



Antagonism of chloride and nitrate inhibits nitrate reductase activity in chloride-stressed maize

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Abstract

Chloride (Cl^-) is required for photosynthesis and regulates osmotic balance. However, excess Cl^- application negatively interacts with nitrate (NO_3^-) uptake, although its effect on NO_3^- metabolism remains unclear. The aim was to test whether Cl^- stress disturbs nitrate reductase activity (NRA). A maize variety (*Zea mays* L. cv. LG 30215) was hydroponically cultured in a greenhouse under the following conditions: control (2 mM CaCl_2), moderate Cl^- (10 mM CaCl_2), high Cl^- (60 mM CaCl_2). To substantiate the effect of Cl^- stress further, an osmotic stress with lower intensity was induced by 60 g polyethylene glycol (PEG) 6000 L^{-1} + 2 mM CaCl_2 , which was 57% of the osmotic pressure being produced by 60 mM CaCl_2 . Results show that high Cl^- and PEG-induced osmotic stress significantly reduced shoot biomass, stomatal conductance and transpiration rate, but NRA was only decreased by high Cl^- stress. The interference of NRA in chloride-stressed maize is supposed to be primarily caused by the antagonistic uptake of Cl^- and NO_3^- .

Keywords Chloride stress · Nitrate · Osmotic stress · Photosynthesis

Introduction

Salt stress caused by, for example, NaCl is a serious abiotic threat that imposes negative impacts on crop yield and the quality of plant products (Abdelaal et al. 2020; Naeem et al. 2020; Zörb et al. 2019). The unfavorable effects on plant growth are attributed to either osmotic stress or ion toxicity (Munns and Tester 2008). Chloride (Cl^-) is the anion of NaCl and has multiple functions in plants (Geilfus 2018a; Wege et al. 2017). Being a micronutrient (μM range), Cl^- acts as a dominant functional element involved

in the oxygen-evolving complex of PSII of photosynthesis (Kawakami et al. 2009) and the regulation of enzymatic activity such as asparagine synthase (Rognes 1980) and V-ATPase in the tonoplast (Geilfus 2018b). When the Cl^- concentration lies on a macronutrient level (low millimolar range), it can be beneficial for enhancing water-use efficiency and net photosynthesis rate (A_N) (Rosales et al. 2020) and, as a consequence, for promoting plant growth and biomass production in citrus (Brumos et al. 2010) and tobacco plants (Franco-Navarro et al. 2019). The enhanced plant growth gives indication that Cl^- might also increase nitrogen use efficiency (NUE) (Rosales et al. 2020). However, if Cl^- application exceeds favorable doses, plants may suffer from disturbed ion homeostasis, photosynthetic disorders, reduced biomass, or even cell death (Geilfus 2018b; Wege et al. 2017).

Nitrate reductase (NR) is a substrate-induced enzyme that catalyzes the conversion of NO_3^- to NO_2^- and the production of nitric oxide (NO) (Chamizo-Ampudia et al. 2017; Crawford and Glass 1998; He et al. 2017). NR plays a pivotal role in massive biological processes such as photosynthesis, molecular biology in chloroplast, starch synthesis and metabolism, and nutrient availability and metabolism by regulating not only the availability of nitrite for nitrogen assimilation but also the homeostasis of the signaling

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molecule NO (Chamizo-Ampudia et al. 2017). As a substrate, the NO_3^- concentration has been well documented as positively correlating with nitrate reductase activity (NRA) (Mengel et al. 1983; Hütsch et al. 2016); however, this correlation can differ depending on the analyzed tissue (Mengel et al. 1983). For instance, the correlation of NO_3^- and NRA exhibits a saturation curve and a sigmoidal curve in maize leaves and roots, respectively (Mengel et al. 1983). Hence, NRA is inevitably regulated by the availability of the resource NO_3^- . Stressful conditions have also been reported to decrease NRA in a dose-dependent manner, as shown with regard to water shortage (Larsson et al. 1989; Munjal et al. 1997) and NaCl stress (Botella et al. 1993; Khan et al. 1995) in various plant species. Reduced NRA is assumed to result from stress-related disturbances of NR expression (Lu et al. 1992) and the conversion of the enzyme to an inactivated form (Munjal et al. 1997).

The monovalent macronutrient anion NO_3^- possesses similar physical properties to Cl^- (Wege et al. 2017). This similarity of physical properties leads to an antagonistic relationship in the uptake between these two anions. Under saline conditions, excess Cl^- may reduce the availability of NO_3^- as a substrate that supports the activity of NR. Moreover, the transport of NO_3^- between the vacuole and cytoplasm is regulated by chloride channels such as AtCLCa (Zhang et al. 2017). The biosynthesis and activity of NR are also thought to be influenced by Cl^- , but no experiments have been conducted to substantiate this speculation (Flores et al. 2000). Nevertheless, a direct causation between Cl^- and the inhibition of NRA remains elusive in many crops such as maize. This might be because the sodium component of NaCl salinity has received more attention over the last few decades.

Maize, as a moderately salt (NaCl)-sensitive crop (Farooq et al. 2015), has recently been established to tolerate Cl^- stress moderately in our previous work (Zhang et al. 2019). From this previous work, a mildly tolerant genotype was selected to answer the following questions: (i) does Cl^- stress or osmotic stress influence maize growth and photosynthesis; (ii) does Cl^- stress or osmotic stress influence on NRA in leaf tissues?

Material and methods

Plant material and cultivation

The maize variety (*Zea mays* L. cv. LG30215 supplied by LG c/o Limagrain GmbH), mildly tolerant to Cl^- stress (Zhang et al. 2019), was hydroponically cultured in 5-l pots (two plants per pot) with four biological replicates for each of the treatments, respectively, in the greenhouse, which means eight plants in total under each condition (Fig. 1a).

Grains were germinated for 13 days in sand and transferred to hydroponic solutions at the two-leaf stage. The initial nutrient solution was one third of full strength and then was stepwise increased to two thirds and final full strength every second day. The full nutrient recipe (Richter et al. 2015; Zörb et al. 2015) contains the macronutrients $\text{Ca}(\text{NO}_3)_2$ 0.66 mM, NH_4NO_3 1.33 mM, KH_2PO_4 0.2 mM, K_2SO_4 1.0 mM, MgSO_4 0.5 mM, CaCl_2 2 mM, and Fe-EDTA 0.2 mM and the micronutrients H_3BO_3 5.0 μM , MnSO_4 2 μM , ZnSO_4 0.5 μM , CuSO_4 0.3 μM , and $(\text{NH}_4)_2\text{Mo}_7\text{O}_{24}$ 0.01 μM . Hydroponic solutions were changed twice a week. The initial pH value was approximately 6.1, which could be kept constant without any precipitation for 4 days.

Maize seedlings were treated under the following conditions: control (2 mM CaCl_2), moderate Cl^- (10 mM CaCl_2), high Cl^- (60 mM CaCl_2), and osmotic stress (60 g L^{-1} PEG 6000 (Carl Roth GmbH+Co. KG, Karlsruhe, Germany) + 2 mM CaCl_2). The reason to define 10 mM CaCl_2 as moderate stress and 60 mM CaCl_2 as high stress is that plant heights by both stresses were always lower than the control and the height of plants treated by 10 mM CaCl_2 were constantly higher than those of 60 mM CaCl_2 (Fig. S1). In order to substantiate the effects of Cl^- , 2 mM CaCl_2 was added to PEG-induced osmotic stress, as in the control. PEG-induced osmotic pressure (130.1 mOsm kg^{-1}) was only 57% of that produced by 60 mM CaCl_2 (228.9 mOsm kg^{-1}). The effect of Ca^{2+} on maize growth can be excluded, because the Ca^{2+} concentration in young leaves was 10 mg g^{-1} DM under 60 mM CaCl_2 , which is located in the reported optimal range (2.1 to 16 mg g^{-1} DM) for maize leaves (Gaj et al. 2018; Johnston and Dowbenko 2004). Thus, the detrimental effects of increased Ca^{2+} concentrations were considered to be marginal. The treatments were firstly applied to the plants in nutrient solutions, with half strength at the 7th day after transfer and were afterwards supplemented to reach full strength at the 9th day. The youngest fully developed leaf blade was selected at the first day of treatment. At the 28th day after transfer of the plants into the hydroponic solution, the whole plant was harvested and separated into four fractions (Fig. 1b): young part, fully developed leaf blade, old part, and root. Among them, the young part and old part were a mixture of corresponding leaf blades and leaf sheaths.

Fresh weight and dry weight

The fresh weight of each plant fraction was immediately obtained at harvest. The dry weight was measured after the leaves had been dried at 60 °C for 4 days in a ventilated oven.

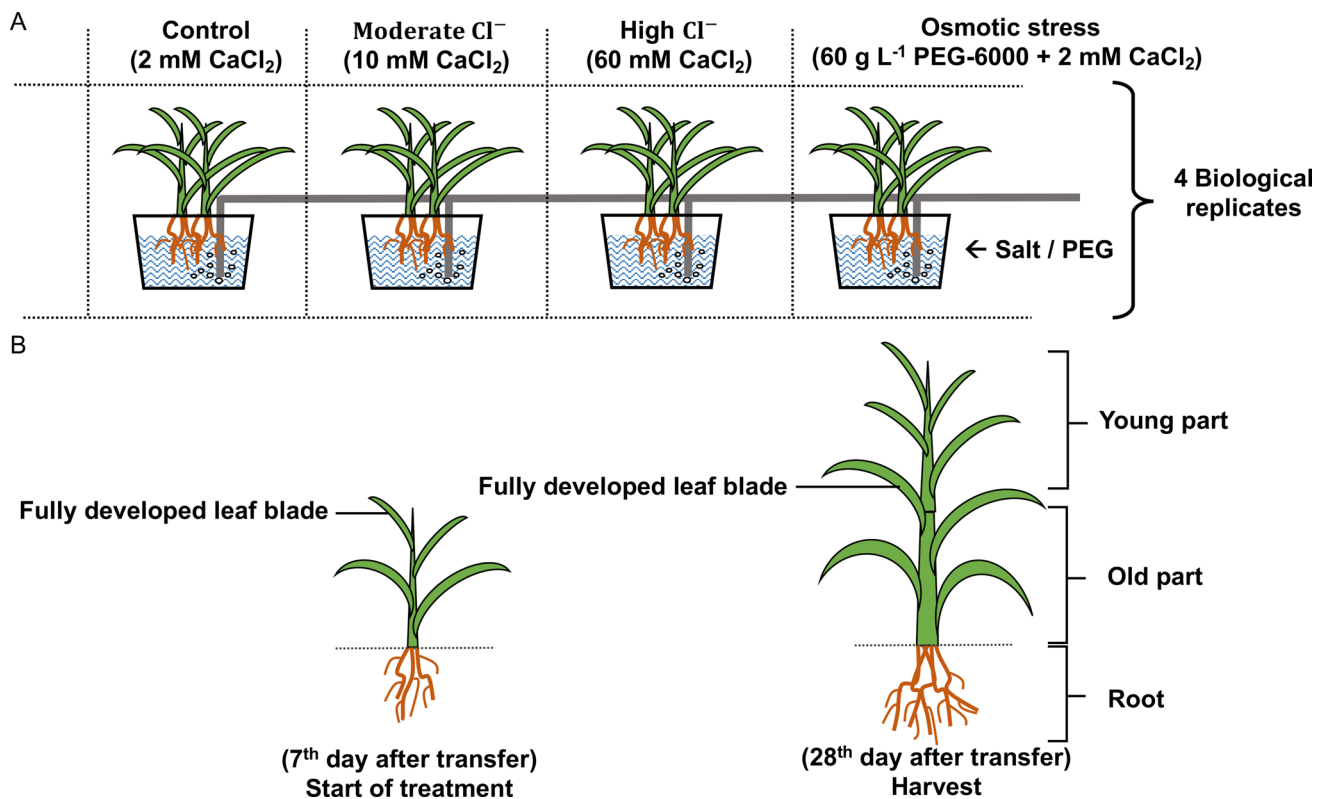


Fig. 1 Experimental design. **a** Experimental set-up of maize plants cultured in hydroponic solution, **b** diagrams of treatment application and harvest

Chloride concentration

Dry materials of each fraction were uniformly ground into fine powder by a mill (Retsch ZM1) equipped with a 0.5 mm sieve mesh. Plant tissue powder (200 mg) was dissolved in 10 mL dH₂O in glass tubes. After being vortexed, the test tubes were covered with glass beads to prevent the escape of water, heated at 80 °C for 15 min, and then quickly cooled for 7 min in the ice bath. The cooled extracts were filtered through pleated filters in 15 mL plastic centrifuge tubes. The Cl⁻ concentration was determined by using a chloride meter (6610, Eppendorf AG, Hamburg, Germany) (Zhang et al. 2019). The extract solution (100 µL) was pipetted into the titration solution (a mixture of 1 mL gelatin plus indicator solution, Biorapid GmbH, Freiburg, Germany) and 15 mL acid buffer (6.4 mL L⁻¹ 65% nitric acid and 57.6 mL L⁻¹ 100% acetic acid). Each sample was measured in three technical replicates.

Nitrate reductase activity

The activity of nitrate reductase (EC 1.7.1.1) was determined according to the method of Hageman and Hucklesby (Hageman and Hucklesby 1971) with slight modification. Frozen leaf material (−80 °C) was ground in liquid nitrogen,

and 0.2 g of this leaf powder was weighed into 2 mL reaction vessels. After the addition of 1 mL digestion buffer (100 mM TRIS / HCl pH 8.0, 1 mM EDTA, 10 mM cysteine) with thorough vortexing, centrifugation was carried out at 4 °C for 10 min at 20,000×g. The supernatant was pipetted into a new reaction vessel and placed on ice. For each sample, two reaction tubes were prepared according to the following scheme: 400 µL protein extract and 600 µL reaction buffer (100 mM KNO₃; 1 mM NADH⁺ H⁺; 1.08 mM K₂HPO₄; 1.47 mM Na₂HPO₄) were mixed (batch reaction). To one of the tubes (reference tube), 200 µL batch stopper (zinc acetate dehydrate; 1 M C₄H₆O₄Zn•2H₂O) was immediately added in order to estimate the initial concentration of NO₂⁻ in leaf samples. This reference tube was subsequently incubated on ice for 30 min. Meanwhile, the second tube with exclusive reaction buffer was also incubated for 30 min at 30 °C before the batch stopper (zinc acetate, 200 µL) was added to stop the reaction. The reaction mixtures were then centrifuged at 20 °C for 2 min at 20,000×g. Aliquots of 500 µL supernatant from these two tubes were each subsequently pipetted into empty tubes, followed by the addition of 500 µL sulfanilamide (25% HCl; 10 mg L⁻¹ and 500 µL naphthyl reagent (N-(1-naphthyl) ethylene diamine dihydrochloride; 0.2 mg L⁻¹) and allowed to stand for 15 min at room temperature for color development. For the blank and the calibration

curve, a similar mixture was prepared in each case with 500 μL ddH₂O or nitrite standards (0.05 mM, 0.1 mM, 0.5 mM, 1 mM, 5 mM, and 10 mM KNO₂) replacing the supernatant. Finally, the absorbance at 540 nm was measured against the blank value with the multimode reader TriStar S LB 942 (Berthold Technologies, Bad Wildbad, Germany). The NRA is described as the weight of the formed reaction product (NO₂⁻) per tissue fresh mass and time ($\mu\text{mol NO}_2^- \text{g}^{-1} \text{FM h}^{-1}$). Three technical replicates were performed.

Nitrate concentration

NO₃⁻ concentration was determined by a UV/Vis spectrophotometer (SPECORD@50 PLUS, Analytik Jena AG, Germany) via the nitration of salicylic acid (Xu et al. 2016) with a downscaling modification. In brief, liquid-ground maize leaf tissue (100 mg) was dissolved in 1 mL dd H₂O and heated at 90 °C for 30 min. After being cooled to room temperature, samples were centrifuged at 14,000×g for 10 min. The extracted supernatant (20 μL) was transferred to new tubes in parallel with the same amount of standard solution and the blank reference. Salicylic acid-sulfuric acid (80 μL) was added to the supernatant, completely mixed, and then incubated at room temperature for 20 min. Subsequently, NaOH (1.9 mL; 80 mg L⁻¹) was added to the tubes in order to generate a high alkaline environment in which samples were able fully to develop the color. These reaction tubes had to be cooled at room temperature for 30 min, since the concentrated NaOH addition induced the release of heat. The absorbance of all samples was measured at the wavelength of 410 nm with the control as reference. Each sample was technically repeated three times.

Estimated chlorophyll concentration

The chlorophyll content of the fully developed leaf blade was estimated by SPAD meter (Model No. 502, Konica Minolta) (Ebert et al. 2002). For the sake of accuracy, leafy cubes from various positions on the fully developed leaf blade were randomly chosen on both sides of the central vein. The final value was averaged over three measurements.

Stomatal conductance, transpiration rate, and net photosynthetic assimilation

The fully developed leaf blade (Fig. 1b) was used for monitoring stomatal conductance (g_s), transpiration rate (E) and net photosynthetic rate (A_N) with a LCi Portable Photosynthesis System installed with a broad chamber (ADC BioScientific Ltd) (Zhang et al. 2019). Illumination of the chamber mimicked natural sunlight ranging from 150 to 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ per leaf area. In the tested leaf blade,

CO₂ diffusion into leaf chamber was 400 ppm, and H₂O flux was 0.17 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Statistical analysis

Data of all four biological replicates are used to make whisker-box plots. The significant difference between the control and other treatments was evaluated at the probability of 0.05 and 0.01 levels by two factor ANOVA and the LSD test with R studio 1.1.456 (R studio Inc, Boston, USA) with library (agricolae) (De Mendiburu 2017). Principal component analysis (PCA) was analyzed and plotted by R studio 1.1.456 (R studio Inc, Boston, USA) with function “prcomp” (Venables and Ripley 2002) and library (ggbiplot) (Vu 2016) on a basis of 16 parameters with biological replicates measured as above. The Cl⁻ concentration, osmolality, NRA, and NO₃⁻ concentration were measured within three technical replicates, respectively.

Results

Plant biomass and plant height

All plant organs showed significantly reduced fresh mass under high Cl⁻ (60 mM CaCl₂) or osmotic stress when compared with the control (Fig. 2a). No significant difference in fresh weight could be observed between moderate Cl⁻ treatment and the control, except for the total shoot (Fig. 2a). In contrast, negative effects of high Cl⁻ and osmotic treatment on dry weight were present in young tissue, old tissue, and shoots but not in roots (Fig. 2b). With the time of exposure to treatments increased, plant heights by 10 mM CaCl₂, 60 mM CaCl₂, and PEG-induced osmotic stress were constantly lower than the control. Among these treatments, 10 mM CaCl₂ led to the highest plant height while osmotic stressed-plant was the lowest (Fig. S1).

Chloride concentration of all fractions, nitrate reductase activity and nitrate concentration in the fully developed leaf blade

Cl⁻ concentrations of all plant tissues significantly rose with increasing Cl⁻ application, except for young tissue under moderate Cl⁻ treatment (Fig. 3a). Maximum of Cl⁻ concentrations, as expected, appeared under high Cl⁻ treatment. The Cl⁻ concentration of all fractions under osmotic treatment was similar to that of the control, except for the fully developed leaf blade, which was slightly higher (Fig. 3a). This increase is attributable to

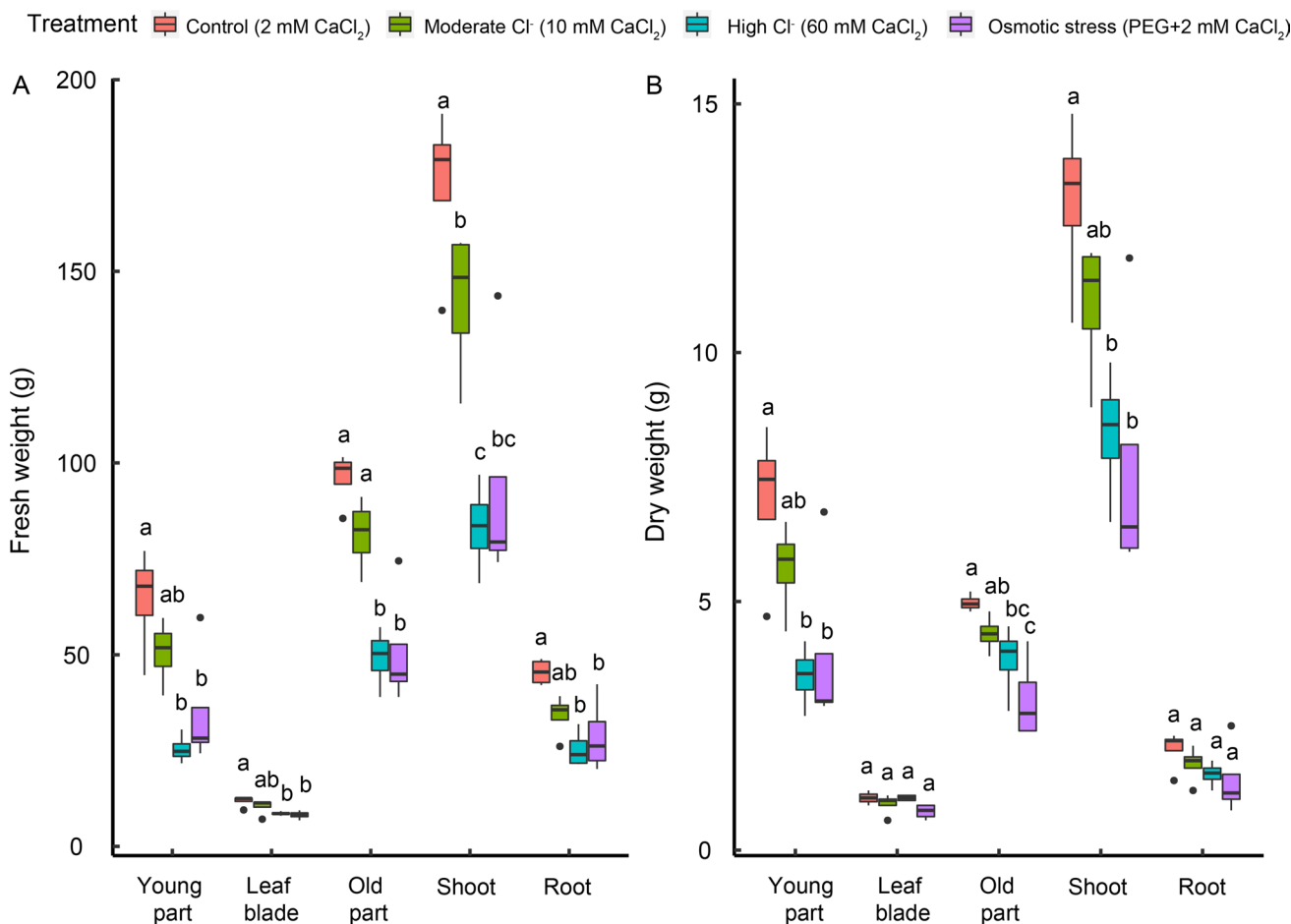


Fig. 2 Fresh and dry biomass of various fractions in maize. **a** fresh weight per pot, **b** dry weight per pot. Small letters represent significant differences ($P < 0.05$, LSD test) in one tissue group under the dif-

ferent treatments ($n = 4$). Shoot represents all above-ground parts, i.e., a mathematic sum of the young part, fully developed leaf blade, and old part

the fact that the treatment termed “osmotic treatment” also included 2 mM CaCl₂. Under moderate to high Cl⁻ application (10 mM to 60 mM CaCl₂), more chloride accumulated in old tissues and roots than in younger tissues (Fig. 3a).

NR exhibited the greatest activity under control conditions (0.37 μmol NO₂⁻ g⁻¹ FM h⁻¹) and moderate Cl⁻ treatment (0.43 μmol NO₂⁻ g⁻¹ FM h⁻¹), but the lowest activity appeared under high Cl⁻ treatment (0.17 μmol NO₂⁻ g⁻¹ FM h⁻¹) (Fig. 3b). No difference was detected between PEG-induced osmotic treatment and control (Fig. 3b).

In accordance with NR, NO₃⁻ concentration showed the similar pattern. It is clear to see that NO₃⁻ concentration (0.0075 mmol g⁻¹ FM) was the lowest under high Cl⁻ treatment and the maximum (0.0265 mmol g⁻¹ FM) was observed in moderate Cl⁻ treatment (Fig. 3c). In comparison with control, osmotic treatment (plus 2 mM CaCl₂) was associated with the same concentration of NO₃⁻ (Fig. 3c).

Estimated chlorophyll concentration, stomatal conductance, transpiration rate, and net photosynthetic rate in the fully developed leaf blade

The chlorophyll concentration per unit of leaf area, as estimated by SPAD readings, gradually increased from 6 to 13 days after treatment (DAT) and afterwards reached a constant level at 18 and 19 DAT in control, moderate Cl⁻ and high Cl⁻ (Fig. 4a). Under the condition of osmotic stress, the chlorophyll concentration always kept constant among the whole period and was also significantly lower than other conditions at 13, 18, and 19 DAT (Fig. 4a). Notably, there were no differences in SPAD readings between different Cl⁻ applications regardless of how long the plants were treated (Fig. 4a).

High Cl⁻ stress and osmotic stress significantly decreased g_s and E (Fig. 4b and c) but did not reduce A_N

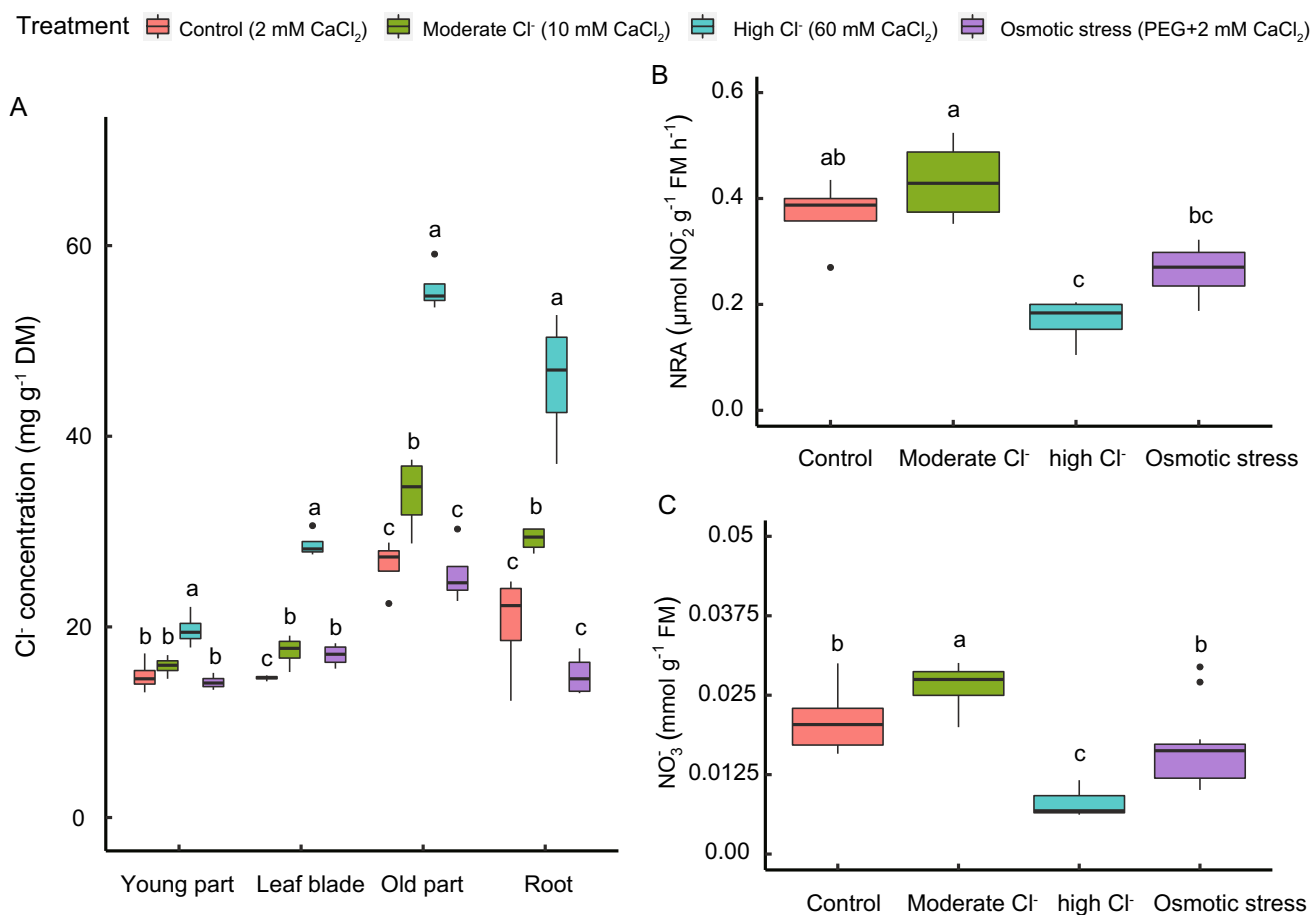


Fig. 3 Chloride concentration of all fractions, nitrate reductase activity and nitrate concentration in the fully developed leaf blade. **a** Cl⁻ concentration of all fractions, **b** nitrate reductase activity (NRA) in the fully developed leaf blade, **c** NO₃⁻ concentration in the fully

developed leaf blade. Small letters represent significant differences ($P < 0.05$, LSD test) in one tissue group under the different treatments (Cl⁻ concentration and NRA, $n = 4$; NO₃⁻ concentration, $n = 12$)

(Fig. 4d) and intercellular CO₂ concentration (Fig. S2) in comparison with the control.

Principal component analysis

In principal component analysis (PCA) (Fig. 5), the high Cl⁻ treatment and osmotic treatment were primarily found on the right side of the first and fourth quadrants of the middle axis, respectively, whereas the control and moderate Cl⁻ treatment generally lay together in the second and third quadrants. All 15 physiological variables in response to the various Cl⁻ applications were comprehensively analyzed, and a large part of the variability could be explained by the first two principal components (PC1 44.6% and PC2 18.4%). In detail, the osmolality (data shown in Fig. S3) of all fractions was allocated in the high Cl⁻ cluster. The fresh and dry biomass of shoots and roots and the SPAD were in the upper left quadrant. Moreover, the gas exchange variables (g_s , E and A_N), NO₃⁻ metabolism parameters (NRA and NO₃⁻

concentration), and ion leakage (data shown in Fig. S4) clustered in the lower left quadrant.

Discussion

Effects of Cl⁻ application and osmotic stress on biomass

The present experiments indicate that Cl⁻ has no direct ionic toxicity on shoot biomass, in which the osmotic component induced by excessive Cl⁻ is responsible for the biomass reduction. This is verified by the biomass data of PEG-induced osmotic stress (Fig. 2), in which a lower osmotic pressure (only 57% of that produced by high Cl⁻) results in a shoot biomass reduction as similar as high Cl⁻ stress for 19 days after treatment. Besides, the close negative correlation between the biomass of whole plants and PEG-induced osmotic stress indicated by the PCA pattern (arrows in opposite directions,

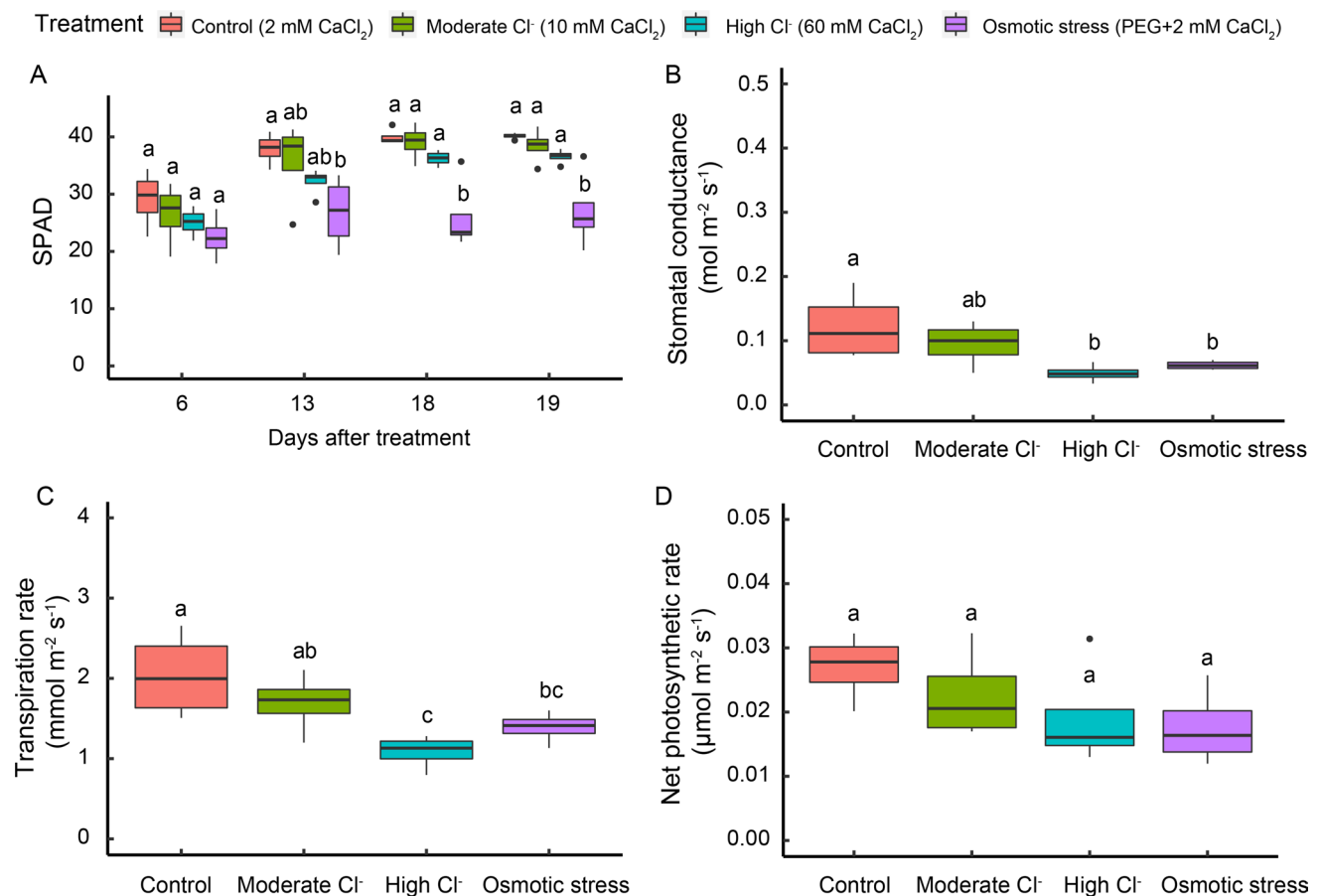


Fig. 4 Estimated chlorophyll concentration, stomatal conductance, transpiration rate, and net photosynthetic rate in the fully developed leaf blade. **a** Estimated chlorophyll concentration as indicated by SPAD, **b** stomatal conductance, **c** transpiration rate and **d** net photosynthetic rate. In subfigure A, small letters represent significant differences ($P < 0.05$, LSD test) under the different treatments at each

time point after treatment ($n = 4$). In subfigures B, C and D, average values of the 6th, 13th, 18th, and 19th days after the treatment are regarded as raw data for whisker-box plotting. Small letters represent significant differences ($P < 0.05$, LSD test) in stomatal conductance, transpiration rate, and net photosynthetic rate under the different treatments ($n = 4$)

Fig. 5) suggest that osmotic stress plays a more vital role in the reduction of plant biomass. Furthermore, the similar visual appearance of small necrotic symptoms on the older leaves (Fig. S5) is observed under both high Cl⁻ stress and the PEG-induced osmotic treatment. The necrotic symptoms of old leaves are believed to be caused by osmotic stress, which was also found in NaCl stressed maize seedlings (Cramer et al. 1994; De Costa et al. 2007). The comparisons of shoot biomass (Fig. 2) and necrotic symptoms (Fig. S5) between PEG-induced osmotic treatment and control further proves that the osmotic-stress-induced growth inhibition or leaf necrosis in maize is not specific to Cl⁻.

Context of Cl⁻ and estimated chlorophyll concentration, stomatal conductance, transpiration rate, and photosynthesis rate

Osmotic stress is a serious threat to chlorophyll concentration indicated by SPAD; indeed, it caused a decrease of 32.6% under PEG-induced osmotic treatment (Fig. 4a) and a clear negative correlation between SPAD and osmotic stress cluster by PCA plots (Fig. 5). This is in agreement with the observation that water stress can reduce the chlorophyll concentration up to 40% without adversely affecting photosynthesis at mid-day in maize (Sanchez et al. 1983).

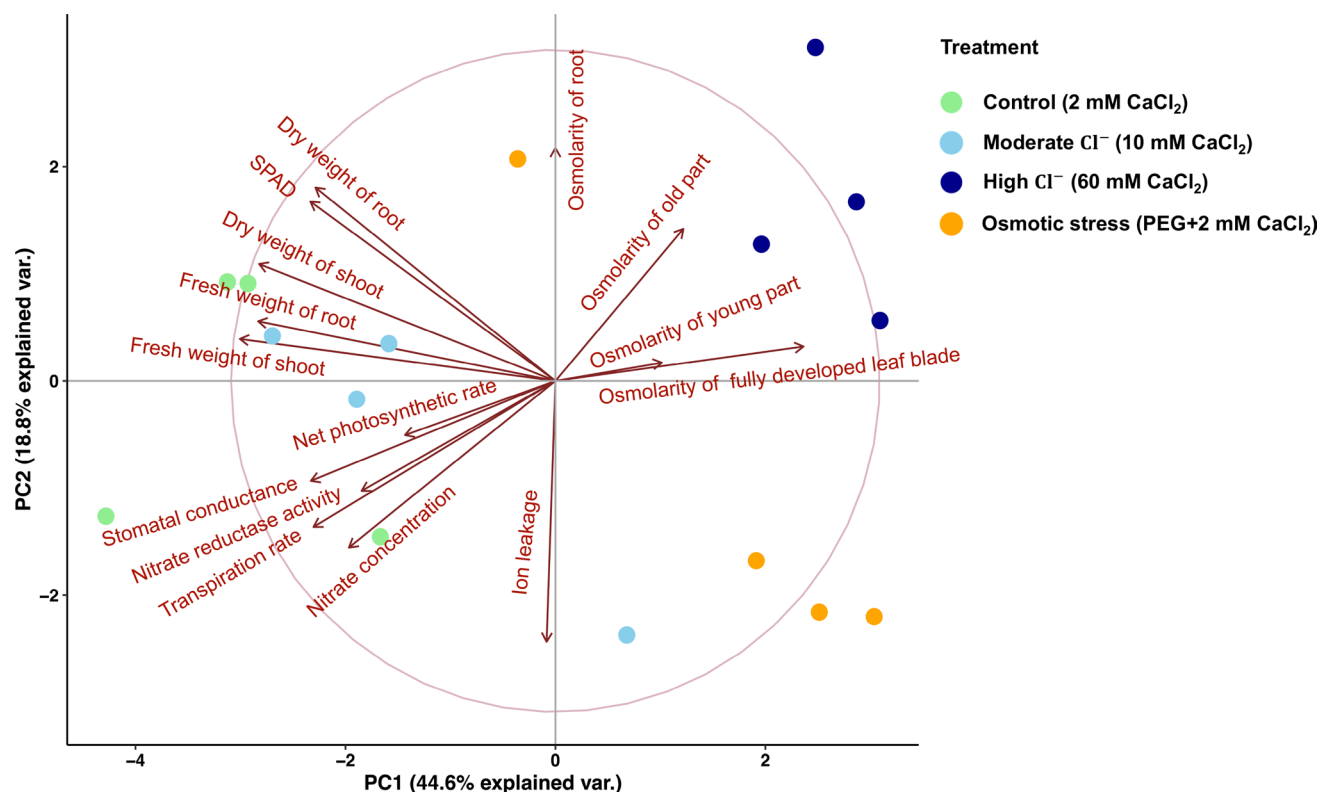


Fig. 5 Principal component analysis of 15 variables in maize responding to Cl^- application or osmotic treatment

In parallel, the uncompromised photosynthesis was also observed in the present experiment (Fig. 4d). Interestingly, chlorophyll reduction did not appear in high Cl^- application with higher osmotic stress (Fig. 4a). It could be resulted from the osmotic adjustment of Cl^- , in which the leaf blade had a considerably higher Cl^- concentration under 60 mM CaCl_2 than PEG-induced osmotic stress (Fig. 3a). In addition, there was no significant difference in SPAD readings between varied Cl^- doses at the whole treatment period. Therefore, reductions of chlorophyll concentration in maize result from osmotic stress rather than from Cl^- ionic toxicity.

The reduced stomatal conductance (g_s) (Fig. 4b) decreased E (Fig. 4c) but did not disturb A_N (Fig. 4d) under high Cl^- stress and PEG-induced osmotic stress. One reason might be that the stomatal regulation predominantly minimizes water loss while only marginally inhibiting CO_2 assimilation (Farquhar and Sharkey 1982). This can be evidenced by the data (Fig. 4b and Fig. S2), which showed that reduced g_s did not influence intercellular CO_2 concentration by high Cl^- stress and osmotic stress.

Inhibitory effects of Cl^- stress on NRA in maize

The negative correlation of NO_3^- metabolism variables (NO_3^- concentration and NRA) and gas exchange parameters (g_s , E , and A_N) with Cl^- stress was stronger than that with osmotic

stress, as shown by the PCA results (Fig. 5). Therefore, the correlation differences indicate that the mechanism of NO_3^- metabolic interference between osmotic stress and Cl^- stress is different (as shown by proposed mechanisms in Fig. 6).

Under PEG-induced osmotic stress (plus 2 mM CaCl_2), the osmotic imbalance caused more Cl^- to enter into the leaf cells (Figs. 3a and 6a) probably because of Cl^- as a ‘cheap’ osmoticum (Wege et al. 2017). Apart from this, Cl^- is also thought to liberate (by means of substitution) other osmotica such as proline, sucrose, malate, K^+ , and NO_3^- for use in other functions (Flowers 1988). The osmotic adjustment of Cl^- is beneficial to help plants adapt the osmotic stress. Although PEG-induced osmotic stress significantly reduced E (Figs. 4c and 6a), the decreased mass flow did not induce a reduction of NO_3^- concentration in the fully leaf blade as well as NRA (Figs. 3b, c and 6a).

The addition of excess Cl^- has considerably reduced NO_3^- concentration in the fully developed leaf blade, and thereby decreasing NRA (Figs. 3 and 6b). It arises a question how the overdose of external Cl^- results in a considerable reduction of NO_3^- concentration in the distant leaves via the competitive uptake with NO_3^- or the reduced NO_3^- translocation by decreased mass flow or both. The antagonistic uptake between Cl^- and NO_3^- has been well documented, because these two monovalent anions share a NO_3^- transporter *ZmNPF6.4*, which is a root-located transmembrane protein

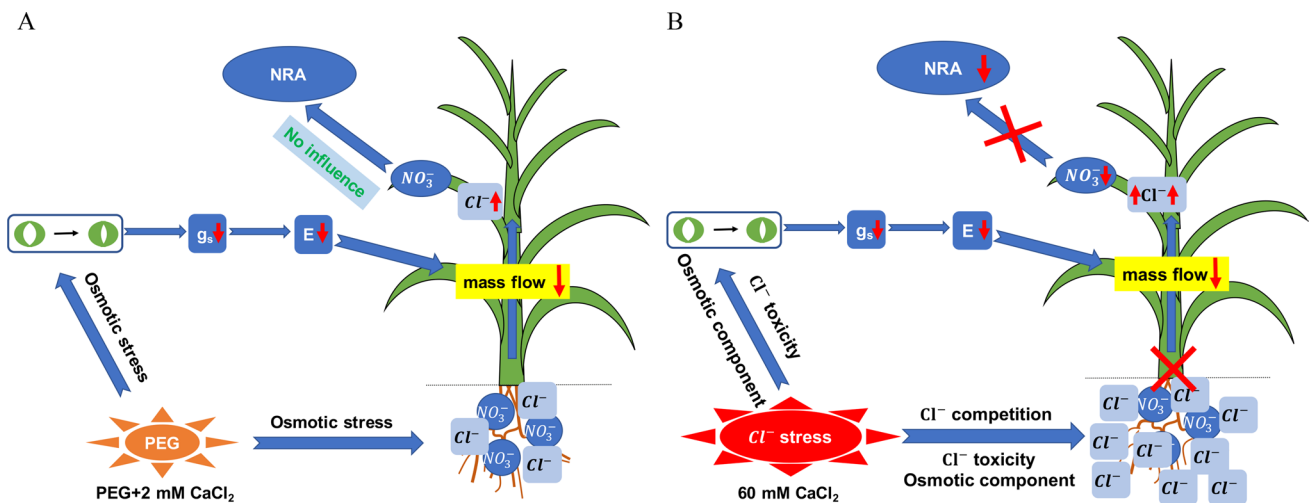


Fig. 6 Proposed mechanisms of NRA interference by Cl^- stress or osmotic stress in maize. **a** PEG-induced osmotic stress + 2 mM CaCl_2 , **b** Cl^- stress (60 mM CaCl_2). Abbreviations are as following: Cl^- chloride, NO_3^- nitrate, NRA nitrate reductase activity, g_s stomatal

conductance, E transpiration rate, “↑”, an increase of the corresponding parameter, “↓”, a decrease of the corresponding parameter, “X” the breakdown of the corresponding biological process

(Wen et al. 2017). Wen et al. (2017) found that an increase of external Cl^- concentration from 0 to 10 mM could facilitate Cl^- uptake of *ZmNPF6.4*. It explains the competition to NO_3^- uptake. Besides, the effect of reduced NO_3^- translocation via mass flow could be excluded or at least very tiny, although the data of NO_3^- xylem translocation was not measured at the present work. It can be well evidenced by transpiration rate data (Fig. 4c), because PEG-induced osmotic stress and 60 mM CaCl_2 stress caused the similar reduction of E , but the decreased mass flow indicated by E did not show a negative effect on leaf NO_3^- concentration under PEG-induced osmotic stress (Figs. 3c and 4c). Therefore, it is reasonably believed that the competitive uptake between Cl^- and NO_3^- is the primary culprit to decrease leaf NO_3^- concentration, and the lack of substrate (NO_3^-) restricts the NRA, since NR is a typical substrate-induced enzyme (Flores et al. 2000).

To compare both stresses, NR activity was significantly decreased by high Cl^- stress due to the antagonism with NO_3^- , but no influence was observed in osmotic stress (Fig. 6). However, the difference in NR activity did not affect biomass production, since both stresses always had identical biomass (Fig. 2). Therefore, this discrepancy is reasonably supposed to be a beneficial aspect of Cl^- , because a higher amount of Cl^- was accumulated in the developed leaf blade under high Cl^- , nearly 1.7 times as high as that of osmotic stress (Fig. 3a). Geilfus (2018a) reported that Cl^- could promote cell growth by regulating osmotic potential and turgor pressure.

In order to overcome this $\text{Cl}^-/\text{NO}_3^-$ uptake competition under saline conditions, genetic modifications have been conducted in crops, e.g., the overexpression of chloride channels (*GmCLC1*, *GsCLC-c2*) in the hairy roots of

soybean which has been found to enhance salt tolerance. The over-expressed transport protein is highly correlated with the significant decrease of Cl^- concentration in shoots and then indirectly maintains a high NO_3^- accumulation in plant tissues. As a result, the ratio of $\text{Cl}^-/\text{NO}_3^-$ remains constant in the stems and leaves of soybean (Wei et al. 2016, 2019). Nevertheless, whether this sole remedy is really sufficient to guarantee the metabolic function of NR in leaves under saline conditions remains unclear, because a preliminary question that if mass flow directly influence NO_3^- xylem translocation under osmotic stress should be firstly answered. If this effect depends on osmotic intensity or crop species, then the genetically modified strategy would not be working efficiently.

Conclusions

Maize is more sensitive to osmotic stress rather than Cl^- ion toxicity. Cl^- has beneficial effects on mitigating the osmotic stress via osmotic adjustment. Osmotic stress did not interfere NRA, since leaf NO_3^- concentration was not reduced. A link between Cl^- stress and NRA has been established in Cl^- -stressed plants: the decreased NRA might be primarily caused by the antagonistic uptake of Cl^- and NO_3^- , finally resulting in the scarcity of metabolically available NO_3^- in leaf tissues.

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Author contributions C-MG and CZ designed the concept and the experiments. XZ and LE conducted the experiments in the greenhouse and laboratory. BF supervised the cultivation of plants and took part in the data interpretation. XZ was responsible for data analysis, figures plotting, results discussion and manuscript drafting.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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