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ORIGINAL PAPER

Discriminating the impact of Na^+ and Cl^- in the deleterious effects of salt stress on the African rice species (*Oryza glaberrima* Steud.)

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Abstract

Salinity resistance of the African rice species (*Oryza glaberrima*) is poorly documented and the specific responses of the plant to Na^+ and Cl^- toxic ions remain unknown. Cultivars TOG5307 and TOG5949 were maintained for 15 days on iso-osmotic nutrient solutions containing 50 mM NaCl, or a combination of Cl^- salts (Cl^--dominant) or Na^+ salts (Na^+-dominant). Plant water status, ion accumulation, gas exchange, fluorescence related parameters, carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios were analyzed. TOG5307 accumulated lower amounts of Na^+ and Cl^- in the shoot (1.63 and 1.49 µmol g^{-1} DW, respectively) than TOG 5949 (2.5 and 2.2 µmol g^{-1} DW). At 50 mM NaCl, TOG5307 also exhibited a higher net carbon assimilation rate (2.51 µmol CO_2 m^{-2} s^{-1}) than TOG5949 (1.51 µmol m^{-2} s^{-1}) and a higher water use efficiency. Most recorded physiological parameters were affected by both Na^+ and Cl^-. The pattern of modification induced by both types of toxicities was similar in the two studied cultivars which thus mainly differ for the quantitative aspects of the response rather than for the qualitative nature of the response. NaCl was the most detrimental treatment, followed by Na^+-dominant treatment while Cl^--treatment had the lowest effect. The two considered cultivars mainly differ for their response to the ionic component of salt stress but not for their osmotic behaviour. The impact of Na^+ and Cl^- on considered parameters are additive, except for mineral nutrition where synergistic interactions were recorded for Na^+ and S accumulation.

Keywords Ion toxicity · NaCl · *Oryza glaberrima* · Rice · Salinity

Introduction

Soil salinization is a major environmental constraint that hampers crop production in more than 5% of agricultural land and 20% of irrigated cultivated areas (Morton et al. 2019; Zörb et al. 2019). The human population is expected to reach 10 billion by 2050, and increasing crop yield in these salt-affected areas constitutes an important challenge for the next decades (Tilman et al. 2011). This is especially important for rice which represents the main staple food for 3 billion peoples and provides 27% of the energy intake in the population of third world countries (Ishwara Lakshmi et al. 2019). Unfortunately, rice is a typical glycophyte species exhibiting a high level of salt-sensitivity at the seedling and the flowering stages (Lutts et al. 1995; Ganie et al. 2019; Yong et al. 2020).

Besides the classical Asian rice species *Oryza sativa* which was domesticated 9000 years ago, an African rice species, *Oryza glaberrima*, was independently domesticated 3000 years ago from the wild rice progenitor *O. barthii* (Choi et al. 2019; Veltlman et al. 2019). Being less productive than *O. sativa*, *O. glaberrima* has been only marginally cultivated but it now appears as a promising source of readily available genetic diversity for rice improvement (Kang and Futakuchi 2019; Wambugu et al. 2019). This is especially valid for resistance to numerous abiotic and biotic constraints since numerous accessions of *O. glaberrima* exhibit

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interesting properties for resistance to high temperatures (Li et al. 2015), drought (Bimpong et al. 2011; Shaibu et al. 2018; Kang and Futakuchi 2019; Kartika et al. 2020), iron toxicity (Majerus et al. 2007; Sikirou et al. 2018; Mayabe et al. 2020) and several diseases (Thiémélé et al. 2010; Petitot et al. 2017; Wambugu et al. 2019). As far as salinity is concerned, *O. glaberrima* received recent attention to Na^+ exclusion (Platten et al. 2013) or microRNA involved in biological pathways of salinity tolerance (Mondal et al. 2018). Accessions displaying contrasting levels of salt-resistance were recently identified (Prodjinoto et al. 2018).

Salinity is a complex environmental constraint that comprises two major components: (i) an osmotic component related to a decrease in the external water potential which compromises plant water absorption and (ii) an ionic component related to the accumulation of toxic ions (Munns and Tester 2008; Munns et al. 2020). Although various salts may contribute to a high soil electrical conductivity in field conditions, most studies devoted to salt stress focus on NaCl toxicity and only consider Na^+ impact on plant response with little regard to Cl^- toxicity. As an essential micronutrient, Cl^- plays important role in plant physiology (Teakle and Tyerman 2010). When present in excess, however, it can disturb numerous metabolic pathways and leads to extensive damages to the plant. Some authors even consider that Cl^- soil concentration is more important than Na^+ concentration in terms of salt impact on the yield of major crops (Dang et al. 2008). In rice, Na_2SO_4 was reported to be less toxic than iso-osmotic solutions of NaCl at both cellular (Lutts et al. 1996) and whole plant levels (Irakoze et al. 2019, 2020). In a short-term experiment, Lefèvre et al. (2001) demonstrated that KCl was unexpectedly more toxic than NaCl although K^+ is a non-toxic element: this could be explained by the higher Cl^- concentration recorded in KCl-treated plants comparatively to NaCl-exposed ones. This supports the hypothesis that Cl^- is an important factor in the salt-induced injuries in rice plants and that a better knowledge of the plant behavior requires to discriminate between the impact of individual ions Na^+ and Cl^-.

The use of Na^+-dominant salt solution, Cl^--dominant solution, and NaCl solution allows to precise the impact of each ion as well as their interaction without inducing major side effects linked to the accumulation of excessive counterions in the used salts (Tavakkoli et al. 2010, 2011). In *Oryza sativa*, such a strategy allowed Khare and co-workers to demonstrate that Na^+ and Cl^- have different impacts on the induction of oxidative stress, proline metabolism, and nitrosative response and that these ions act in an additive way when added as NaCl in the root medium (Khare et al. 2015; Kumar and Khare 2016; Khare et al. 2020).

The nature and the extent of interactions between Na^+ and Cl^- may differ among species (Bhuiyan et al. 2017). To the best of our knowledge, no data are available on discrimination between Na^+ and Cl^- toxicities in the African rice *Oryza glaberrima*. The aim of the present work was therefore to compare the impacts of Na^+, Cl^- and their simultaneous presence as NaCl on two cultivars of *O. glaberrima* differing in salinity resistance. Plant behavior was analyzed in relation to mineral nutrition, plant water status, and photosynthetic properties.

Material and methods

Plant material and growth conditions

Seeds of *Oryza glaberrima* Steud. were obtained from Africa Rice (Bouaké, Ivoiry Coast). Previous researches demonstrated that accession TOG5307 (AccNumber WAB0021855) is salt-tolerant while TOG5949 (AccNumber WAB0020144) is salt-sensitive (Prodjinoto et al. 2018). Seeds of each cultivar were germinated in glass vessels on two layers of filter papers (Whatman 85 mm, Grade 1) moistened with 10 mL of sterile deionized water. Glass vessels were placed in a germination chamber (LEEC Plant Germination Cabinets SL3) at 25 °C under a 16 h daylight period (150 µmol m^{-2} s^{-1}). Daytime humidity was 70% and illumination was provided by Sylvania fluorescent tubes (F36W/840-T8, cool white).

Ten days old seedlings of uniform size from the two cultivars were transferred into a phytotron and fixed on polystyrene plates floating on Yoshida's nutritive solution (YNS) (Yoshida et al. 1976). For each treatment, 18 seedlings of each cultivar were distributed per tank containing 16 L of YNS. Three tanks per accession and treatment were considered. All tanks were randomly rearranged during the experiment and solutions were renewed each week. The temperature was maintained at 29 °C during the day and 26 °C during the night and the illumination was provided by PHILIPS metal iodide lamp (HPIT/400W) for 16 h day^{-1} with a photon flux density of 300 µmol m^{-2} s^{-1}. Daytime humidity was maintained between 65% and 80%. After acclimatization during two weeks, Na^+, Cl^- or NaCl stresses were applied as described by Tavakkoli et al. (2011). The used solutions consisted in:

- Control (YNS, no amendments);
- 50 mM of Na^+-dominant salts (YNS plus 7.5 mM Na_2SO_4, 7.5 mM Na_2HPO_4, 20 mM $NaNO_3$);
- 50 mM of Cl^--dominant salts (YNS plus 7.5 mM $CaCl_2.2H_2O$, 7.5 mM $MgCl_2{\cdot}6H_2O$, 20 mM KCl)
- NaCl (YNS plus 50 mM NaCl)

The control nutrient solution had an osmotic potential (Ψs) of -0.12 ± 0.002 MPa and electric conductivity (EC) of 0.87 ± 0.11 mS cm^{-1} while all salt treatments

(Na^+-dominant, Cl^--dominant and NaCl) had similar EC (5.83 ± 0.11 mS cm^{-1}). The NaCl solution had a Ψs of -0.31 ± 0.002 MPa while both Na^+-dominant and Cl^--dominant exhibited a similar Ψs of -0.28 ± 0.009 MPa. The pH of all treatments was daily maintained at about 6.7 and the experiment was conducted for two weeks as a randomized complete block design.

Plant growth, water content, and osmotic potential

At the end of stress exposure, the length of the shoot was estimated from the root-shoot junction to the tip of the longest leaf and the length of the longest root was measured. Plants were then harvested and roots were rinsed for 30 s in deionized water to remove ions from the root surface and the free spaces. For mineral analysis, 12 plants per treatment per cultivar were kept. Roots and shoots were separated and weighed for fresh weight (FW) estimation. Samples were then dried at 70 °C for 72 h in an oven until reaching a constant dry weight (DW).

For the measurement of Ψs, roots and leaves of 8 plants per treatment per accession were cut into small segments. Then, samples were quickly introduced in an Eppendorf tube perforated with four small holes. Eppendorf tubes were rapidly frozen in liquid nitrogen for 30 s and warmed at room temperature for 5 min in order to break the cellular membrane (Lutts et al. 1999). After 3 cycles of frozen/warmed, tubes were then encased in a second unperforated Eppendorf tube. Samples were centrifuged at 15,000×*g* for 15 mins at 4 °C. The supernatant corresponding to the collected tissular sap was used to assess the osmolarity (c). Osmolarity was assessed with a vapour pressure osmometer (Wescor 5520) and converted from mosmol K g^{-1} to MPa using the formula: $\Psi s\ (MPa) = -c\ (mosmol\ kg^{-1}) \times 2.58 \times 10^{-3}$ according to the Van't Hoff equation.

Ion content

For shoots and roots of each cultivar per treatment, *ca.* 20 mg DW were digested with 4 mL of 0.5% of nitric acid at 80 °C. After complete evaporation, residues were dissolved with HNO_3 (68%) + HCl_{cc} (1:3, *v/v*) and incubated under gentle agitation of 80 rpm for 48 h. The solution was then filtered using a layer of filter paper (Whatman 85 mm, Grade 1). The filtrate was used to estimate the concentrations of Na^+ and K^+ by flame emission using an Atomic Absorption Spectrometer (Thermo scientific S series model AAS4, Thermo Fisher Scientific Waltham, MA, USA). Anions were extracted according to Hamrouni et al. (2011). The concentrations of Cl^- and S^{2-} were determined by liquid chromatography (HPLC-Dinex ICS2000, Dionex Corporation, Sunnyvale, California, U.S.A.) using an AS15/AG15 column/precolumn system and 20–38 mM KOH as eluent for 40 min.

Proline, total soluble sugar, malondialdehyde, flavonoids, and total phenolics determination

For proline quantification, 200 mg FW of roots and shoots were ground in liquid nitrogen in a mortar containing 10 mL of 3% sulfosalicylic acid. Samples were centrifuged at 1000×*g* for 5 min and 2 mL of the supernatant were incubated at 100 °C in the presence of 2 mL ninhydrin and 2 mL acetic acid. After extraction with toluene (2 mL), proline was quantified at 520 nm with a Beckman DU640 spectrophotometer using proline standards (Sigma Aldrich) as controls (Bates et al. 1973).

For total soluble sugar estimation, a portion of leaves (*ca.* 300 mg FW) were mixed with 7 mL of ethanol 70% (w/v) for 5 min on ice and centrifuged at 8000×*g* at 4 °C; 200 µL of the supernatant then reacted with 1 mL of anthrone solution (0.5 g anthrone, 250 mL 95% H_2SO_4 and 12.5 mL distilled water). The absorbance was read at 625 nm according to Yemm and Willis (1954). A calibration curve was established using glucose as the standard.

Malondialdehyde (MDA, a product of lipid peroxidation) was measured as the 2-thiobarbituric acid-reactive substances (TBARS) according to Boaretto et al. (2014): 250 mg FW were ground in liquid nitrogen with the solution of 1.25% glycerol and 5% trichloroacetic acid. Samples were then centrifuged at 6700×*g* for 10 min at 4 °C and 2 mL of supernatant were mixed with 2 mL of 0.67% thiobarbituric acid. After incubation at 100 °C for 30 min, samples were cooled on ice. Absorbance was read at 532 nm and values related to non-specific absorption (600 nm) were subtracted. A molar extinction coefficient of 155 mM^{-1} cm^{-1} was used to calculate MDA concentration.

Flavonoids and total phenolics were extracted from frozen leaves of each cultivar using methanol 80%. The concentration of total phenolics in this methanolic extract was estimated using the Folin–Ciocalteu method (Slinkard and Singleton 1977). An aliquot (20 µL) of sample was added to 1.58 mL of deionized water and 100 µL of the Folin–Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min, before adding 300 µL of 2% sodium carbonate (Na_2CO_3) solution. After incubation for 2 h at room temperature in the dark, the absorption was measured at 765 nm. Total phenolic contents were expressed as milligrams of gallic acid equivalents per gram of fresh weight (mg of GAE g^{-1} FW) through the calibration curve with gallic acid.

In the same methanolic extract, flavonoids were quantified at 510 nm following the method of Dewanto et al. (2002) using phosphomolybdic-phosphotungstic reagent and catechin as standard. An aliquot (250 µL) of the sample was mixed with 1.25 mL of deionized water and 75 µL of 5

% $NaNO_2$. After 6 min, 150 µL of 10% aluminum chloride ($AlCl_3$) and 500 µL of 1M NaOH were added to the mixture. Finally, the mixture was adjusted to 2.5 mL with deonised water. The absorbance of the mixture was determined at 510 nm. Standard curve was established using 0 to 450 µg ml^{-1} catechin as standard. Total flavonoid content was expressed as mg (+)-catechin equivalent per gramme of fresh weight (mg Catechins. 10^{-2} g^{-1} of FW).

Total soluble protein concentrations were determined according to Bradford (1976).

Chlorophyll fluorescence, gas exchange, and chlorophyll content

Photosynthetic-related parameters and gas exchange were determined on the middle part of the second youngest fully expanded leaf (basipetal numbering) of 8 plants per treatment per cultivar. The chlorophyll fluorescence was determined by using a portable pulse-modulated chlorophyll fluorimeter (FMS2, Hansatech, King's Lynn, UK). Leaf portions were acclimated to darkness for 30 min and the minimal fluorescence level (F_0) was estimated by measuring the modulated light (0.1 µmol m^{-2} s^{-1}) in the dark-adapted middle part of leaves. The maximal fluorescence level (F_m) with all photosystem II (PSII) reaction centers closed was determined by a 0.8 s saturation pulse at 18,000 µmol m^{-2} s^{-1}. Then, the leaf was continuously illuminated with white actinic light (600 µmol m^{-2} s^{-1}) for 3 min. The steady-state value of fluorescence (F_s) was recorded and a second saturating pulse at 18,000 µmol m^{-2} s^{-1} was imposed to determine maximal fluorescence level in the light-adapted state (F'_m). The actinic light was removed and the minimal fluorescence level in the light-adapted state (F'_0) was determined by illuminating the leaf with a 3 s pulse of far-red. Using both light and dark fluorescence parameters, the maximal efficiency of PSII photochemistry in the dark-adapted state (F_v/F_m), the photochemical quenching coefficient (qP), the non-photochemical quenching (NPQ), the electron transport rate (ETR), and the actual PSII efficiency (Φ_{PSII}) were calculated according to Maxwell and Johnson (2000).

Gas exchange was recorded on the same leaf with an infrared gas analyzer (LCA4; ADC Bioscientific, Hoddesdon, Hertfordshire, UK) using a PLC Parkinson leaf cuvette for 1 min (20 records/min) and an airflow of 300 mL min^{-1}. Air taken from the external atmosphere was sent to a chamber into which a leaf portion of 6.25 cm^2 was introduced. The net carbon assimilation rate (mmoles CO_2 m^{-2} s^{-1}, *A*) was measured under constant photosynthetic photon flux. The stomatal conductance (g_s) was assessed using porometer (type AP4-UM-3, Delta T-devices, UK). The intercellular CO_2 content (µmoles mol^{-1}, *C*i) and the instantaneous transpiration (mmoles H_2O m^{-2} s^{-1}, *E*) were estimated using a water vapor analyzer (LCA 28.7, ADC, Great Amwell, England) and an air supply unit (ASU 10.87, ADC, Hertfordshire, UK). All measurements were performed between 10:00 am and 2:00 pm. The efficiency of electron transport (ETR/*A*), the efficiency of the instantaneous carboxylation (*A*/*C*i), the intrinsic (A/g_s), and instantaneous (*A*/*E*) water use efficiency were calculated.

Pigments (chlorophyll a (*Chl* a), chlorophyll b (*Chl* b), and total carotenoids) were extracted in the dark from leaf segments ground in the presence of 8 mL of cold acetone 80%. The extract was centrifuged at 1000×*g* for 10 min at 4 °C and the absorbance was read at 663.2, 646.8, and 470 mm. The pigment concentration was then calculated (µg per mL solution) according to Lichtenthaler (1987):

$$Chl\text{a} = 12.25A_{663.2} - 2.79A_{646.8}$$

$$Chl\text{b} = 21.50A_{646.8} - 5.10A_{663.2}$$

$$\text{Total carotenoid} = \left(1000A_{470} - 1.82Chl\text{a} - 85.02Chl\text{b}\right)/198$$

Carbon and nitrogen isotopic measurements

Dry matter of roots and shoots were ground to homogenous powder and *ca*. 2 mg were weighed and placed in tin capsules (3 × 5 mm) before elemental and stable isotope analysis. Carbon and nitrogen stable isotope measurements were performed on each sample analyzed with an elemental analyzer (Vario MICRO Cube, Elementar, Hanau, Germany) coupled to a continuous-flow isotope-ratio mass spectrometer (IsoPrime100, Elementar UK, Cheadle, United Kingdom). Carbon and nitrogen elemental composition was reported in % of the dry weight of the sample. Stable isotope ratios (SIR) of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) were expressed conventionally in delta notation as parts per thousand (‰) according to the following equation:

$$\delta X = \left[(\text{R sample/R standard}) - 1\right] \times 1000$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$. Carbon and nitrogen ratios were expressed with the V-PDB (Vienna Peedee Belemnite) standard and atmospheric nitrogen respectively. Pure gases of CO_2 and N_2 were used as calibration against certified reference materials, i.e., sucrose (IAEA-CH6) and ammonium sulfate (IAEA-CH6). These latter were obtained from the International Atomic Energy Agency (IAEA, Vienna, Austria). The analytical precision was assessed by procedural blanks, internal replicates (i.e., glycine and one of rice sample), and isotopic certified material (i.e., IAEA-CH6 and IAEA-CH6). The standard errors on replicated measurements from a single sample were ± 0.08‰ for $\delta^{13}C$, ±

0.11‰ for $\delta^{15}N$, ± 0.23% for C elemental composition, and ± 0.05 for N elemental composition.

As far as ^{13}C is concerned, although δ provides information on the $^{13}C/^{12}C$ of the tissues, it does not fully clarify the effect of the biological discrimination process that causes variation in this ratio (Shaheen and Hood-Nowotny 2005). Discrimination ($\Delta^{13}C$) is thus considered and defined as

$$\Delta = (\delta a - \delta p)/(1 + \delta)$$

where δa and δp are the carbon isotope composition of source air and plant material, respectively, relative to the international standard Pee Dee Belemnite.

Ionic toxicity index

Ion toxicities applied in our study are expected to change the behavior of treated plants compared to control ones. To quantify the relative impact of stress-induced changes comparatively to control, we quantified for each parameter and each type of toxicity (Na^+-dominant, Cl^--dominant or NaCl) an Ion Toxicity Index (ITI) defined as:

$$ITI_y = |(V_y/V_c) - 1|$$

where y is the type of toxicity (Na, Cl, or NaCl), V_y the value recorded for a given parameter in plants exposed to toxicity y, and V_c is the value recorded for control plants.

The tested hypothesis is that for each cultivar, $ITI_{NaCl} = ITI_{Na} + ITI_{Cl,}$ considering that Na^+ and Cl^- toxicities acted in a strict additive way.

Statistical analysis

Statistical analyses were performed using JMP Pro 14 software. The analysis of the main effects of cultivars and stresses was based on the variance analysis. Means were compared utilizing Tukey's HSD all-pairwise comparisons at the $P = 0.05$ as a post-hoc test. Person chi-squared test using Yate's correction for continuity was used the test the validity of the additive model for ITI_{NaCl}.

Results

Plant growth and water status

The two tested cultivars exhibited similar shoot and root dry weight under control conditions (Fig. 1). All stress treatments significantly reduced plant growth: Cl^--dominant treatment was the less damaging, followed by Na^+-dominant, while NaCl was the most deleterious treatment for plant growth. As far as the shoot part is concerned, the salt-sensitive TOG5949 was always more affected by the treatments

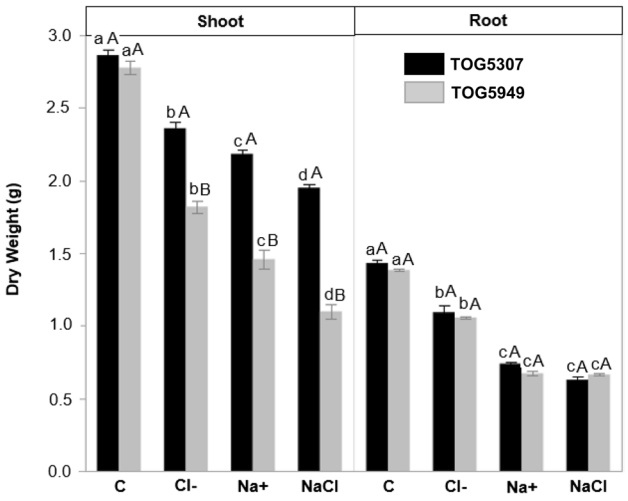

Fig. 1 Dry weight (DW) of shoot and roots of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 (white bars) cultivated during 2 weeks in control conditions (C) or in the presence of 50 mM of either Cl^- (chloride-dominant), Na^+ (sodium-dominant) or NaCl. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments followed by the same lowercase letter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a particular condition do not differ statistically

than the salt-resistant TOG5307. In contrast, no difference between cultivars has been recorded for roots. TOG5949 exhibited a shorter stature than TOG5307 under control conditions (Table 1). Shoot length was inhibited by all treatments but no significant difference was recorded between Na^+- dominant and NaCl treatment. Chloride-dominant treatment had no impact on root length in TOG5307 but reduced root elongation in TOG5949. The ratio root/shoot DW remained unaffected by the applied treatments and was similar for the two cultivars (Table 1).

The shoot water content (WC) remained unaffected in Cl^--dominant and Na^+-dominant treatment but NaCl reduced WC in the shoot of the two cvs. (Table 2). Root WC was reduced by Na^+-dominant and by NaCl treatments in TOG5307 and only by NaCl in TOG5949. In the two considered cultivars, shoot Ψs decreased following the same trend: the lowest decrease was observed for Cl^--dominant treatment, and the highest for NaCl treatment, Na^+-dominant being intermediate. In all cases, however, including in control conditions, the shoot Ψ_s of TOG5949 was significantly lower than the values recorded for TOG5307. A similar difference was also observed for the root Ψs (Table 2).

Mineral nutrition

As shown in Fig. 2, Na^+ accumulated in response to Na^+-dominant and NaCl treatments in shoots and roots

Table 1 Shoot length, root length and dry weight root:shoot ratio of African rice (*Oryza glaberrima* Steud.) seedlings from cv. TOG5307 and TOG5949 cultivated during 2 weeks in control (C) conditions or in the presence of 50 mM of either Cl^- (chloride dominant), Na^+ (sodium dominant) or NaCl

Cultivars	Treatments	Shoot length (cm)	Root length (cm)	(DW) Root:shoot ratio
TOG5307	C	62.67 ± 1.33^{aA}	32.00 ± 1.15^{aB}	0.23 ± 0.01^{aA}
	Cl^-	52.33 ± 0.88^{bA}	30.33 ± 0.88^{aA}	0.23 ± 0.01^{aA}
	Na^+	36.67 ± 1.86^{cA}	32.33 ± 1.20^{aA}	0.20 ± 0.03^{aA}
	NaCl	33.33 ± 1.45^{cA}	18.33 ± 0.88^{bA}	0.21 ± 0.04^{aA}
TOG5949	C	57.33 ± 1.33^{aB}	42.00 ± 1.15^{aA}	0.24 ± 0.02^{aA}
	Cl^-	41.67 ± 1.86^{bB}	27.00 ± 0.58^{bB}	0.22 ± 0.01^{aA}
	Na^+	31.67 ± 1.45^{cA}	22.33 ± 0.88^{cB}	0.18 ± 0.03^{aA}
	NaCl	27.00 ± 2.08^{cA}	21.67 ± 0.88^{cA}	0.22 ± 0.03^{aA}

Treatments followed by the same lowercase latter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase latter in a particular condition do not differ statistically

Table 2 Water content and osmotic potential (Ψs) of African rice (*Oryza glaberrima* Steud.) seedlings from cv. TOG5307 and TOG5949 cultivated during 2 weeks in control (C) conditions or in the presence of 50 mM of either Cl^- (chloride dominant), Na^+ (sodium dominant) or NaCl

Treatments	Water content (%)				Ψs (MPa)			
	Shoot		Root		Shoot		Root	
	TOG5307	TOG5949	TOG5307	TOG5949	TOG5307	TOG5949	TOG5307	TOG5949
C	90.20 ± 1.00^{aA}	90.52 ± 0.24^{aA}	93.11 ± 0.31^{aA}	92.68 ± 0.59^{aA}	-1.20 ± 0.03^{aA}	-1.45 ± 0.02^{aB}	-0.56 ± 0.01^{bA}	-0.71 ± 0.03^{aB}
Cl^-	91.43 ± 0.47^{aA}	91.00 ± 0.41^{aA}	92.50 ± 0.22^{aB}	93.26 ± 0.54^{aA}	-1.70 ± 0.03^{bA}	-1.81 ± 0.01^{bB}	-0.52 ± 0.01^{aA}	-0.79 ± 0.01^{bB}
Na+	88.33 ± 0.61^{aA}	89.89 ± 0.31^{aA}	91.45 ± 0.28^{bB}	92.69 ± 0.17^{aA}	-1.82 ± 0.01^{cA}	-2.08 ± 0.02^{cB}	-0.70 ± 0.03^{dA}	-0.81 ± 0.01^{bB}
NaCl	84.78 ± 0.60^{bA}	85.23 ± 0.99^{bA}	88.42 ± 0.81^{bA}	88.22 ± 0.24^{bA}	-2.38 ± 0.04^{dA}	-2.64 ± 0.03^{dB}	-0.62 ± 0.02^{cA}	-0.74 ± 0.02^{aB}

Treatments followed by the same lowercase latter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase latter in a particular condition do not differ statistically

(Fig. 2a). The salt-sensitive TOG5949 exhibited a higher Na^+ concentration than the salt-resistant TOG5307. As far as shoots are concerned, Na^+ accumulated to a higher extent in response to NaCl than in response to Na^+-dominant treatment in TOG5949 while no difference was recorded between treatments for TOG5307. The root Na^+ concentration was higher in response to NaCl than in response to Na^+-dominant treatment.

In control conditions, TOG5307 contained higher concentrations of K^+ than TOG5949. An excess of Na^+ in the medium induced a drastic decrease in the shoot K^+ concentration: under stress conditions, K^+ concentration remained higher in TOG5307 than in TOG5949. The shoot K^+ decrease was more marked in response to NaCl than to Na^+-dominant treatment in TOG5949 but not in TOG5307. An excess of Na^+ also strongly reduced K^+ concentration in the roots. It has to be noticed that Cl^--dominant treatment also induced a significant decrease in root K^+ concentration in both cultivars.

Chloride accumulated in response to Cl^--dominant and NaCl treatments and to a higher extent in the shoots than in the roots. The shoot chloride concentration was higher than the root concentration in TOG5949. The shoot Cl^- the content was higher in TOG5949 than in TOG5307 while an opposite trend was recorded for the roots. For both cultivars, shoot Cl^- concentration was significantly higher in response to NaCl than in response to Cl^--dominant treatment.

Under control conditions, sulfur concentration was higher in TOG5307 than in TOG5949. NaCl treatment drastically increased S concentration in both cultivars while neither Na^+-dominant nor Cl^--dominant treatment had similar effects. A NaCl-induced increase in S concentration was also recorded at the root level.

Proline, total soluble sugar, malondialdehyde, flavonoids, and total phenolics quantification

Malondialdehyde (Fig. 3a), as a marker of lipid peroxidation, increased to similar values in response to Cl^--dominant and Na^+ dominant treatment in TOG5949, while the recorded values were lower for Cl^--dominant in TOG5307. The highest MDA concentration was recorded in NaCl-treated plants and remained lower in TOG5307 than in TOG5949.

Proline concentration (Fig. 3b) increased in response to all types of ionic toxicities and, once again, exhibited

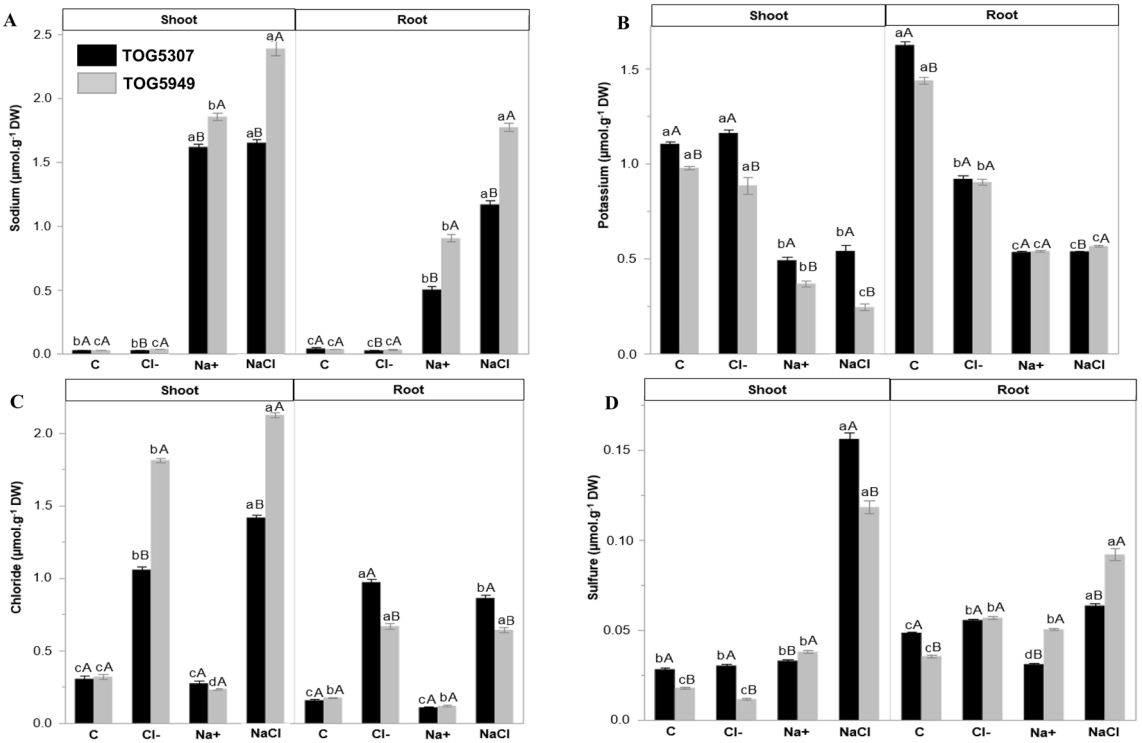

Fig. 2 **a** Sodium, **b** potassium, **c** chloride, and **d** chloride concentration in shoots and roots of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 (grey bars) cultivated during 2 weeks in control conditions (C) or in the presence of 50 mM of either Cl^- (chloride dominant), Na^+ (sodium dominant) or NaCl. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments followed by the same lowercase letter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a particular condition do not differ statistically

the maximal values in response to NaCl. The two studied cultivars accumulated similar proline concentrations when exposed to Cl^--dominant treatment while proline concentration was higher in TOG5307 than in TOG5949 in the presence of Na^+ excess (Na^+-dominant and NaCl). In TOG5307, the total soluble sugar concentration increased in response to NaCl only (Fig. 3c) while these compounds significantly accumulated in response to all types of toxicities in TOG5959, their concentration is always higher in the salt-sensitive TOG5959 than in the salt-resistant TOG5307.

Total flavonoids concentration (Fig. 3d) exhibited similar values in the two cultivars in the absence of stress. It increased to a similar extent in the two cultivars exposed to Cl^--dominant treatment, and to higher values in response to Na^+ toxicities. In plants exposed to Na^+-dominant and NaCl, flavonoids concentrations were higher in TOG5307 than in TOG5949. Total phenolics concentration (Fig. 3e) was also similar in the two considered cultivars maintained in the absence of stress and increased to similar values in response to Cl^--dominant solution. In plants exposed to Na^+-dominant solution, total phenolics accumulated to a similar concentration than in response to Cl^- in TOG5307 while they did not accumulate and remained similar to controls in TOG5949. The maximal total phenolics concentration was recorded in NaCl-treated plants with a higher value in TOG5307 than in TOG5949. The total soluble protein was marginally affected by Cl^- dominant toxicity but decreased in response to Na^+ excess: total soluble protein remained significantly higher in TOG5307 than in TOG5949 and was, for both cultivars, the lowest in NaCl-treated plants.

Gas exchange and photosynthesis-related parameters

Chlorophyll a concentration (Fig. 4a) was lower in TOG5949 than in TOG5307 in control plants. It decreased in response to ion toxicities, once again according to the following order: Cl^--dominant > Na^+-dominant > NaCl. A similar trend was observed for *Chl*b (Fig. 4b), except that no difference was recorded between Na^+-dominant and NaCl treatments; for these two types of toxicity, TOG5949 was significantly more affected than TOG5307. The *Chl*a/*Chl*b ratio, however (Fig. 4c) remained the same in the two cultivars, and for all types of toxicity, *Chl*a/*Chl*b being significantly higher than in control, except for NaCl-treated plants. Carotenoid concentration (Fig. 4d) also decreased in response to ionic toxicities. Cl^--dominant solution, was the less deleterious treatment, and the two

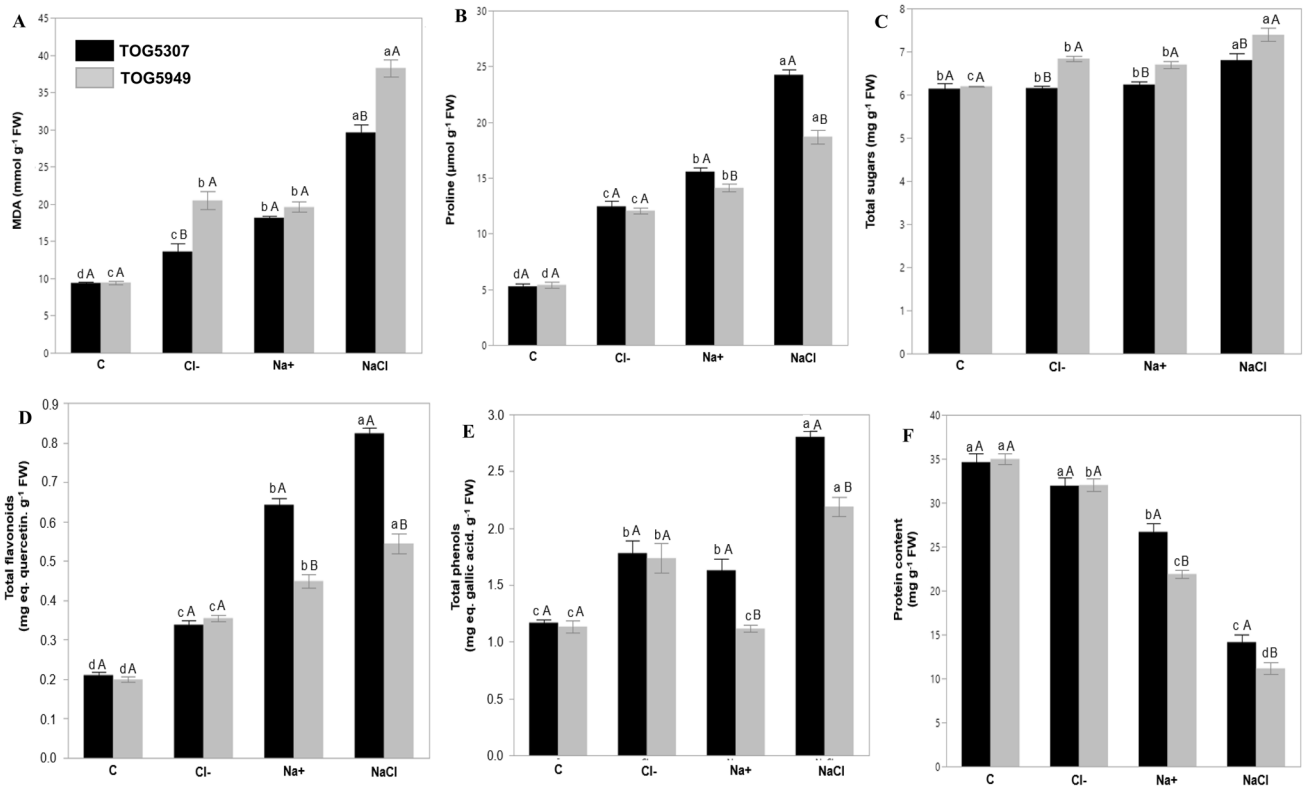

Fig. 3 **a** Malondialdehyde, **b** proline, **c** total sugars, **d** total flavonoids, **e** total phenols, and **f** soluble protein concentration of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 (grey bars) cultivated during 2 weeks in control conditions (C) or in the presence of 50 mM of either Cl^- (chloride dominant), Na^+ (sodium dominant) or NaCl. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments followed by the same lowercase letter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a particular condition do not differ statistically

studied cultivars displayed similar carotenoid concentration in this case. The presence of a high concentration of Na^+ was by far more toxic, especially or TOG5949 which exhibited similar carotenoid concentration in response to Na^+-dominant and NaCl treatment, the value being in both cases lower than in TOG5307.

Data related to chlorophyll fluorescence parameters are provided in Fig. 5. The Na^+-dominant and NaCl treatments significantly decreased F_v/F_m (Fig. 5a) and Φ_{PSII} (Fig. 5b) in the two cultivars but to a higher extent in TOG5949 than in TOG5307. Photochemical quenching (qP) was not affected by Cl^--dominant treatment but was reduced in response to Na^+-dominant and NaCl treatment in TOG5307 and response to NaCl treatment only in TOG5949. Similarly, Cl^--dominant treatment had no impact on NPQ values (Fig. 5d) while Na^+-dominant solution increased NPQ to similar values for the two cultivars. Non-photochemical quenching was even higher in response to NaCl than in response to Na^+-dominant solution in TOG5949 while values remained similar in TOG5307.

Net photosynthesis (*A*; Table 3) significantly decreased in response to all ionic treatments according to the following order $Cl^- > Na^+ >$NaCl. Although both genotypes exhibited similar *A* values in control conditions, TOG5307 always presented higher *A* values than TOG5949 in response to all ion toxicities. Instantaneous transpiration (*E*; Table 3) also decreased in response to ion stress, NaCl being once again the most deleterious treatment for the two cultivars. The two genotypes exhibited similar *E* values, except for the NaCl treatment, with higher values recorded in TOG5307 than in TOG5949. The Cl^--dominant treatment had no impact on *C*i values while Na^+ -dominant and NaCl treatments significantly reduced *C*i values in the two genotypes. Stomatal conductance also decreased in the two cultivars according to $Cl^- > Na^+ >$ NaCl. The two genotypes presented similar g_s values, except under Na^+-dominant treatment where stomatal conductance was slightly higher in TOG5307 than in TOG5949.

The instantaneous water use efficiency (*A*/*E*; Fig. 6a) increased in response to Na^+-dominant and NaCl treatment but was not affected in Cl^--dominant treatment. In the presence of Na^+ excess or NaCl, WUE_i was higher in TOG5307 than in TOG5949. The A/C_i ratio (Fig. 6b) decreased in response to all ion toxicities, Cl^-dominant treatment being

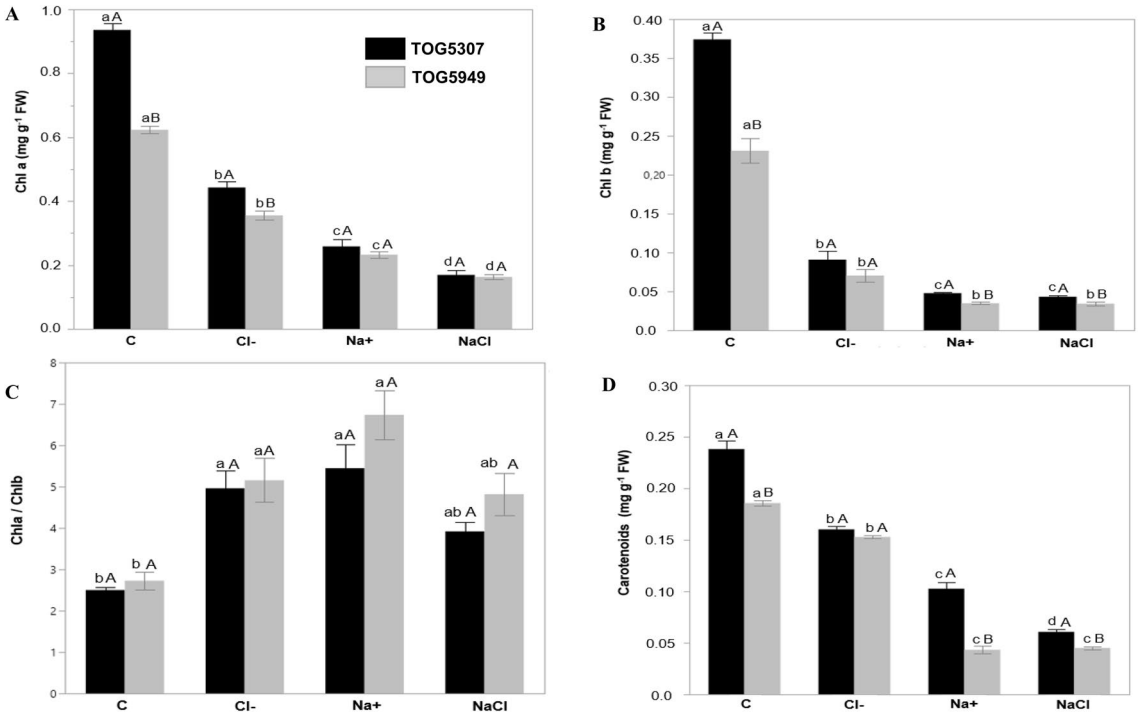

Fig. 4 **a** Chlorophyll a, **b** chlorophyll b, **c** *Chl* a/*Chl* b, and **d** carotenoids concentration of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 cultivated during 2 weeks in control conditions (C) or in the presence of 50 mM of either Cl^- (chloride dominant), Na^+ (sodium dominant), or NaCl. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments followed by the same lowercase letter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase latter letter in a particular condition do not differ statistically

the less damaging treatment while NaCl was the most deleterious one. In all cases except control, the A/C_i ratio was higher in TOG5307 than in TOG5949. In TOG5307, the A/g_s ratio remained constant in all treatments while it significantly decreased in TOG5949 in response to NaCl stress only (Fig. 6c). In TOG5307, the ETR/*A* ratio (Fig. 6d) increased in response to NaCl only; in the salt-sensitive TOG5949, it slightly increased in response to Cl^--dominant or Na^+-dominant treatments and strongly increased by 500% in the NaCl treatment. In all toxic treatments, the ETR/*A* ratio was higher in TOG5949 than in TOG5307.

Carbon and nitrogen isotope ratios and concentrations

Carbon isotope discrimination ($\Delta^{13}C$) (Table 4) was always significantly lower in TOG5949 comparatively to TOG5307, even in controls. The toxic ion-induced decrease in $\Delta^{13}C$ was similar in response to Cl^- - dominant, Na^+-dominant, and NaCl treatment in the salt-sensitive TOG5949. As far as salt-resistant TOG5307 is concerned, Na^+ toxicities (Na^+-dominant and NaCl) induced a lower $\Delta^{13}C$ value than Cl^--dominant treatment. Nitrogen isotope ratio ($\delta^{15}N$; Table 4) strongly decreased in response to Cl^--dominant solution and exhibited similar values in the two cultivars in response to this treatment. The $\delta^{15}N$ value recorded in response to the Na^+-dominant solution was higher than in response to Cl^- in TOG5307 while the difference between the two treatments was quite lower in TOG5949. In response to NaCl, the $\delta^{15}N$ value recorded for TOG5307 was lower than for TOG5949.

Elemental composition (Table 4) indicated that %C decreased in response to all ionic toxicities. The minimal value was recorded for Na^+ treatment in TOG5307 and Cl^- and NaCl treatments in TOG59949. In plants exposed to Na^+-dominant solution, the %C was higher in TOG5949 than in TOG5307 while an inverse trend was observed for all other treatments. The highest total N content was recorded in control plants. It was lower in Cl^--dominant and NaCl treatments than in Na^+-dominant solution in both cultivars. In the control and Na^+-dominant solution, TOG5949 contained more N than TOG5307 while an inverse trend was observed for Cl^--dominant and NaCl treatments.

Ion toxicity indexes

The ITI values are presented in Table 5. Ion toxicities were first determined for Na^+ and Cl^- accumulation in plants, which should be regarded as a direct consequence of plant exposure to high external concentrations of those ions which

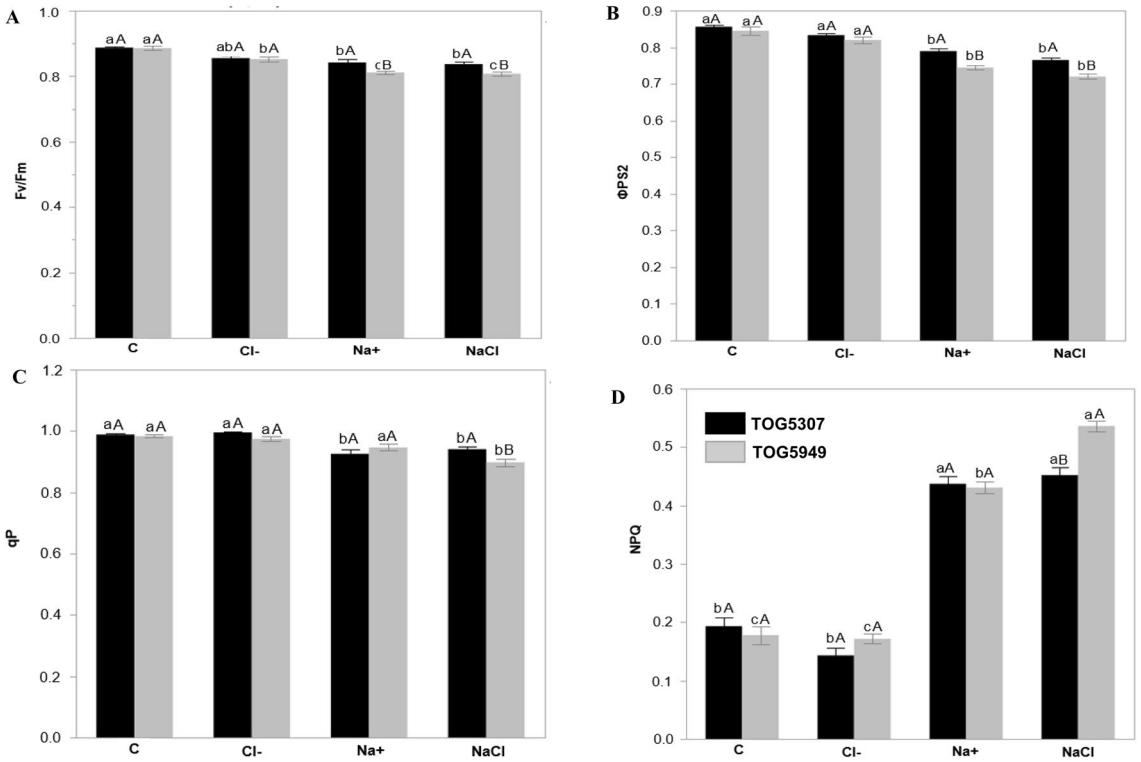

Fig. 5 **a** F_v/F_m, **b** Φ_{PS2}, **c** qP and **d** NPQ of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 (grey bars) cultivated during 2 weeks in control conditions (C) or in the presence of 50 mM of either Cl^- (chloride dominant), Na^+ (sodium dominant) or NaCl. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments followed by the same lowercase letter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a particular condition do not differ statistically

Table 3 Net carbon assimilation rate (*A*), instantaneous transpiration (*E*), intercellular CO_2 concentrations (C_i) and stomatal conductance (g_s) of African rice (*Oryza glaberrima* Steud.) seedlings cultivated during 2 weeks in control conditions or in the presence of 50 mM of either Cl^- (chloride dominant) or Na^+ (sodium dominant) or NaCl

Cultivars	Treatments	*A* (µmol CO_2 m^{-2} s^{-1})	*E* (mmol m^{-2} s^{-1})	C_i (µmol mol^{-1})	g_s (mmol H_2O m^{-2} s^{-1})
TOG5307	C	6.48 ± 0.12^{aA}	3.49 ± 0.05^{aA}	339 ± 4^{aA}	461 ± 6^{aA}
	Cl^-	5.58 ± 0.14^{bA}	3.20 ± 0.10^{bA}	342 ± 3^{aA}	334 ± 7^{bA}
	Na^+	4.07 ± 0.06^{cA}	1.56 ± 0.04^{cA}	320 ± 2^{bA}	249 ± 7^{cA}
	NaCl	2.51 ± 0.07^{dA}	0.86 ± 0.03^{dA}	315 ± 2^{bA}	162 ± 13^{dA}
TOG5949	C	6.42 ± 0.08^{aA}	3.45 ± 0.04^{aA}	348 ± 2^{aA}	454 ± 10^{aA}
	Cl^-	4.73 ± 0.13^{bB}	2.88 ± 0.07^{bA}	341 ± 3^{aA}	324 ± 6^{bA}
	Na^+	3.46 ± 0.08^{cB}	1.52 ± 0.05^{cA}	303 ± 2^{bB}	217 ± 9^{cB}
	NaCl	1.51 ± 0.08^{dB}	0.65 ± 0.02^{dB}	310 ± 2^{bA}	163 ± 7^{dA}

Treatments followed by the same lowercase latter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase latter in a particular condition do not differ statistically

are absorbed by the roots and progressively accumulated in the tissues. ITI_{Na} recorded for Na^+ accumulation in roots and shoots of plants exposed to Na^+-dominant and NaCl solutions were higher than ITI_{Cl} recorded for Cl^- accumulation in plants exposed to Cl^--dominant and NaCl solutions. ITI_{Na} for Na^+ accumulation was lower in TOG5307 than in TOG5949. ITI_{Cl} for root Cl^- accumulation was however higher in TOG5307 than in TOG5949 while an inverse trend was recorded for the shoot Cl^- concentration.

Besides Na^+ and Cl^- concentrations, other parameters were modified as a consequence of the accumulation of toxic ions (and external osmotic constraints which are supposed to be similar since all used nutrient solutions displayed similar Ψs values). Table 5 presents the ITI values for morphological

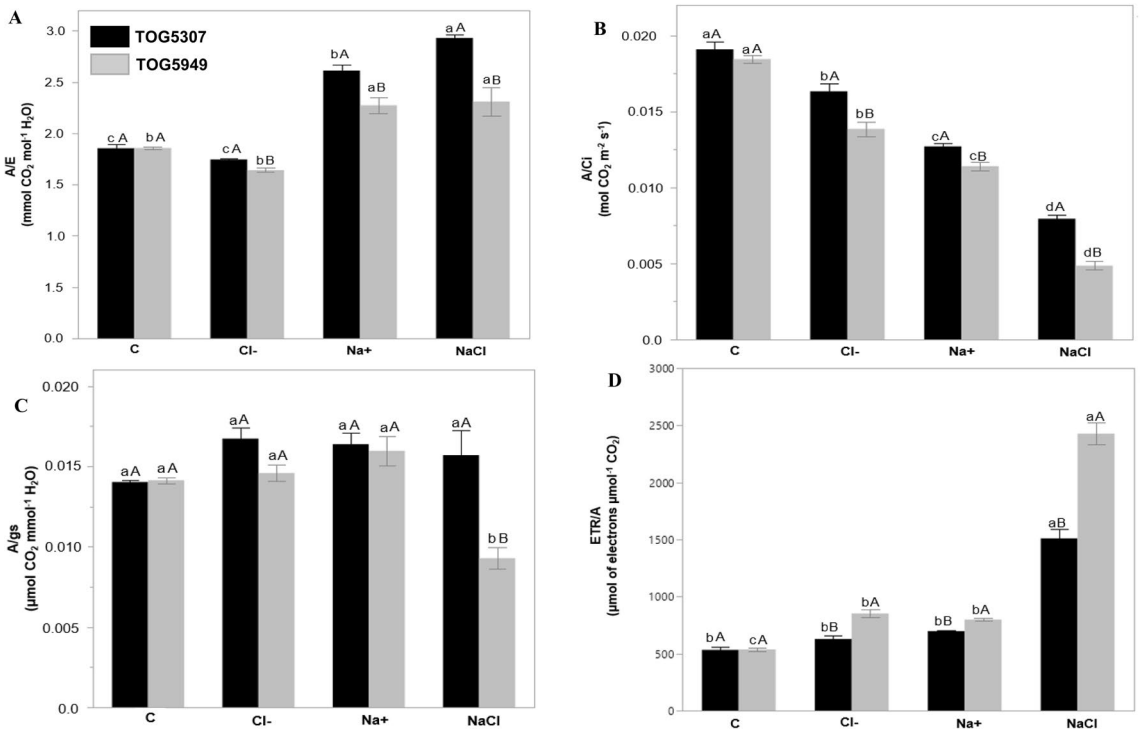

Fig. 6 **a** *A*/*E*, **b** *A*/*C*i, **c** A/g_s, and **d** ETR/*A* of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 (grey bars) cultivated during 2 weeks in control conditions (C) or in the presence of 50 mM of either Cl^- (chloride-dominant), Na^+ (sodium-dominant) or NaCl. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments followed by the same lowercase letter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a particular condition do not differ statistically

Table 4 $\Delta^{13}C$ $\delta^{15}N$, and C and N elemental content (%C and %N) of African rice (*Oryza glaberrima* Steud.) seedlings of cv TOG5307 (salt-resistant) and YOG5949 (salt-sensitive) cultivated during 2 weeks in control conditions or in the presence of 50 mM of either Cl^- (chloride dominant) or Na^+ (sodium dominant) or NaCl

Varieties	Treatments	$\Delta^{13}C$	$\Delta^{15}N$	%C	%N
TOG5307	C	27.24 ± 0.05^{aA}	-6.56 ± 0.06^{aA}	40.99 ± 0.13^{aA}	4.60 ± 0.03^{aB}
	Cl^-	25.87 ± 0.05^{bA}	-12.01 ± 0.06^{cA}	37.11 ± 0.13^{bA}	4.06 ± 0.03^{cA}
	Na^+	24.22 ± 0.05^{cA}	-9.21 ± 0.06^{bA}	35.66 ± 0.13^{cB}	4.39 ± 0.03^{bB}
	NaCl	24.46 ± 0.05^{cA}	-11.74 ± 0.06^{cB}	36.32 ± 0.13^{bA}	3.69 ± 0.03^{dA}
TOG5949	C	25.97 ± 0.05^{aB}	-7.71 ± 0.06^{aB}	40.11 ± 0.13^{aB}	4.86 ± 0.03^{aA}
	Cl^-	23.88 ± 0.05^{bB}	-12.05 ± 0.06^{dA}	36.67 ± 0.13^{cB}	3.81 ± 0.03^{cB}
	Na^+	24.67 ± 0.05^{bA}	-11.71 ± 0.06^{cB}	38.55 ± 0.13^{bA}	4.72 ± 0.03^{bA}
	NaCl	23.87 ± 0.05^{bB}	-10.78 ± 0.06^{bA}	35.35 ± 0.13^{cB}	3.55 ± 0.03^{dB}

Treatments followed by the same lowercase latter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase latter in a particular condition do not differ statistically

parameters, plant water status (including water use efficiency determined by $\Delta^{13}C$), photosynthetic parameters, nutritive parameters (C%, N%, as well as K and S concentration in roots and shoots), and biochemical parameters (flavonoids, total phenolics, MDA, $\delta^{15}N$, shoot proline, total soluble protein concentration).

For almost all recorded parameters, both ITI_{Na} and ITI_{Cl} were significantly higher in TOG5949 than in TOG5307: the major exceptions are the instantaneous water use efficiency (*A*/*E*), the carotenoid concentration, $\delta^{15}N$, NPQ and shoot Ψ_s for Cl^- toxicity as well as *A*/*E*, A/g_s, C%, N%, flavonoids, and total phenolics concentration and $\Delta^{13}C$ for Na^+ toxicity. As far as NaCl is concerned, the ITI_{NaCl} was lower in TOG5949 than in TOG 5307 for *A*/*E*, ETR values, flavonoids, and total phenolics concentration as well as $\delta^{15}N$ and root Ψ_s. In the two considered cultivars, ITI_{Na} was higher than ITI_{Cl}, confirming that Na^+ had higher impacts than Cl^- regarding most of the considered properties, as indicated by ITI_{Na}/ITI_{Cl} ratio higher than 1. One noticeable exception for TOG5949 was the concentration of the total phenolic

Table 5 Ion toxicity index (ITI) for plants of *Oryza glaberrima* Steud exposed to Na^+-dominant solution (ITI_{Na}), Cl^--dominant solution (ITI_{Cl}) or NaCl (50 mM) (ITI_{NaCl}). Plants of two distinct cultivars (TOG5949: salt-sensitive and TOG5307: salt-resistant) were exposed to ion toxicities for 2 weeks. For ITI_{NaCl}, a distinction was established between « observed » ITI and predicted ITI calculated on the basis of $ITI_{Na} + ITI_{Cl}$

Parameters	TOG5949					TOG5307				
	ITI_{Na}	ITI_{Cl}	ITI_{NaCl}obs	ITI_{NaCl}pred	ITI_{Na}/ITI_{Cl}	ITI_{Na}	ITI_{Cl}	TI_{NaCl}obs	ITI_{NaCl}pred	ITI_{Na}/ITI_{Cl}
Na^+ and Cl^-										
Root Na^+	23.67	0.09	47.40	23.77	263	11.55	0.36	28.19	11.91	32.08
Shoot Na^+	67.11	0.38	86.71	67.49	176	55.73	0.02	56.90	55.75	27.86
Root Cl^-	0.32	2.78	2.63	3.10	0.11	0.31	5.14	4.45	5.45	0.06
Shoot Cl^-	0.26	4.65	5.62	4.91	0.06	0.10	2.46	3.63	2.56	0.04
Morphology										
Root DW	0.82	0.63	0.89	1.45	1.30	0.75	0.47	0.82	1.22	1.60
Shoot DW	0.77	0.59	0.89	1.36	1.31	0.71	0.46	0.81	1.17	1.54
Root length	0.47	0.36	0.48	0.83	1.31	0.01	0.05	0.43	0.06	0.20
Shoot length	0.45	0.27	0.53	0.72	1.67	0.49	0.28	0.54	0.77	1.75
Root/Shoot	0.24	0.08	0.05	0.32	3.0	0.15	0.02	0.08	0.17	7.50
% DW root	0.76	0.63	0.79	1.39	1.21	0.62	0.36	0.73	0.98	1.72
% DW shoot	0.55	0.49	0.52	1.04	1.12	0.55	0.39	0.49	0.94	1.41
Water status										
Root WC	0.06	0.05	0.06	0.11	1.20	0.04	0.03	0.05	0.07	1.33
Shoot WC	0.13	0.12	0.13	0.25	1.08	0.13	0.09	0.12	0.22	1.44
g_s	0.52	0.29	0.64	0.81	1.79	0.46	0.28	0.65	0.74	1.64
E	0.56	0.17	0.81	0.73	3.29	0.55	0.08	0.75	0.63	6.88
Ψs root	0.15	0.12	0.05	0.27	1.25	0.23	0.08	0.1	0.31	2.88
Ψs shoot	0.43	0.24	0.82	0.67	1.79	0.52	0.42	0.99	0.94	1.24
$\Delta^{13}C$	0.05	0.08	0.08	0.13	0.63	0.11	0.05	0.1	0.16	2.20
Photosynthesis										
A	0.46	0.26	0.77	0.72	1.77	0.37	0.14	0.61	0.51	2.64
A/Ci	0.38	0.25	0.74	0.63	1.52	0.33	0.14	0.58	0.47	2.36
A/*E*	0.22	0.12	0.24	0.34	1.83	0.41	0.06	0.58	0.47	6.83
A/gs	0.13	0.03	0.34	0.16	4.33	0.17	0.19	.12	0.36	0.89
Carot.	0.77	0.18	0.76	0.95	4.28	0.57	0.33	0.74	0.90	1.73
Chla	0.63	0.43	0.74	1.06	1.47	0.72	0.53	0.82	1.25	1.36
Chlb	0.85	0.69	0.85	1.54	1.23	0.87	0.76	0.88	1.63	1.14
Chla/Chlb	1.47	0.89	0.77	2.36	1.65	1.17	0.98	0.57	2.15	1.19
C_i	0.13	0.02	0.11	0.15	6.50	0.06	0.01	0.07	0.07	6.0
ETR	0.2	0.17	0.06	0.37	1.18	0.18	0.01	0.01	0.19	18.0
ETR/*A*	0.49	0.59	3.53	1.08	0.83	0.31	0.18	1.84	0.49	1.72
Fv/Fm	0.08	0.04	0.09	0.12	2.0	0.05	0.04	0.06	0.09	1.25
NPQ	1.42	0.04	2.01	1.46	35.50	1.26	0.26	1.34	1.52	4.85
ΦPSII	0.12	0.03	0.15	0.15	4.0	0.08	0.03	0.11	0.11	2.67
qP	0.04	0.01	0.09	0.05	4.0	0.06	0.01	0.05	0.07	6.0
Nutrition										
Root K	0.9	0.37	0.88	1.27	2.43	0.92	0.43	0.91	1.35	2.14
Shoot K	0.62	0.09	0.75	0.71	6.89	0.55	0.05	0.51	0.6	11.0
Root S	0.42	0.6	1.59	1.02	0.57	0.36	0.15	0.31	0.51	2.40
Shoot S	1.12	0.35	5.62	1.47	3.20	0.17	0.07	4.52	0.24	4.28
C (%) Shoot	0.04	0.09	0.12	0.13	0.44	0.13	0.09	0.11	0.22	1.44
N (%) Shoot	0.03	0.22	0.27	0.25	0.14	0.04	0.12	0.2	0.16	0.33
Biochemsitry										
Proline	1.61	1.23	2.46	2.84	1.31	1.95	1.36	3.6	3.31	1.43

Table 5 (continued)

Parameters	TOG5949					TOG5307				
	ITI_{Na}	ITI_{Cl}	ITI_{NaCl}obs	ITI_{NaCl}pred	ITI_{Na}/ITI_{Cl}	ITI_{Na}	ITI_{Cl}	TI_{NaCl}obs	ITI_{NaCl}pred	ITI_{Na}/ITI_{Cl}
Flavonoids	1.25	0.78	1.73	2.03	1.60	2.06	0.61	2.92	2.67	3.38
Total phenolics	0.01	0.53	0.93	0.54	0.02	0.39	0.52	1.4	0.91	0.75
Leaf soluble sug.	0.08	0.01	0.19	0.18	0.80	0.02	0.01	0.11	0.03	2.0
$\delta^{15}N$	0.52	0.56	0.40	1.08	0.93	0.4	0.83	0.79	1.23	0.48
Leaf proteins	0.37	0.08	0.68	0.45	4.63	0.23	0.08	0.54	0.31	2.87

which was more affected by Cl^- than by Na^+ in this genotype. In TOG5307, a higher relative impact of Cl^- comparatively to Na^+ was recorded mainly for $\delta^{15}N$, root length, N%, and total phenolics. If we except Na^+ and Cl^- concentration in plants were exposed to NaCl, the highest ITI_{NaCl} value was recorded for shoot S concentration, suggesting that NaCl strongly modified the sulfur metabolism in both cultivars. Besides S concentration, the highest ITI_{NaCl} was found for ETR/*A* as well as for leaf MDA and proline concentrations in both cultivars.

If we do not consider Na^+ or Cl^- concentrations, a significant correlation (Fig. 7) was found between ITI_{Na} and ITI_{Cl}, which suggests that, from a global point of view, the parameters significantly affected by Na^+ were also affected by Cl^-, even if the recorded correlation was better for TOG5307 (Fig. 7a; $r^2 = 0.61$) than for TOG5949 (Fig. 7b; $r^2 = 0.49$). Similarly, ITI_{Na} for TOG5949 was significantly correlated to ITI_{Na} in TOG5307 (Fig. 7c; $r^2 = 0.65$) while ITI_{Cl} for TOG5949 was correlated to ITI_{Cl} in TOG5307 (Fig. 7d; $r^2 = 0.74$): this indicates that the pattern of modification induced by both types of toxicities was similar in the two studied cultivars which thus mainly differ for the quantitative aspect of the response rather than for the qualitative nature of the response.

One of the tested hypotheses was that the two considered toxic ions acted in an additive way and not in a synergistic or antagonist interaction. This implies that $ITI_{NaCl} = ITI_{Na} + ITI_{Cl}$ and this could be checked, for the two genotypes, by analyzing the observed ITI_{NaCl} and compare it with "predicted" ITI_{NaCl} corresponding to the sum ITI_{Na} +

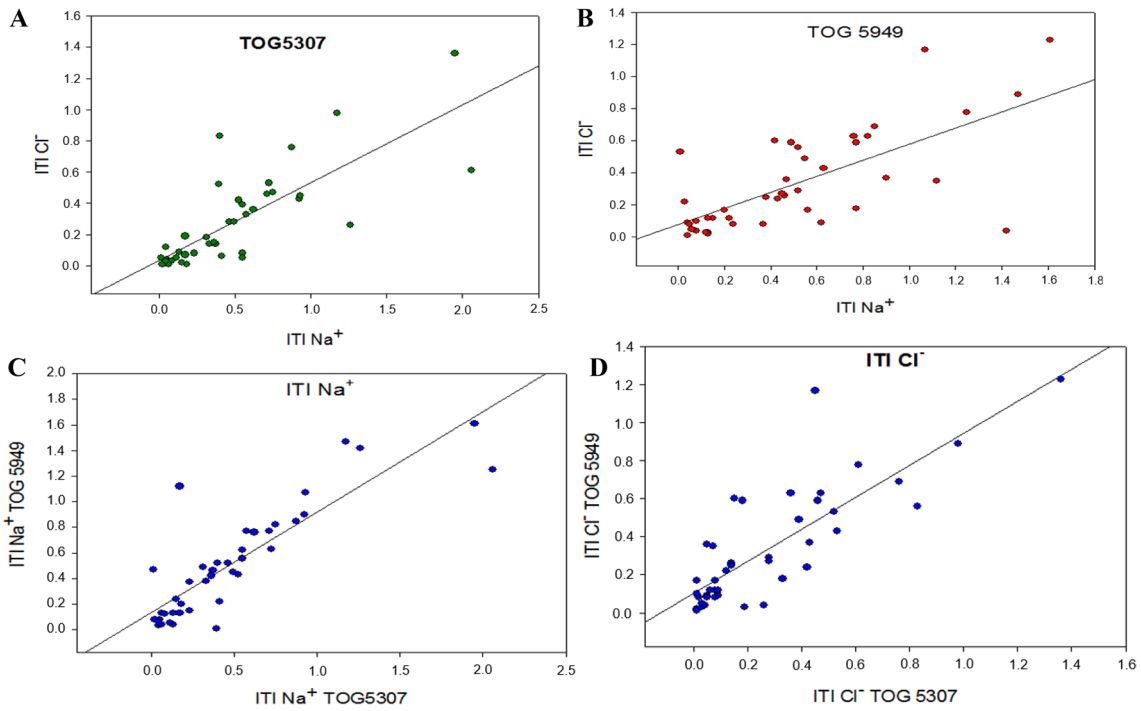

Fig. 7 Correlations of ion toxicity index (ITI) on **a** sodium and **b** chloride of African rice seedlings (*Oryza glaberrima* Steud.) from cv. **c** TOG5307 and **d** TOG5949 cultivated during 2 weeks in control conditions (C) or in the presence of 50 mM of either Cl^- (chloride dominant), Na+ (sodium dominant) or NaCl

ITI_{Cl} (Table 5). It is noteworthy that for root Na^+ accumulation in TOG5307 and root and shoot Na^+ accumulation in TOG5949, the observed ITI_{NaCl} was quite higher than the predicted ITI_{NaCl}. For Cl^- accumulation, the observed ITI_{NaCl} was lower than predicted ITI_{NaCl} for roots while an inverse trend was recorded for shoots. As far as morphological parameters are concerned, the observed ITI_{NaCl} was lower than the predicted ITI_{NaCl}, suggesting that the negative consequences of the two ions on plant growth were not strictly additive. For the other parameters, however, the additivity model was confirmed for all parameters in both genotypes according to Person chi-squared test using Yate's correction for continuity, except for ETR/*A* and shoot S concentration in both TOG5307 and TOG5949. In the two cases, the "observed" ITI_{NaCl} was significantly higher than the "predicted" ITI_{NaCl} and this suggests that for these two parameters (especially for shoot S concentration), the two toxic ions interact synergistically and that both ions Na^+ and Cl^- must accumulate in the plant to induce the recorded changes.

Discussion

Differences of salinity resistance between cultivars

Though the African rice species *Oryza glaberrima* displays a high level of intraspecific variability (Choi et al. 2019; Ishwara Lakshmi et al. 2019; Mayabe et al. 2020), it has rarely been characterized for its behaviour in the presence of NaCl. The present work demonstrates that the two tested cultivars exhibited contrasting levels of salinity resistance to salt stress: in the presence of 50 mM NaCl, TOG5307 accumulated lower concentrations of Na^+ in shoots and roots comparatively to TOG5949 while Cl^- content was lower in the shoots but higher in the roots of TOG5307. This suggests that efficient regulation of Na^+ absorption and Cl^- translocation from root to shoot may be involved in salinity resistance in *O. glaberrima*. Until recently, the physiological impact of Cl^- accumulation has often been neglected in studies devoted to salt stress but some data confirm that reduced net xylem loading of Cl^- contributes to salt stress resistance in cereal species (Teakle and Tyerman 2010).

The salt-induced decrease in K^+ concentration is a well-known process. It is commonly considered that Na^+ and K^+ ions present similar hydrated radii and that Na^+ is consequently absorbed by poorly selective K^+-transporters (Munns and Tester 2008; Morton et al. 2019). The salt-induced decrease in K^+ content was less marked in TOG5307 than in TOG5949. The overall consequence is that after 15 days of NaCl treatment, the K^+/Na^+ ratio, which is often considered as the major criteria conditioning plant response to NaCl (Almansouri et al. 1999; Lefèvre et al. 2001; Roshandel and Flowers 2009; Ganie et al. 2019) decreased to 0.10 in TOG5949 but remained at 0.33 in TOG5307. This better $K^+ >< Na^+$ discrimination in TOG5307 may at least partly explain the better photosynthetic behaviour recorded in NaCl-treated plants of TOG5307 in terms of *A* (Table 3) and *A*/*C*i (Fig. 6), as well as the lower oxidative stress as suggested by the lower leaf concentration of MDA (Fig. 3).

Ionic versus osmotic stress

Salinity is a complex environmental constraint that exhibits a water stress component resulting from a decrease in the external osmotic potential. According to the biphasic model (Munns et al. 1995; Munns et al. 2020), this water stress component induced by external ions (phase I) occurs before any ion-specific impact which requires the progressive build-up of toxic ion within plant tissues (phase II). It may thus be expected that salt-resistant cultivars more efficiently regulate their plant water status than salt-sensitive ones in relation to stomatal closure allowing to reduce water losses by transpiration and/or to osmotic adjustment contributing to maintain a favourable water potential gradient for water absorption (Lutts et al. 1999; Morton et al. 2019; Munns et al. 2020). The two considered cultivars, however, did not strongly differ for plant water status-related parameters: both shoot WC and leaf g_s were similar in NaCl-treated plants of TOG5307 and TOG5949. It has been demonstrated that the first phase is rather short-lived in rice in comparison to other plant species because of the rapid build-up of toxic ions in rice shoot (Yeo et al. 1991; Lefèvre et al. 2001; Roshandel and Flowers 2009) so that the contrasting behaviour of TOG5307 and TOG5949 could be due to different rates of ion accumulation and to different tolerance mechanisms adopted by the two cultivars to cope with internal accumulated Na^+ and Cl^- rather than the capacity of water status regulation.

In *Oryza sativa*, Wang et al. (2018) considered that stomatal limitation of photosynthesis mainly reflects the osmotic component of salinity but our data on *O. glaberrima* provide a different picture since for similar external Ψs, the g_s value was the lowest in NaCl-treated plants and the highest in Cl^--treated ones, demonstrating that the ionic component plays a key role in the decrease of stomatal conductance in African rice.

Na^+ versus Cl^- toxicity

The ionic component of salt stress is itself a complex environmental constraint since it is related to Na^+ and Cl^- accumulation. Most studies consider Na^+ impact while Cl^- effects were rarely considered until recent years. There is no proof however that the two ions act on similar physiological targets and in the same manner. In order to distinguish the Na^+ and the Cl^- effects, the present study compares

the impact of NaCl solution with the impact of Na^+ or Cl^--enriched solution, as recommended in previous studies (Tavakkoli et al. 2010, 2011; Khare et al. 2015, 2020). This method inevitably led to differences in the concentration of balancing ions. It has to be mentioned that Na^+-dominant solution did not induce S or N over-accumulation despite the use of sulfate or nitrate salts in this solution. This suggests that the accumulation of essential elements used as balancing ions was rather limited compared to the accumulation of the tested toxic ions, confirming the results of Tavakkoli et al. (2010, 2011). Similarly, the Cl^--dominant solution surprisingly induced a decrease in the root K^+ concentration in both cultivars, despite a high concentration of KCl in the external medium. This suggests that Cl^- may impair K^+ transporters involved in root absorption, even in the absence of Na^+. Possible targets for Cl^-/K^+ interaction are members of the cation chloride co-transporter (CCC) family which may be involved in K^+/Cl^- symport and an excess of Cl^- may trigger transcriptional downregulation of some of those transporters, thus leading to a decrease in K^+ absorption (Wu and Li 2019). An impact of Cl^- excess in rice on K^+-inward channels (OsAKTs) and high-affinity K^+ transporters (OsHAKs) (Yong et al. 2020) could also not be ruled out and requires further investigations. Because a similar process occurred in two cultivars that accumulated different amounts of Na^+, it is tempting to speculate that NaCl-induced decrease in K^+ concentration was not necessarily only the consequence of $K^+ >< Na^+$ competition. From a quantitative point of view, NaCl-treated plants accumulated Na^+ and Cl^- in similar ranges of concentration but it has to be mentioned that Na^+ accumulation in roots and shoots of TOG5949 was higher in plants exposed to NaCl than in those exposed to Na^+-dominant solution. This suggests that Cl^- accumulation may increase Na^+ uptake (while it was previously postulated that it decreased K^+ uptake!).

As an essential element, Cl^- may be absorbed by a complex array of passive and active transporters and both high and low-affinity systems occur (Teakle and Tyerman 2010). As far as the shoot is concerned, Cl^- also accumulated to a higher extent in NaCl-treated plants than in plants exposed to Cl^--dominant solution. The fact that $ITI_{NaClobs}$ was higher than $ITINaCl_{pred}$ supports the hypothesis that Na^+ and Cl^- may synergistically influence the absorption of each other and a deeper analysis of membrane transporters behavior could provide useful information on the molecular basis of these processes. Besides the quantitative aspects of ion accumulation at the whole organ level, ion distribution, and especially vacuolar compartmentation, is an important component of salinity resistance in plants (Munns et al. 1995, Munns and Tester 2008). This is especially valid for Na^+ which is toxic for the cytosolic enzyme, even at very low concentration, and only very few species such as *Theobroma cacao* can partially replace a small proportion of K^+ with Na^+ for metabolic functions (Gattward et al. 2012).

Synergistic interaction between Na^+ and Cl^- was noticed in *O. glaberrrima* for S accumulation in the shoot (Fig. 2). This accumulation indeed occurred in response to NaCl but not in response to Na^+-dominant or Cl^--dominant solution: $ITINaCl_{obs}$ culminated at 5.62 and 4.52 in TOG5949 and TOG5307, respectively, while $ITI_{NaClpred}$ was only 1.42 in the former and 0.24 in the latter. Hence, NaCl-treated plants have a specific physiological status triggering S accumulation only when both toxic ions are simultaneously present. Salt-induced increase in sulfur has been reported for mungbean (Hussain et al. 2019) or sorghum (de Andrade et al. 2018) while NaCl had no impact on S content in onion (Aghajanzadeh et al. 2019). In *Oryza sativa*, Irakoze et al. (2019) did not detect a NaCl-induced increase in S content. Khare et al. (2015) reported that oxidative stress culminated in response to NaCl, comparatively to Na^+ or Cl^- enriched treatments and the salt-induced increase in S absorption reflect an increased demand in this element for the synthesis of glutathione involved in ROS quenching (Hussain et al. 2019; Nazar et al. 2011). Another synergistic interaction between Na^+ and Cl^- ions was reported by Martin and Koebner (1995) but concerns chlorophyll-fluorescence-related parameter and not mineral nutrition.

The ion toxicity index (Table 5) quantified the relative changes for each parameter when plants were exposed to NaCl, to Na^+-dominant solution, or Cl^--dominant solution comparatively to plants cultivated on control nutrient solution in the absence of ion toxicity. It does provide information regarding the quantitative importance of a modification but it does not specify if the recorded modification should be considered as a symptom of injury (as it was the case for *A* decrease or MDA accumulation (Abdelaal et al. 2020a) or as a strategy of resistance (as it was the case for the synthesis of the osmoprotectant osmolytes proline and soluble sugars (Abdelaal et al. 2020b) or the non-enzymatic antioxidant polyphenol). Our data on *O. glaberrima* indicate that i) the behaviour of TOG5949 was more affected than the behaviour of TOG5307 for most parameters and all types of ion toxicities ii) that Na^+ was usually more toxic than Cl^- and iii) the highest toxicity was recorded for NaCl. These data corroborate the results obtained by Kumar and Khare (2016) and Khare et al. (2015, 2020) on *O. sativa* and Tavakkoli et al. (2011) in barley.

The positive correlation recorded between ITI_{Na} and ITI_{Cl} for both cultivars indicates that the two ions act similarly on similar parameters. Hence, if we do not consider mineral content, the effect of Na^+ and Cl^- are rarely « ion-specific » *sensu stricto*. Since Cl^- accumulated to the same range of concentration as Na^+, the fact that ITI_{Cl^-} was usually lower than ITI_{Na} demonstrated that Cl^- had a lower toxicity level. Except for mineral properties (see above) for which

synergistic interaction may occur, and for morphological-related parameters for which antagonist interaction may be observed, pure additivity was commonly observed and ITI_{NaCl} correspond to $ITI_{Na} + ITI_{Cl}$: it thus implies that each ion did not affect the recorded parameter at a « saturation » level, or that the two ions act on distinct targets for the same parameter.

Ion toxicity and photosynthesis

Chloride assumes key functions in the regulation of photosynthesis (Dukic et al. 2019) but its over-accumulation in chloroplasts has been reported to specifically alter photosynthetic processes through non-stomatal effects (chlorophyll degradation and reduction of the actual quantum yield of PSII electron transport), while Na^+ mainly acts on stomatal conductance (Tavakkoli et al. 2010, 2011). Our data only partly corroborate this view: in both cultivars, g_s was indeed more reduced by Na^+-dominant and NaCl treatments than by Cl^--treatments (Table 3) but chlorophyll content also appeared more affected by Na^+ and NaCl toxicity; Φ_{PSII}, qP, and NPQ remained unaffected by Cl^--treatment, although Na^+ and NaCl reduced F_v/F_m, qP and Φ_{PSII} and drastically increased NPQ. Hence, in *O. glaberrima*, Na^+-induced inhibition of photosynthesis may be due to both stomatal and non-stomatal causes.

A decrease in C_i leading to a decrease in C_i/C_a in response to Na^+ and to NaCl treatments causes loss of PSII efficiency by reducing CO_2 availability for photosynthesis and will depress the amounts of electron-accepting $NADP^+$ as the carbon reduction cycle slows. Similarly, an increased NPQ may result from the fact that a decrease in CO_2 assimilation reduces the demand for the products of electron transport, thus increasing the dissipation of light energy. According to Wang et al. (2018), *A* values in salt-stressed rice may also be affected by mesophyll conductance, by the capacity of electron transport, and by RuBP regeneration. The ETR/*A* ratio remained unaffected in response to Na^+ and Cl^- toxicity and increased in response to NaCl only. Electron transport rate did not appear as a limiting factor in salt-treated *O. glaberrima* and the recorded increase in ETR/*A* indicates that alternative sinks (Mehler reaction, photorespiration, …) may replace photosynthesis for electron transfer.

Isotope discrimination as a tool to compare Cl^- and Na^+ toxicities

In contrast to instantaneous water use efficiency (*A*/*E*) and to intrinsic water use efficiency (A/g_s) which should be regarded as WUE at the time of measurement, $\Delta^{13}C$ provides pertinent information regarding the plant behaviour during the whole period from stress imposition to final harvest (Xu et al. 2007; Gao et al. 2018). In Cl^- dominant treatment, the *A*/*E* ratio did not increase but $\Delta^{13}C$ was significantly reduced, as was the case for g_s values. The fact that C_i was unaffected in those plants despite a significant decrease in g_s suggests that mesophyll conductance and/or Rubisco activities were affected to some extent. According to Gouveia et al. (2019), however, $\Delta^{13}C$ does not automatically show a significant association with water use efficiency.

The two considered cultivars differed for $\Delta^{13}C$ values in control conditions, reflecting an intraspecific variability already noticed for *O. sativa* (Shaheen and Hood-Nowotny 2005; Gao et al. 2018) and other cereals (Xu et al. 2007). It is interesting to notice that $\Delta^{13}C$ in TOG5307 remained higher in Cl^--treated plants than in those exposed to Na^+ toxicity while an opposite trend was observed for TOG5949. After 15 days of treatment, however, C_i and g_s were higher in response to Cl^- than in response to Na^+ for both cultivars. The kinetics of stress development is extremely important for plants and maybe a direct function of the rate of toxic ion accumulation (Almansouri et al. 1999). In the present work, the measure performed at the end of stress exposure did not fully reflect the kinetics of stress development on a time-scale basis and the very low $\Delta^{13}C$ values recorded in Cl^--treated plants of TOG5949 might be explained by a faster accumulation of Cl^- in this cultivar. The significant positive correlation ($r^2 = 0.64$) between $\Delta^{13}C$ and g_s supports the hypothesis that stomatal conductance was not affected according to similar kinetics in the two tested cultivars.

According to Yousfi et al. (2009), $\delta^{15}N$ is even more directly related to salinity resistance than $\Delta^{13}C$. It however appears as a complex parameter influenced by a myriad of factors such as uptake, efflux, enzyme activities involved in N assimilation, N release as NO and NH_3 at the leaf level, and reallocation between organs (Evans 2001; Saud et al. 2020). In both genotypes, $\delta^{15}N$ exhibited minimal values in response to Cl^- treatment. Chloride reduces NO_3^- absorption (Britto et al. 2004) and total N content in the shoot in *O. glaberrima* was more reduced by Cl^- than by Na^+ toxicity (Table 4). In the present study, N is mainly afforded as NH_4NO_3 and the $\delta^{15}N$ value of the used salt was -2.0 ± 0.1 ‰. It is however not possible to determine if NO_3^- and NH_4^+ ions contained similar proportions of ^{15}N. It is frequently considered that discrimination is higher when nitrate constitutes the main source of nitrogen and that NH_4^+ transporters poorly discriminate between ^{15}N and ^{14}N (Evans 2001). In the present study, $\delta^{15}N$ was determined for the shoot part only, while N assimilation also occurs at the root level (especially for NH_4^+) and discrimination occurring in the roots strongly influence the proportion of ^{15}N reaching photosynthetic tissues

Conclusions

It is concluded that the differential impact of NaCl on two African rice (*Oryza glaberrima*) cultivars is mainly related to the ionic rather than to the osmotic component of salinity. Sodium appeared more toxic than Cl^- on a wide range of parameters, but act on the same targets, although with a different quantitative impact. In most cases, Na^+ and Cl^- acted in an additive way, although an antagonist effect was suggested for some morphological parameters and an additive effect for shoot S concentration. Isotope discrimination data suggest that the time-course of stress development at the plant level might be an important aspect for understanding plant behaviour.

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Author contributions HP performed the experiment and analyzed the data; WI helps with the statistical treatment, GL performed the isotope discrimination analysis; SL and CG conceived the experiment and managed the research; SL and HP wrote the first draft of the manuscript. All the authors prepared the final version and approved submission

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Declarations

Conflict of interest The authors declare no conflict of interest.

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