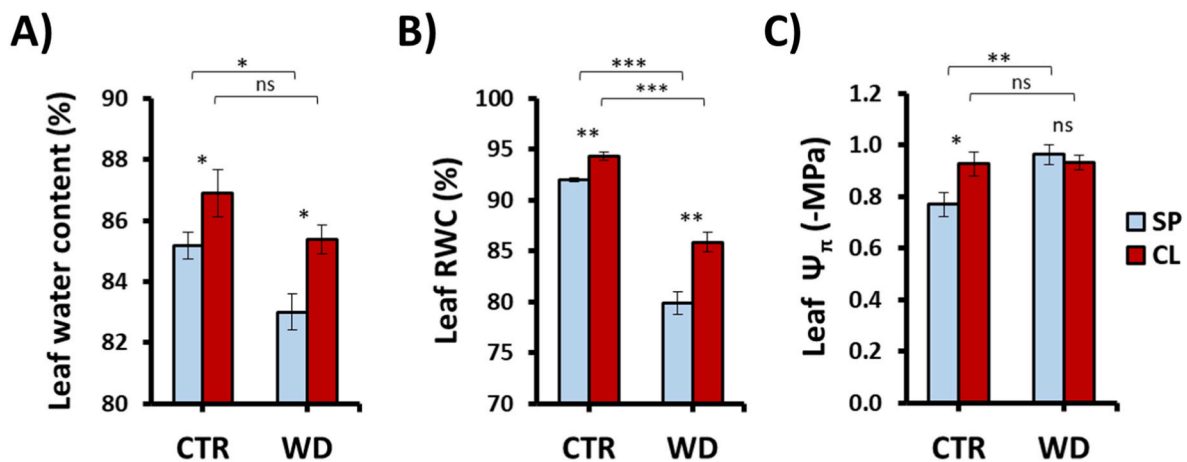


Fig. 2. Effect of Cl^- nutrition and sustained water deficit on water status. Tomato plants were grown under two nutritional treatments: SP, a control treatment with Cl^- at micronutrient levels (0.075 mM Cl^-); and CL, with Cl^- supplied at macronutrient levels (5 mM Cl^-), maintaining an equivalent cationic balance in both cases. At 13 days after sowing, two irrigation treatments were applied for 25 days: 100% field capacity (CTR, well-watered control) and 60% field capacity (WD, water deficit). (A) Leaf water content; (B) leaf relative water content (RWC); (C) leaf osmotic potential (Ψ_{π}). Data represent mean $\pm$ SE ($n = 5-6$). Statistical significance was assessed by ANOVA. Asterisks denote significance levels: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns, not significant ($P > 0.05$).

drought-induced physiological stress symptoms in tomato plants, by improving leaf water status, maintaining photosynthetic performance, and reducing oxidative damage, thereby promoting sustained growth under water-limiting conditions.

3.4. Chloride enhances photosynthetic pigment stability and antioxidant capacity under water deficit

To identify physiological traits contributing to drought resistance, beyond the water-related mechanisms previously characterized in tobacco plants (Franco-Navarro et al., 2021), we analyzed biochemical responses in tomato, including the accumulation of photosynthetic pigments, total phenolic compounds and flavonoids, and antioxidant capacity through FRAP, DPPH, and Reducing Power assays. Under well-watered conditions, Cl^- treatment did not cause significant changes in photosynthetic pigments levels compared with SP plants (Table 2), which is consistent with the similar photosynthetic performance observed between both nutritional treatments (Fig. 3B). However, WD caused a significant reduction in chlorophyll *a* and total chlorophyll (*a* + *b*) content in SP plants, whereas CL-treated plants showed no significant alterations in these pigments (Table 2). Regarding carotenoids, WD induced more than a threefold increase in both SP and CL plants; however, their accumulation was significantly higher in SP plants (Table 2). As a result of these pigment changes under drought, SP plants exhibited a lower chlorophyll/carotenoid ratio compared with CL plants (Table 2). Anthocyanin levels were also reduced under WD, with similar decreases in both nutritional treatments (Table 2).

With respect to antioxidant responses, no significant differences were observed between SP and CL plants under well-watered conditions (Table 2). In contrast, under WD, CL-treated plants exhibited significantly higher contents of total phenolic compounds and flavonoids, together with greater antioxidant activity across all three assays (FRAP, DPPH, and Reducing Power). These findings indicate that Cl^- nutrition enhances the antioxidant potential of tomato plants under drought conditions.

3.5. Chloride nutrition enhances nutrient use efficiency under water deficit

We next evaluated whether these Cl^- -dependent changes in ionic composition translated into improved nutrient-use efficiency parameters under WD (Fig. 5). Under drought conditions, all nutrient efficiency indices were consistently higher in CL plants compared with SP plants,

although the relative contribution of uptake and utilization components varied among nutrients. For N, both overall NUE and NUpE were markedly enhanced in CL plants, whereas NUpE was significantly reduced. This indicates that the superior N efficiency of CL plants primarily derives from enhanced internal utilization rather than increased uptake. A similar pattern was observed for K, Ca and Mg. In all three cases, uptake efficiencies (KUpE, CaUpE and MgUpE) did not differ from SP, while utilization efficiencies (KUE, CaUE and MgUE) increased significantly in CL plants under WD. Consequently, total nutrient-use efficiencies (KUE, CaUE, MgUE) reached approximately 130–180% of SP values (Fig. 5).

Because the exact amount of nutrient solution applied could only be quantified under WD conditions, nutrient-use efficiency parameters are presented exclusively for drought-treated plants and expressed as relative values (CL normalized to SP = 100%). The corresponding absolute values for all parameters and both irrigation treatments are provided in Table S1.

3.6. Multivariate analysis of physiological and nutritional responses to Cl^- nutrition and water deficit

To further integrate the physiological and nutritional effects of Cl^- under drought conditions, principal component analysis (PCA) was performed using both physiological (Fig. 6A) and nutritional (Fig. 6B) traits. The first two principal components explained 74.2% of the total variance in the physiological dataset (PC1: 59.6%; PC2: 14.6%) and 87.6% in the nutritional dataset (PC1: 63.2%; PC2: 24.4%).

In the physiological PCA (Fig. 6A), PC1 was mainly driven by variables associated with growth, water status and photosynthetic performance, including total, leaf, root and flower dry weight, RWC, A_N , ETR, Qy, SPAD and WUE. In contrast, Ψ_{π} showed a relatively small loading on PC1 and loaded predominantly on PC2, indicating that variation in osmotic potential accounted mainly for within-treatment variability rather than for the separation between SP-WD and CL-WD plants. Along this axis, SP-WD plants (blue circles) clustered on the negative side of PC1, close to oxidative stress indicators (i.e. MDA, carotenoids), reflecting a stress-dominant response. In contrast, CL-WD plants (red circles) were clearly separated on the positive side of PC1, co-localizing with variables reflecting improved hydration status, photosynthetic performance and biomass production, thus confirming the strong integrative effect of Cl^- nutrition under drought. The g_s loaded mainly on PC2, indicating within-treatment variability rather than a major contribution to treatment separation.

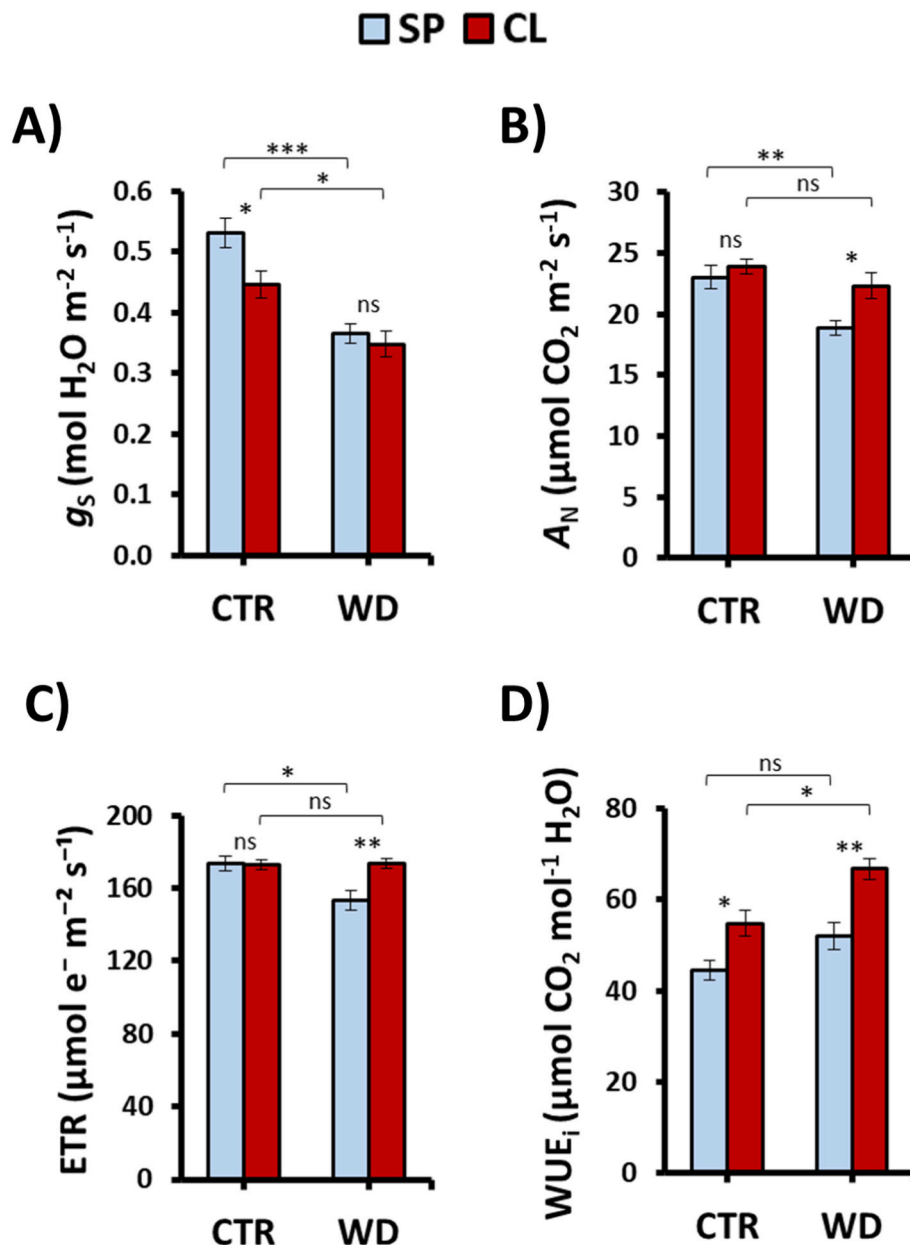


Fig. 3. Effect of Cl^- nutrition and sustained water deficit on gas-exchange parameters. Tomato plants were grown under two nutritional treatments: SP, a control treatment with Cl^- at micronutrient levels (0.075 mM Cl^-); and CL, with Cl^- supplied at macronutrient levels (5 mM Cl^-), maintaining an equivalent cationic balance in both cases. At 13 days after sowing, two irrigation treatments were applied for 25 days: 100% field capacity (CTR, well-watered control) and 60% field capacity (WD, water deficit). (A) stomatal conductance (g_s); (B) net photosynthetic rate (A_N); (C) electron transport rate (ETR); and (D) intrinsic water use efficiency (WUE_i). Data represent mean $\pm$ SE ($n = 5-6$). Statistical significance was assessed by ANOVA. Asterisks denote significance levels: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns, not significant ($P > 0.05$).

In the nutritional PCA (Fig. 6B), PC1 was dominated by variables related to nutrient-use efficiency and internal nutrient utilization. The NUE and NUTe, together with K, Ca and Mg use and utilization efficiencies (KUE, CaUE, MgUE, KUTE, CaUTE, and MgUTE), showed strong positive loadings on PC1 and were associated with CL-WD plants. Conversely, NUpE loaded negatively on PC1 and was linked to SP-WD plants, indicating that Cl^- nutrition under drought primarily improves internal nutrient utilization rather than nutrient acquisition. Uptake efficiencies of K, Ca and Mg (KUpE, CaUpE, MgUpE) showed weaker contributions to PC1, indicating a secondary role in treatment separation.

To further support the relationships revealed by the PCA, a comprehensive correlation analysis including all physiological, nutritional and biochemical parameters (Fig. S2A–D) was generated. This

analysis showed strong positive associations between biomass accumulation, Ψ_n , RWC and nutrient-use efficiency, together with negative correlations between these traits and oxidative stress markers. Together, the PCA and correlation analyses highlight that Cl^- nutrition triggers a coordinated physiological and nutritional adjustment under drought, integrating enhanced osmotic regulation, improved water status and more efficient nutrient utilization.

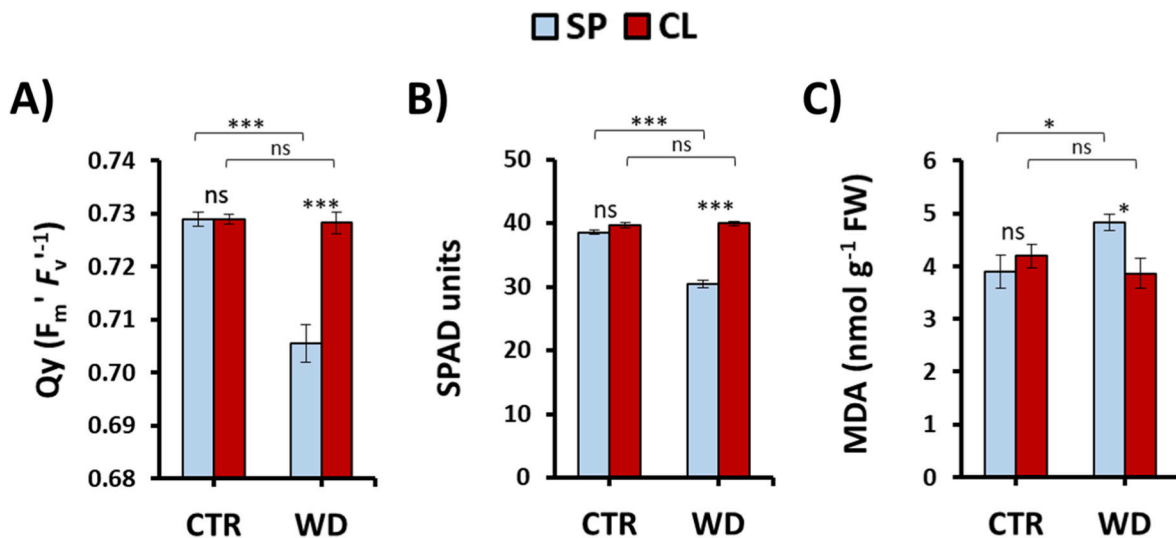


Fig. 4. Effect of Cl^- nutrition and sustained water deficit on physiological stress markers. Tomato plants were grown under two nutritional treatments: SP, a control treatment with Cl^- at micronutrient levels (0.075 mM Cl^-); and CL, with Cl^- supplied at macronutrient levels (5 mM Cl^-), maintaining an equivalent cationic balance in both cases. At 13 days after sowing, two irrigation treatments were applied for 25 days: 100% field capacity (CTR, well-watered control) and 60% field capacity (WD, water deficit). (A) Maximum quantum efficiency of PSII (F_v/F_m ; Qy); (B) SPAD values indicating relative chlorophyll content (arbitrary units); and (C) malondialdehyde (MDA) content measured as an indicator of lipid peroxidation. Data represent mean $\pm$ SE ($n = 5-6$). Statistical significance was assessed by ANOVA. Asterisks denote significance levels: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns, not significant ($P > 0.05$).

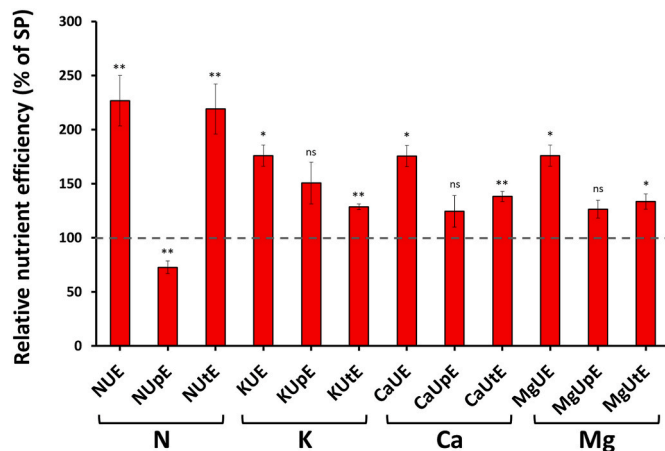


Fig. 5. Effect of Cl^- nutrition on nutrient use efficiency under water deficit conditions. Tomato plants were grown under two nutritional treatments: SP, a control treatment with Cl^- at micronutrient levels (0.075 mM Cl^-), and CL, with Cl^- supplied at macronutrient levels (5 mM Cl^-), maintaining equivalent cationic balance in both cases. At 13 days after sowing, irrigation was adjusted to 60% field capacity (WD) for 25 days, as described in Fig. 1. Bars represent the relative values of Cl^- -treated plants (CL) normalized to the corresponding SP plants (set as 100%) for each nutrient efficiency parameter: NUE, NUpE, NUtE, KUE, KUpE, KUtE, MgUE, MgUpE, MgUtE, CaUE, CaUpE, and CaUtE. Data represent mean $\pm$ SE ($n = 4-6$). Statistical significance was assessed by ANOVA on absolute values. Asterisks denote significance levels: * $P \leq 0.05$; ** $P \leq 0.01$; ns, not significant ($P > 0.05$). Absolute values for all parameters and both irrigation treatments are provided in Table S1.

4. Discussion

4.1. Chloride mitigates drought-induced growth inhibition by promoting vegetative and reproductive development, adaptive root responses, and improved water status

Chloride (Cl^-) has traditionally been regarded as a potentially harmful ion in agriculture due to its involvement in salinity stress and its

presumed antagonism with NO_3^- uptake. However, increasing evidence supports its reclassification as a beneficial macronutrient in higher plants when accumulated at millimolar concentrations, enhancing plant growth through improved efficiency in the use of water, carbon, and N (Franco-Navarro et al., 2016, 2019; Colmenero-Flores et al., 2019; Rosales et al., 2020; Peinado-Torrubia et al., 2023, 2025). In this context, Cl^- should no longer be viewed merely as a passive osmoticum or potential stressor, but rather as a versatile macronutrient that promotes growth, stress resilience, and more sustainable fertilization strategies (Franco-Navarro et al., 2021; Lucas et al., 2024). The tomato cultivar Ailsa Craig was selected here because it is a genetically stable and widely used model background in tomato physiology, nutrition and stress biology. Unlike many commercial hybrids, whose heterozygosity introduces substantial phenotypic variability, Ailsa Craig ensures high reproducibility and facilitates mechanistic interpretation of nutritional effects. Importantly, the beneficial functions of Cl^- are not restricted to this cultivar. Previous studies from our group and others have demonstrated Cl^- -dependent improvements in growth, water relations, and NUE across species including tobacco, lettuce, Swiss chard, spinach, Arabidopsis, olive and mandarin (Rosales et al., 2020; Franco-Navarro et al., 2016, 2019, 2021; Lucas et al., 2024). This evidence supports that the Cl^- -mediated mechanisms described here are conserved among higher plants. In this study, we demonstrate that Cl^- nutrition mitigates drought-induced growth inhibition in tomato plants. During WD, Cl^- -treated plants maintained higher total and organ biomass than SP plants (Fig. 1), confirming previous results in tobacco (Franco-Navarro et al., 2021) and revealing a conserved Cl^- -dependent mechanism across species.

Although the role of Cl^- in plant responses to salinity has been widely explored, much less is known about the specific physiological mechanisms by which Cl^- contributes to drought resistance in crop plants. The application of mild salt stress, which promotes the accumulation of high levels of saline ions in leaves, has been shown to improve water status and enhance drought tolerance in several species, including citrus (Pérez-Pérez et al., 2007; Colmenero-Flores et al., 2020), wild barley (Ahmed et al., 2013), and the xerophyte *Zygophyllum xanthoxylum* (Ma et al., 2012). More recently, Franco-Navarro et al. (2021) demonstrated that Cl^- acts as an active osmotic component improving water relations, photosynthesis, and turgor maintenance in

Table 2
Effect of Cl[−] nutrition and sustained water deficit on photosynthetic pigments and antioxidants.

	Treatments	CTR	WD	P value
Chlorophyll a (mg g ^{−1} FW)	SP	1.222 ± 0.135	0.818 ± 0.050	*
	CL	1.109 ± 0.064	0.988 ± 0.050	ns
	P-value	ns	*	
Chlorophyll b (mg g ^{−1} FW)	SP	0.713 ± 0.086	0.523 ± 0.036	ns
	CL	0.670 ± 0.025	0.619 ± 0.017	ns
	P-value	ns	*	
Chlorophyll a+b (mg g ^{−1} FW)	SP	1.935 ± 0.210	1.341 ± 0.084	*
	CL	1.778 ± 0.084	1.608 ± 0.059	ns
	P-value	ns	*	
Carotenoids (mg g ^{−1} FW)	SP	28.905 ± 3.121	98.205 ± 1.578	***
	CL	23.892 ± 2.100	88.348 ± 2.518	***
	P-value	ns	*	
Chlorophylls/carotenoids	SP	0.067 ± 0.001	0.014 ± 0.001	***
	CL	0.076 ± 0.006	0.018 ± 0.001	***
	P-value	ns	**	
Anthocyanins (μg g ^{−1} FW)	SP	8.338 ± 0.812	6.400 ± 0.262	**
	CL	10.068 ± 1.015	5.708 ± 0.554	**
	P-value	ns	ns	
Phenols (mg/100 g FW)	SP	0.125 ± 0.012	0.104 ± 0.019	ns
	CL	0.124 ± 0.011	0.143 ± 0.012	ns
	P-value	ns	*	
Flavonoids (mg/100 g FW)	SP	0.163 ± 0.009	0.082 ± 0.015	**
	CL	0.133 ± 0.014	0.133 ± 0.011	ns
	P-value	ns	*	
FRAP (mM/g FW)	SP	12.402 ± 1.131	12.352 ± 0.941	ns
	CL	11.404 ± 1.305	15.552 ± 0.920	*
	P-value	ns	*	
DPPH (%)	SP	89.823 ± 3.281	88.858 ± 2.859	ns
	CL	86.159 ± 2.791	93.403 ± 0.895	*
	P-value	ns	*	
Reducing power (% activity equivalent to ascorbic acid)	SP	2171.44 ± 152.03	1929.90 ± 79.310	ns
	CL	1946.60 ± 176.95	2354.20 ± 118.20	*
	P-value	ns	*	

Tomato plants were grown under two nutritional treatments: SP, a control treatment with Cl[−] at micronutrient levels (0.075 mM Cl[−]); and CL, with Cl[−] supplied at macronutrient levels (5 mM Cl[−]), maintaining an equivalent cationic balance in both cases. At 13 days after sowing, two irrigation treatments were applied for 25 days: 100% field capacity (CTR, well-watered control) and 60% field capacity (WD, water deficit). Data represent mean ± SE (n = 5–6). Statistical significance was assessed by ANOVA. Asterisks denote significance levels: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ns, not significant (P > 0.05).

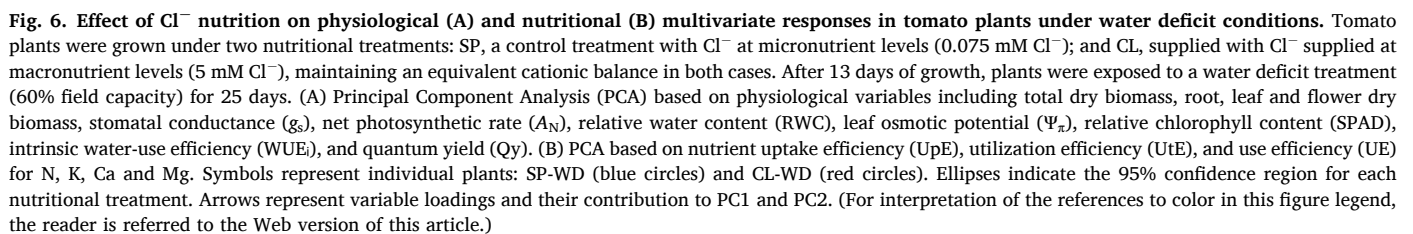
tobacco under drought stress conditions. Building upon that evidence, our results now show that this Cl[−]-dependent response is also evident in tomato, a major crop species, and extends beyond the regulation of water status to include additional impacts on growth and nutrient efficiency, as supported by the coordinated ionic and physiological adjustments observed in our study. Specifically, WD triggered a notable

increase in leaf Cl[−] accumulation in SP plants (Table 1), indicating that Cl[−] is actively mobilized as part of the adaptive response. Similar Cl[−]-dependent osmotic adjustment has been reported in other species such as papaya (Mahouachi et al., 2006), faba bean (Shabala et al., 2000), and the halophytic species *Pugionium cornutum* (Cui et al., 2020). In tomato, this mechanism appears to be cultivar-dependent, as the drought-tolerant variety ‘Zarina’ also exhibited enhanced Cl[−] uptake and leaf accumulation under moderate water limitation compared with more sensitive cultivars (Sánchez-Rodríguez et al., 2010a).

One of the most relevant mechanisms by which Cl[−] nutrition improves drought resistance is its contribution to osmotic adjustment. By accumulating in the vacuole as a non-metabolizable anion, Cl[−] contributes to a more negative osmotic potential and consequently, to greater turgor and dehydration tolerance (Colmenero-Flores et al., 2019; Cakmak et al., 2023). This osmotic contribution has been clearly demonstrated in tobacco, where Cl[−] accounted for a major fraction of leaf Ψ_π and was associated with higher RWC, leaf succulence and turgor maintenance under WD (Franco-Navarro et al., 2021). In tomato, a similar mechanism has been reported in the drought-resistant cultivar ‘Zarina’, where increased Cl[−] accumulation was associated with improved leaf water status (Sánchez-Rodríguez et al., 2010b). Consistent with these observations, Cl[−]-treated tomato plants in our study showed significantly higher leaf WC and RWC under WD (Fig. 2A–B). Importantly, CL plants exhibited a more negative Ψ_π under well-watered conditions, and was maintained during WD (Fig. 2C). In contrast, SP plants showed a marked decline in Ψ_π only under drought, reaching values similar to CL. This pattern indicates that Cl[−] nutrition establishes a constitutively lower Ψ_π that supports tissue hydration and turgor maintenance under drought, rather than inducing a stronger osmotic adjustment in response to stress. In addition, CL plants displayed a distinct ionic profile under WD, characterized by sustained Cl[−] accumulation together with coordinated reductions in SO₄^{2−} and PO₄^{3−}, consistent with anion substitution processes (Colmenero-Flores et al., 2019; Cakmak et al., 2023). Notably, Ca²⁺ concentrations increased in CL plants but declined sharply in SP plants (Table 1), suggesting that Cl[−] nutrition helps preserve Ca²⁺ homeostasis, a key determinant of membrane stability and drought signalling (Knight et al., 1997; Sanders et al., 2002). Altogether, the combined changes in Ψ_π, water status and ion composition provide physiological evidence that Cl[−] contributes predominantly to inorganic osmotic adjustment in tomato under WD. This direct determination of Ψ_π provides integrative physiological evidence of osmotic adjustment, complementing studies of individual organic osmolytes such as proline or soluble sugars.

The CL plants not only maintained greater total biomass under WD but also preserved flower biomass at levels comparable to well-watered conditions (Fig. 1D). In contrast, SP plants exhibited a drastic reduction in floral biomass, likely due to stress-induced flower abortion. This differential response highlights the capacity of Cl[−] nutrition to sustain reproductive development under drought conditions, likely by maintaining sufficient water status and carbon availability to support floral organ retention. Flower abortion is a well-documented consequence of WD, as reproductive development is particularly sensitive to stress-induced disruptions in carbon allocation and hormonal balance (Ji et al., 2012). Therefore, the ability of CL-treated plants to maintain flower production under WD may have important implications for fruit set and yield stability under drought conditions.

Additionally, CL plants exhibited a clear shift in biomass allocation, characterized by enhanced root growth and a significantly higher root-to-shoot ratio compared with SP plants (Fig. 1E–F). While this increase is a common adaptive response to water limitation (Zhu et al., 1998; Comas et al., 2013), the stronger effect observed in CL plants indicates that Cl[−] may promote adaptive plasticity in resource partitioning. To our knowledge, this provides novel evidence that Cl[−] nutrition may promote a drought-induced shift in root allocation in cultivated tomato, supporting its role in enhancing adaptive plasticity under WD, although the influence of pot volume on root growth should be considered when



Chloride has been shown to improve WUE_i in tobacco plants under well-watered conditions by reducing g_s without compromising A_N , due to compensatory increases in g_m (Franco-Navarro et al., 2019; Maron, 2019; Peinado-Torrubia et al., 2025). A similar response was observed in tomato plants in the present study (Fig. 3), consistent with previous reports linking reduced transpiration and stable photosynthesis with improved WUE (Flexas et al., 2013; Lawson and Blatt, 2014). Under WD, SP plants exhibited declines in g_s , A_N and ETR, resulting in significantly lower WUE_i compared with CL plants (Fig. 3). This pattern represents a typical drought avoidance strategy based on stomatal closure, which conserves water but limits CO_2 uptake (Roig-Oliver et al., 2020). In contrast, CL-treated plants maintained A_n and showed only a moderate decline in g_s , leading to significantly higher WUE_i . These results indicate

that Cl^- enables a more favorable balance between water conservation and carbon assimilation. Although g_m was not directly measured, the maintenance of A_N is consistent with preserved or enhanced mesophyll diffusion to CO_2 capacity, which has been identified as a key modulator of WUE_i under drought conditions and can be influenced by nutritional or structural factors (Tholen et al., 2012; Flexas et al., 2013). The concurrent increase of Ca^{2+} , K^+ , and Mg^{2+} concentrations in CL plants under WD (Table 1) may further support improved photosynthetic performance, as K^+ plays essential roles in stomatal regulation, osmotic adjustment, and protection against oxidative stress under drought (Nieves-Cordones et al., 2019), while Mg^{2+} serves as the central ion in chlorophyll molecules and is critical for maintaining thylakoid membrane structure, electron transport, and photochemical efficiency (Tränkner et al., 2018). The concurrent stabilization of Ca^{2+} may also contribute to chloroplast membrane integrity and ionic homeostasis, as well as to stress-induced signaling responses (White and Broadley, 2003). This enhanced efficiency of CO_2 assimilation per unit water lost may explain the greater biomass production observed in CL plants under WD (Fig. 1). Together, these findings support the conclusion that Cl^- nutrition enhances drought resilience in tomato by optimizing water-use dynamics and sustaining photosynthetic capacity.

Under sustained WD, SP plants showed strong reduction in biomass, accompanied by pronounced declines in Qy and SPAD values and increased MDA accumulation (Fig. 4), indicating oxidative and photochemical damage. Similar responses have been described in drought-sensitive tomato genotypes and other species such as common bean (Sánchez-Rodríguez et al., 2010b; Rosales et al., 2011, 2012). In contrast, CL-treated plants exhibited a coordinated attenuation of these responses, maintaining higher Qy and SPAD values and accumulating significantly less MDA under drought (Fig. 4). These findings suggest that Cl^- nutrition confers photochemical stability under WD, which is consistent with its positive effects on pigment integrity and ionic balance.

The integrated analysis of stress indicators, pigment profiles, and oxidative markers revealed that Cl^- nutrition triggers a broad protective response (Table 2). Chlorophyll *a* declined under WD, but the reduction was notably smaller in CL plants, likely contributing to their improved photosynthetic performance. Both nutritional treatments exhibited an increase in carotenoid content under WD; however, SP plants accumulated significantly higher levels, reflecting a more pronounced stress-induced photoprotective response. In contrast, the lower carotenoid levels and reduced MDA content in CL plants indicate a lower oxidative load and a diminished requirement for photoprotective mechanisms. The maintenance of foliar Ca^{2+} concentrations in CL plants under WD may further contribute to membrane structural stability and support Ca^{2+} -mediated stress signalling (White and Broadley, 2003), thereby potentially limiting oxidative damage propagation. Moreover, CL plants accumulated higher concentrations of total phenolics and flavonoids and showed enhanced antioxidant activity under WD, reinforcing cellular redox homeostasis and limiting ROS-induced damage. These observations are consistent with those of Sánchez-Rodríguez et al. (2010a), who reported that drought-tolerant tomato cultivars exhibited higher antioxidant enzyme activities (particularly those of the ascorbate–glutathione cycle) than sensitive genotypes under similar stress conditions, further supporting that Cl^- nutrition contributes to improved antioxidant capacity as part of a coordinated drought-tolerance mechanism. Therefore, Cl^- nutrition appears to enhance antioxidant capacity as part of a coordinated drought-tolerance mechanism integrating osmotic regulation, photosynthetic stability, and redox balance. Altogether, these findings highlight the role of Cl^- in strengthening both structural and biochemical defences, enhancing the plant's capacity to withstand oxidative stress during water-limiting conditions. It is also important to note that our interpretation of oxidative stress mitigation is based on an integrative set of indicators, including total antioxidant capacity (FRAP, DPPH, reducing power), phenolic and flavonoid contents, as well as stress-related physiological

markers such as MDA, SPAD and Qy. Together, these complementary metrics provide a robust assessment of oxidative status even in the absence of specific enzymatic or metabolite analyses.

4.3. Chloride nutrition improves nitrogen assimilation and cation utilization during drought

A central outcome of this study is that Cl^- nutrition enhances both N and cation use efficiency in tomato plants, particularly under WD (Fig. 5). Consistent with previous observations under optimal conditions (Peinado-Torrubia et al., 2023), CL plants exhibited lower foliar NO_3^- but higher NH_4^+ concentrations, a pattern that persisted under drought (Table 1). These shifts were accompanied by enhanced NUE and NUtE, indicating a more efficient assimilation and internal utilization of available N forms. It should also be considered that NUpE is expressed per unit root biomass; therefore, the markedly larger root systems of CL plants under WD distribute N uptake across more root tissue, mathematically lowering NUpE even when absolute N acquisition is maintained or increased. Consequently, the reduction in NUpE does not indicate impaired NO_3^- uptake but reflects the stimulation of root growth in CL plants. The greater whole-plant biomass achieved by CL plants under WD further supports that N acquisition was not limiting and was efficiently converted into structural and metabolic components. These increases in NUE and NUtE are consistent with enhanced internal NO_3^- reduction and metabolic flux toward amino acid synthesis under Cl^- nutrition, as reported for other crops under osmotic stress (Xu et al., 2012). Similar improvements in N assimilation have been associated with enhanced drought tolerance in rice under nutritional and genetic strategies (Reguera et al., 2013), reinforcing the physiological relevance of these results.

Our findings also align with earlier studies showing that partial substitution of NO_3^- by Cl^- improves NUE (Rosales et al., 2020; Lucas et al., 2024) and reduces physiological sensitivity to WD, particularly under conditions where water, rather than N, constrains productivity (Franco-Navarro et al., 2021). Importantly, the coordinated decrease in NO_3^- and increase in NH_4^+ , together with enhanced NUE, NUtE, and growth, strongly suggests that Cl^- promotes N utilization and assimilation rather than N storage. These patterns are consistent with our previous evidence showing that Cl^- and NO_3^- transport can be at least partially uncoupled at the molecular level, as channels such as SLAH1 and SLAH3 facilitate Cl^- loading into the xylem without directly affecting NO_3^- transport (Cubero-Font et al., 2016). Moreover, the SLAH3/SLAH1 regulatory module provides a mechanistic basis for the differential partitioning of these anions, as SLAH1-dependent gating selectively enhances Cl^- translocation while maintaining NO_3^- fluxes. Under stress or ABA signalling, repression of SLAH1 reduces Cl^- delivery to the shoot without affecting NO_3^- , illustrating that both anions can be differentially regulated rather than transported through a strictly competitive mechanism (Cubero-Font et al., 2016). Although transporter expression was not examined here, these regulatory principles support the coordinated Cl^- - NO_3^- adjustments observed in our plants under drought. Moreover, Cl^- transport is mediated by multiple transporter families operating at different cellular membranes, including plasma membrane and tonoplast-localized systems, and is subject to transcriptional, post-transcriptional and post-translational regulation (Colmenero-Flores et al., 2019). This multilevel control highlights the complexity of Cl^- homeostasis under stress conditions and supports the interpretation that integrated physiological responses provide a robust framework for understanding its functional role under drought. In a previous study (Peinado-Torrubia et al., 2023), Cl^- nutrition was shown to stimulate photorespiration, leading to a moderate accumulation of NH_4^+ derived from glycine decarboxylation. A similar mechanism may contribute to the higher NH_4^+ levels observed here in CL plants, although the enhanced NUtE indicates that this NH_4^+ is efficiently reassimilated rather than accumulating to detrimental levels. In contrast, the increase in NH_4^+ detected in SP plants under WD likely reflects stress-induced

fluxes associated with photorespiratory metabolism, together with a limited reassimilation capacity, consistent with their lower NUTe. Although drought-driven increases in photorespiration are generally associated with improved metabolic adjustment in tolerant genotypes (Sánchez-Rodríguez et al., 2011; Rosales et al., 2012), SP plants appear unable to exploit this pathway effectively. Thus, while NH_4^+ accumulation arises from distinct metabolic contexts in CL and SP plants, only CL plants incorporate this N source efficiently into biomass, contributing to their higher N-use efficiency under drought.

In addition to its effects on N metabolism, Cl^- nutrition markedly influenced cation homeostasis under WD. Thus, foliar concentrations of K^+ , Ca^{2+} and Mg^{2+} were higher in CL plants in response to WD compared with SP plants (Table 1), indicating that Cl^- nutrition helps maintain or even enhance the retention of these essential cations during drought. In SP plants, the marked decline in Ca^{2+} and the lower levels of K^+ compared with CL plants are consistent with drought-induced disruptions in ion homeostasis. In contrast, the ability of CL plants to increase cation concentrations under WD likely contributes to their improved photosynthetic performance, membrane stability and metabolic function. Notably, despite their higher cation concentration, CL plants also showed significantly greater KUTe, CaUTe and MgUTe, demonstrating that increased availability was accompanied by more efficient metabolic utilization towards growth rather than passive accumulation. This pattern reveals a more effective incorporation of cations into structural and metabolic biomass components. Consequently, overall nutrient-use efficiencies (KUE, CaUE, and MgUE) increased in CL plants compared with SP plants (Fig. 5). Because the exact amount of nutrient solution applied could only be quantified under WD, these parameters are presented exclusively for drought-treated plants and expressed as relative values (CL normalized to SP = 100%). The corresponding absolute values for both irrigation treatments are provided in Table S1.

Overall, the ability of CL plants to maintain high nutrient-use efficiency during WD is particularly relevant from an agronomic perspective, as it enables crops to maximize resource utilization while minimizing nutrient losses and external input dependency. These findings indicate that Cl^- nutrition helps maintain mineral nutrient homeostasis during drought by optimizing internal nutrient utilization and supporting the retention and balanced distribution of key cations. This improved nutritional coordination contributes to sustaining physiological performance and biomass production under water-limiting conditions, thereby enhancing drought resilience in tomato plants.

4.4. Integrative physiological coordination underlying Cl^- -mediated drought resilience

The multivariate analyses further support the integrative role of Cl^- nutrition under drought conditions (Fig. 6; Fig. S2A–D). In the physiological PCA, variables describing improved water status (particularly RWC), photosynthetic performance (A_n , ETR, Qy, SPAD) and biomass accumulation loaded together on PC1 and were closely associated with CL-WD plants, whereas SP-WD plants clustered with oxidative stress markers (particularly MDA). The Ψ_π contributed mainly to PC2, consistent with the more moderate differences in Ψ_π between treatments compared with the much larger contrasts in water status and growth. Importantly, both Ψ_π and g_s loaded predominantly on PC2 rather than on PC1, which accounted for most of the variance separating SP-WD and CL-WD plants. This indicates that, although osmotic adjustment and stomatal regulation are integral components of drought responses, differences in Ψ_π and g_s alone do not explain the primary multivariate separation between nutritional treatments. Instead, the dominant axis of variation was more strongly associated with sustained photosynthetic performance, biomass production and water status. Notably, WUE_i was also aligned with this primary axis, reinforcing that the improved efficiency observed in CL plants cannot be attributed solely to altered stomatal behaviour but is linked to the maintenance of photosynthetic

functionality under drought.

This pattern suggests that the beneficial effect of Cl^- under drought extends beyond basic osmotic adjustment or stomatal behaviour and reflects a broader coordination of metabolic and structural processes. The maintenance of A_n together with higher Qy, SPAD and lower MDA in CL plants supports the preservation of photosynthetic structural integrity and redox stability under WD. Although g_m was not directly measured in this study, the maintenance of A_n despite only moderate variation in g_s is consistent with the possibility of preserved internal CO_2 diffusion capacity and stable photosynthetic functionality (Franco-Navarro et al., 2019). In agreement with this interpretation, previous work has shown that macronutrient Cl^- supply enhances PSII photochemical efficiency, increasing ETR and Y(II) while reducing non-regulated energy dissipation, and promotes ultrastructural adjustments in chloroplasts that accelerate photosynthetic maturation and improve energy partitioning under well-watered conditions (Peinado-Torrubia et al., 2025). Such improvements in photochemical balance and redox control, even under non-stress conditions, provide a physiological basis for the enhanced stability of Qy and the lower oxidative damage observed here under drought. A similar integrative response has been described under low- NO_3^- conditions, where Cl^- supply enhanced NUE and N utilization efficiency, maintained photosynthetic performance and antioxidant capacity, and alleviated metabolic stress symptoms associated with N limitation (Lucas et al., 2024). Together, these findings indicate that Cl^- nutrition promotes a coordinated reconfiguration of water relations, carbon assimilation and nitrogen metabolism that improves plant performance under distinct environmental constraints.

In the nutritional PCA, CL-WD plants were associated with higher N and cation use and utilization efficiencies, while SP-WD plants aligned with lower NUPe and reduced cation acquisition. The correlation analysis (Fig. S2A–D) further supports these patterns by showing strong positive associations among Ψ_π , RWC, nutrient-use efficiencies and biomass, and negative correlations with oxidative stress indicators. Together, these multivariate patterns indicate that Cl^- nutrition promotes a coordinated adjustment involving water maintenance, ionic balance, photosynthetic capacity and nutrient utilization under drought. Collectively, these findings indicate that Cl^- nutrition stabilizes the functional integration of water relations, photosynthetic performance and nutrient utilization under drought. This coordinated physiological configuration, rather than the amplification of a single stress-response mechanism, underlies the improved growth and resilience observed in Cl^- -treated tomato plants.

5. Conclusions

Overall, our results demonstrate that Cl^- supplied at macronutrient levels enhances drought resistance in tomato through a multifaceted mechanism that extends well beyond its classical roles in osmotic adjustment and WUE. Cl^- nutrition is associated with coordinated modulation of water relations and gas exchange, mitigates oxidative stress, attenuates pigment and photochemical impairment, enhances N assimilation, and promotes more efficient utilization of essential cations under WD. Multivariate analyses further support that these processes form a coordinated and functionally integrated physiological and nutritional response promoted by Cl^- nutrition under drought.

This integrative response highlights Cl^- as a key contributor of plant performance under abiotic stress, with potential implications extending beyond tomato. Our results suggest that optimizing the $\text{Cl}^-:\text{NO}_3^-$ ratio in fertilization programs according to irrigation regimes may enhance crop WUE and resilience to drought, while potentially reducing N inputs and environmental NO_3^- pollution. Incorporating Cl^- into nutrient management strategies therefore represents a cost-effective and sustainable approach to support crop productivity under increasingly frequent water-limiting conditions.

Altogether, these findings call for a broader reassessment of Cl^- , not

as a passive osmotic ion, but as an active macronutrient with important roles in plant stress physiology, nutrient balance, and agricultural sustainability. Future studies should examine the underlying molecular and anatomical adjustments, including direct measurements of mesophyll conductance, ionic distribution dynamics, and nutrient transporter activity, to further refine the mechanistic basis of Cl^- -mediated drought resilience.

CRediT authorship contribution statement

Marta Lucas: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Francisco J. Moreno-Racero:** Writing – review & editing, Methodology, Investigation. **Alba Delgado-Vaquero:** Methodology, Investigation. **Antonio Diaz-Espejo:** Supervision, Resources, Methodology, Funding acquisition. **José M. Colmenero-Flores:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Miguel A. Rosales:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

A generative AI tool was used for translation into English. The authors reviewed and edited the content and take full responsibility for the manuscript.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jose M. Colmenero-Flores reports financial support was provided by Spanish Ministry of Science and Innovation. Miguel A. Rosales reports financial support was provided by European Union (Horizon, 2020). Alba Delgado-Vaquero reports financial support was provided by Government of Andalusia. Marta Lucas reports financial support was provided by Spanish National Research Council. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jplph.2026.154744>.

Data availability

Data will be made available from the corresponding author upon reasonable request.

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