# *Oryza glaberrima* response strategies to salinity : distinction between osmotic and ionic components

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A thesis submitted in fulfilment of the requirements for the degree of **Doctor of Philosophy (PhD) in Sciences**

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# Dédicace

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*Mieux vaut vivre un jour comme un lion que cent ans comme un mouton.*

Proverbe italien

*Croque la vie à pleines dents !*

Hermann PRODJINOTO

# List of achievements

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1. **Prodjinoto, H.**, Irakoze, W., Gandonou, C., & Lutts, S. (2021). *Inhibitors of Na+/H+ antiporter and cation-chloride cotransporters have contrasting effects on two cultivars of Oryza glaberrima Steud. differing in salinity resistance. Journal of Soil Science and Plant Nutrition.* https://doi.org/10.1007/s42729-021-00603-z
2. **Prodjinoto, H.**, Irakoze, W., Gandonou, C., Lepoint, G., & Lutts, S. (2021). *Discriminating the impact of Na+ and Cl− in the deleterious effects of salt stress on the African rice species (Oryza glaberrima Steud.). Plant Growth Regulation, 1-19. https://doi.org/10.1007/s10725-021-00709-5*
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3. Using genetic resource data for breeding. The G2P-SOL Advanced Training School II (2021).
4. Plant Session: Exploiting the natural genetic variation in photosynthesis and CO2 assimilation. SEB 2021 Annual Conference.
5. Plant Science for Climate Emergency (PSCE) and IPBO Satellite - "Applied Climate Research in Africa" (2021).

6. ELI-day (2021). Earth & Life Institute. UCLouvain.
7. 1st International Electronic Conference on Agronomy (IECAG2021). Session: Sustainable Management Practices for Soil Health and Food Security (2021).
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9. XVIIIe édition des Journées Scientifiques Internationales de Lomé (JSIL 2018) sous le thème: "L'Afrique face aux ODD: quelles recherche et innovation pour un développement durable?", Campus universitaire de Lomé – Togo.
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# List of abbreviations

| | |
|---|---|
| $\Psi_S$ : | Osmotic potential |
| AFLP : | Amplified Fragment Length Polymorphism |
| AKT : | Arabidopsis $K^+$ transporters |
| ANOVA : | Analysis of variance |
| AOAD : | Antioxidant activity measured in dichloromethane extract |
| AOAM : | Antioxidant activity measured in methanol extract |
| CAPS : | Cleaved Amplified Polymorphic Sequences |
| CCC : | Cation/chloride cotransporter |
| cDNA : | Complementary deoxyribonucleic acid |
| CEC : | Cation Exchange Capacity |
| Chla : | Chlorophyll a |
| Chlb : | Chlorophyll b |
| CHX : | Cation $H^+$ exchanger |
| CLC : | Chloride channels |
| *cv* : | Cultivar |
| DNA : | Deoxyribonucleic acid |
| DW : | Dry weight |
| EC : | Electrical Conductivity |
| EF1α : | Elongating factor 1 alpha |
| ESP : | Exchangeable sodium percentage |
| ETR : | Electron transport rate |
| EL : | Electrolyte leakage (in %) |
| FAO : | Food and Agriculture Organization |
| FAOSTAT : | Food and Agriculture Organization Statistics |
| FW : | Fresh weight |
| $g_s$ : | Stomatal conductance |
| HAK : | High affinity $K^+$ uptake transporter |
| HKT : | High-affinity $K^+$ transporters |
| HPLC : | High-Performance Liquid Chromatography |
| HSD: | Honestly Significance Difference |
| IAEA : | International Atomic Energy Agency (Vienna, Austria) |
| ITI : | Ion toxicity index |
| LCT : | Low affinity cation transporter |
| LR : | Leaching requirement |
| MDA : | Malondialdehyde |

| | |
|---|---|
| mRNA : | Messenger ribonucleic acid |
| NaCl : | Sodium chloride |
| NHA : | $Na^+/H^+$ antiporter (SOS1) |
| NHX : | $Na^+/H^+$ exchanger |
| NSCC : | Nonselective cation channels |
| NRT : | Nitrate transporter |
| PCR : | Polymerase Chain Reaction |
| PEG : | Polyethylene glycol |
| pH : | Hydrogen potential |
| PIP2 : | Plasma membrane intrinsic protein (aquaporin) |
| PSII : | Photosystem II |
| qPCR : | Quantitative Polymerase Chain Reaction |
| QTL : | Quantitative Trait Loci |
| RAD-Seq : | Restriction site-associated DNA sequencing |
| RAPD : | Random Amplified Polymorphic DNA |
| RFLP : | Restriction fragment length polymorphism |
| RNA : | Ribonucleic acid |
| ROS : | Reactive Oxygen Species |
| RT-PCR : | Reverse Transcriptase- Polymerase Chain Reaction |
| SAR : | Sodium adsorption ratio |
| SIR : | Stable isotope ratio |
| SOS : | Salt Overly Sensitive |
| SSR : | Simple Sequence Repeat |
| TDS : | Total dissolved solutes |
| TSS : | Total soluble salts |
| UAC : | Université d'Abomey-Calavi |
| VNTR : | Variable Number Tandem Repeat |
| WC : | Water content |

# Table of contents

**LIST OF ACHIEVEMENTS** ..... **II**

**LIST OF ABBREVIATIONS** ..... **VI**

**TABLE OF CONTENTS** ..... **VIII**

**GENERAL INTRODUCTION AND OBJECTIVES** ..... **1**

**BIBLIOGRAPHIC REVIEW** ..... **9**

1.1 SALINITY ..... 9

*1.1.1 Definition and classification* ..... *9*

*1.1.2 Salinity assessment and distribution* ..... *13*

*1.1.3 Causes of salinity* ..... *15*

*1.1.4 Consequences of salinity* ..... *16*

*1.1.5 Management and reclamation of salt-affected soils* ..... *18*

1.1.5.1 Mechanical method ..... 18

1.1.5.2 Chemical methods ..... 19

1.1.5.3 Biological methods ..... 20

1.2 EFFECT OF SALT STRESS ON PLANTS ..... 22

*1.2.1 Notion of stress: concepts and definitions* ..... *22*

*1.2.2 Salt stress: two contrasts in the world' flora* ..... *23*

*1.2.3 Components of salt stress* ..... *24*

1.2.3.1 Osmotic component ..... 25

1.2.3.2 Ionic component ..... 27

1.2.3.2.1 Sodium ..... 28

1.2.3.2.2 Chloride ..... 31

*1.2.4 Effect of salinity on plasma membrane and some organelles of cell* ..... *33*

*1.2.5 Plant-holistic responses to salt stress* ..... *34*

1.2.5.1 Morpho-agronomic adaptations to salinity ..... 35

1.2.5.2 Impact of salt stress on photosynthesis ..... 37

1.2.5.3 Impact of salt stress on water status, osmotic adjustment and dehydration tolerance ..... 38

1.2.5.3.1 Desiccation ..... 40

1.2.5.4 Salt stress and oxidative stress ..... 40

1.2.5.5 Salt stress and hormonal status ..... 42

1.3 RICE PLANT ..... 45

*1.3.1 Productivity and economic importance in Benin* ..... *45*

*1.3.2 Major Rice Production Systems in Africa* ..... *46*

*1.3.3 Genus Oryza* ..... *47*

*1.3.4 Oryza sativa L.* ..... *50*
*1.3.5 Oryza glaberrima Steud.* ..... *50*
1.3.5.1 Origin and distribution ..... 50
1.3.5.2 Description ..... 51
1.3.5.3 Resistance to biotic and abiotic stresses ..... 54
1.3.5.4 Cultural and nutritional importance ..... 56
1.3.5.5 Interest of *Oryza glaberrima* for improving rice ..... 57
*1.3.6 NERICA: An introgression of Oryza glaberrima Steud. into Oryza sativa L* ..... *60*
1.4 BIBLIOGRAPHIC REFERENCE ..... 61

**CHAPTER I. SCREENING FOR SALINITY TOLERANCE AT THE YOUNG SEEDLING STAGE IN *ORYZA GLABERRIMA* STEUD.: A MORPHOPHYSIOLOGICAL APPROACH ..... 89**

ABSTRACT: ..... 90
INTRODUCTION ..... 91
MATERIAL AND METHODS ..... 93
RESULTS ..... 96
DISCUSSION ..... 111
REFERENCES ..... 115

**CHAPTER II. IMPACT OF SALINITY ON YIELD-RELATED PARAMETERS OF *ORYZA GLABERRIMA* ..... 125**

ABSTRACT ..... 126
INTRODUCTION ..... 127
MATERIAL AND METHODS ..... 128
RESULTS ..... 133
DISCUSSION ..... 138
REFERENCES ..... 140

**CHAPTER III. DISCRIMINATING THE IMPACT OF $NA^+$ AND $CL^-$ IN THE DELETERIOUS EFFECTS OF SALT STRESS ON THE AFRICAN RICE SPECIES (*ORYZA GLABERRIMA* STEUD.). ..... 149**

ABSTRACT ..... 150
INTRODUCTION ..... 151
MATERIAL AND METHODS ..... 153
RESULTS ..... 159
DISCUSSION ..... 180
CONCLUSIONS ..... 187
REFERENCES ..... 188

**CHAPTER IV. INHIBITORS OF $NA^+/H^+$ ANTIPORTER AND CATION-CHLORIDE-COTRANSPORTER HAVE CONTRASTING EFFECTS ON TWO CULTIVARS OF *ORYZA GLABERRIMA* STEUD. DIFFERING IN SALINITY RESISTANCE. ..... 201**

ABSTRACT ..... 202
INTRODUCTION ..... 203
MATERIAL AND METHODS ..... 204
RESULTS ..... 206
DISCUSSION ..... 209
CONCLUSIONS ..... 214
REFERENCES ..... 215

**CHAPTER V. COMPARISON OF THE EFFECTS OF WATER STRESS, SALINITY AND MIXED TREATMENT (WATER STRESS + SALINITY) ON THE PHYSIOLOGICAL BEHAVIOR OF TWO CULTIVARS OF THE AFRICAN RICE (*ORYZA GLABERRIMA* STEUD.) DIFFERING IN SALINITY RESISTANCE. ..... 223**

ABSTRACT ..... 224
INTRODUCTION ..... 225
MATERIAL AND METHODS ..... 227
RESULTS ..... 234
DISCUSSION ..... 255
CONCLUSION ..... 260
REFERENCES ..... 261

**CHAPTER VI. COMPARISON OF THE IMPACT OF OSMOTIC, NACL AND MIX TREATMENTS ON THE EXPRESSION OF GENES CODING FOR ION TRANSPORTERS IN THE AFRICAN RICE SPECIES *ORYZA GLABERRIMA* STEUD. ..... 277**

ABSTRACT ..... 278
INTRODUCTION ..... 279
MATERIAL AND METHODS ..... 281
RESULTS ..... 288
DISCUSSION ..... 305
REFERENCES ..... 310

**GENERAL DISCUSSION ..... 321**

**CONCLUSIONS AND PERSPECTIVES ..... 341**

# General introduction and objectives

Major goal for the next decades are to eliminate poverty in the world, end hunger, achieve food security, improve nutrition and promote sustainable agriculture (Caron and Châtaigner, 2017). In fact, the Global Hunger Index (GHI) shows a score of 20.0 in 2019. This means that the global level of hunger and undernutrition is on the borderline of the moderate and severe categories. For example, 13.1% of the population is undernourished while more than 25% of children under five years old are hungry (Nigam, 2019). The human population is expected to reach 10 billion by 2050. As consequence, compared to 2005/07 demand, the world food supply would have to increase up to 70% by 2050s (Alexandratos and Bruinsma, 2012; Grafton et al., 2015). This is a real challenge, because the areas likely to be cultivated under optimal conditions are already being exploited. The only solution, then, is to use marginal lands that are not (or not efficiently) cultivated today. Marginal lands are often characterized by degraded soils, which are the consequences of several environmental constraints among which salinity is especially important. The rehabilitation and the exploitation of these salt-affected soils would thus be useful tools to increase food supply and to combat desertification, halting and reversing the soil degradation process (response to 15$^{th}$ SDG).

Salinity is one of the major environmental constraints that greatly reduces crop yields in many regions of the world. The proportion of soils that cannot be cultivated due to excessive salinity is increasing each year in part due to climate change and to unadapted irrigation practices. This is especially the case in arid or semi-arid areas, where the use of poor quality water combined with significant evapotranspiration causes the accumulation of salts in the soils. Recent statistics show that global salt-affected soils went from 915.5 Mha in 1986 to 1069.3 Mha in 2016 (Ivushkin et al., 2019). While salinization affects currently 7% of the world's total land area, 20% of word's cultivated lands, and 33% of world's irrigated lands are prone to salinization and many researches are predicting that 50% of the world's arable lands will become salt-affected by 2050s (Butcher et al., 2016; Cuevas et al., 2019). The problem is all the more urgent as only 1% of the world flora consists in halophytes plant species able to cope with high concentration of salt. The remaining 99%, called "glycophytes species" are sensitive to salt stress and are unable to achieve their whole development cycle in the presence of salinity. This is why

the FAO has rightly chosen " **Halt soil salinization, boost soil productivity**" as the theme for the celebration of World Soil Day 2021 (WSD-2021). However, salinity is a complex constraint that has two major components: osmotic and ionic constraints (Munns and Tester, 2008). Osmotic component is related to a decrease in the external water potential which compromises plant water absorption. In fact, the high build-up of salt ions in the soil solution makes it harder for the roots to extract water. Ionic component is related to the accumulation of toxic ions within the plant, which can be detrimental to cell metabolism. Depending on the intensity of the salt stress, germination, growth, and yield of glycophytes are severely impacted. All cultivated crops (except quinoa and amaranthus) behave as typical glycophytes.

Among cereals, rice exhibits a high level of salt-sensitivity at the seedling and the flowering stages (Hoang et al., 2016; Lutts et al., 1995). Rice is a major source of nutrition for about two-thirds of mankind (Vaughan et al., 2003). It is an energy food of good nutritional value with a predominance of carbohydrate. Rice is cultivated in 114 of the world's 193 countries on six continents. Only two species of rice are cultivated: the Asian species, *Oryza sativa* is by far the most cultivated one. The African rice species, *Oryza glaberrima* was independently domesticated 3000 years ago from the wild rice progenitor *O. barthii*. While *Oryza sativa* is renowned for its high yields, it is however very sensitive to environmental stresses. On the other hand, *Oryza glaberrima* gives a lower yield but resists and tolerates several biotic and abiotic constraints. As consequence, African rice now appears as a promising source of readily available genetic diversity for rice improvement (Kang et Futakuchi, 2019; Ndjiondjop et al., 2018). As far as salinity is concerned, however, *O. glaberrima* received only little attention. Recently, data started to become available for $Na^+$ exclusion (Platten et al., 2013) or microRNA involved in biological pathways of salinity tolerance (Mondal et al., 2018). To our knowledge, no study has really looked into the identification and the selection of salinity tolerant varieties of *Oryza glaberrima*. In addition, there is no information on how the ionic and osmotic components of salinity impact African rice. Understanding the mechanisms responsible for the salinity resistance of plants however requires to precisely identify the impact of the two components as well as their interaction on the plant physiology.

**The general objective of our thesis is therefore to highlight the response strategies of *Oryza glaberrima* to salinity while clearly distinguishing the**

**effect of the osmotic component and that of the ionic component of salt stress on cultivars differing for their mean level of salt resistance.**

To respond to this general objective, this thesis intends to provide an answer to the four specific following questions:

1. Is it possible to identify a cultivar exhibiting similar level of salinity resistance at the vegetative seedling stage on the one hand, and at the reproductive stage on the other hand?

2. What are the respective impacts of $Na^+$ and $Cl^-$ of salt stress on *Oryza glaberrima* Steud's and do these ions act on similar physiological parameters?

3. What are the impacts of the osmotic component of salt stress on *Oryza glaberrima* Steud salt tolerance comparatively to the ionic component and is the interaction between these two components similar in a salt-sensitive and in a salt-resistant cultivar?

4. What is the effect of salt on the expression of genes coding for ion transporters in two contrasting cultivars of *Oryza glaberrima* Steud and could osmotic component have an impact on this expression?

This document is made up of two main parts. The first part presents a literature review summarizing the knowledge related to the problem of soil salinization, its distribution, its causes, its consequences, and the strategies of management and reclamation. Next, we present the effects of salt stress on plants. We have placed particular emphasis on the osmotic and ionic components of salt stress. We also highlighted the strategies that plants may trigger to cope with salt stress. Finally, we introduce the rice with a broad description of the characteristics of *Oryza glaberrima*.

The second part presents the results of various experimental studies carried out throughout our PhD thesis. It is organized in six separated chapters (Figure 1). The first chapter reports the screening of *Oryza glaberrima* seedlings in order to identify two contrasting cultivars exhibiting contrasting levels of salinity resistance at the seedling stage. The purpose of the second chapter was to check that salinity resistance expressed at the vegetative phase of development is still present during the reproductive stage, and to identify the main yield-related parameters affected by salinity stress under African

climatic. The third chapter discriminates the impact of $Na^+$ and $Cl^-$ in the deleterious effects of salt stress on the African rice species. The fourth chapter relates the effects of some of inhibitors of $Na^+/H^+$ antiporter and cation-chloride-cotransporter on these two cultivars of *Oryza glaberrima*. Chapter V focuses on the impact of the osmotic component of salt stress on *Oryza glabberrima* and uses a non-penetrating osmotic component (PEG) to analyze plant response to osmotic stress independently of ion toxicity. The sixth chapter focuses on the expression of genes coding for ion in response to salt stress and non-ionic osmotic stress. For Chapter V and VI, a mix treatment associating NaCl and PEG was also used to assess putative interactions between the two component of salt stress.

Data obtained during this experimental part will be discussed in a global perspective at the end of the thesis in a "General Discussion".

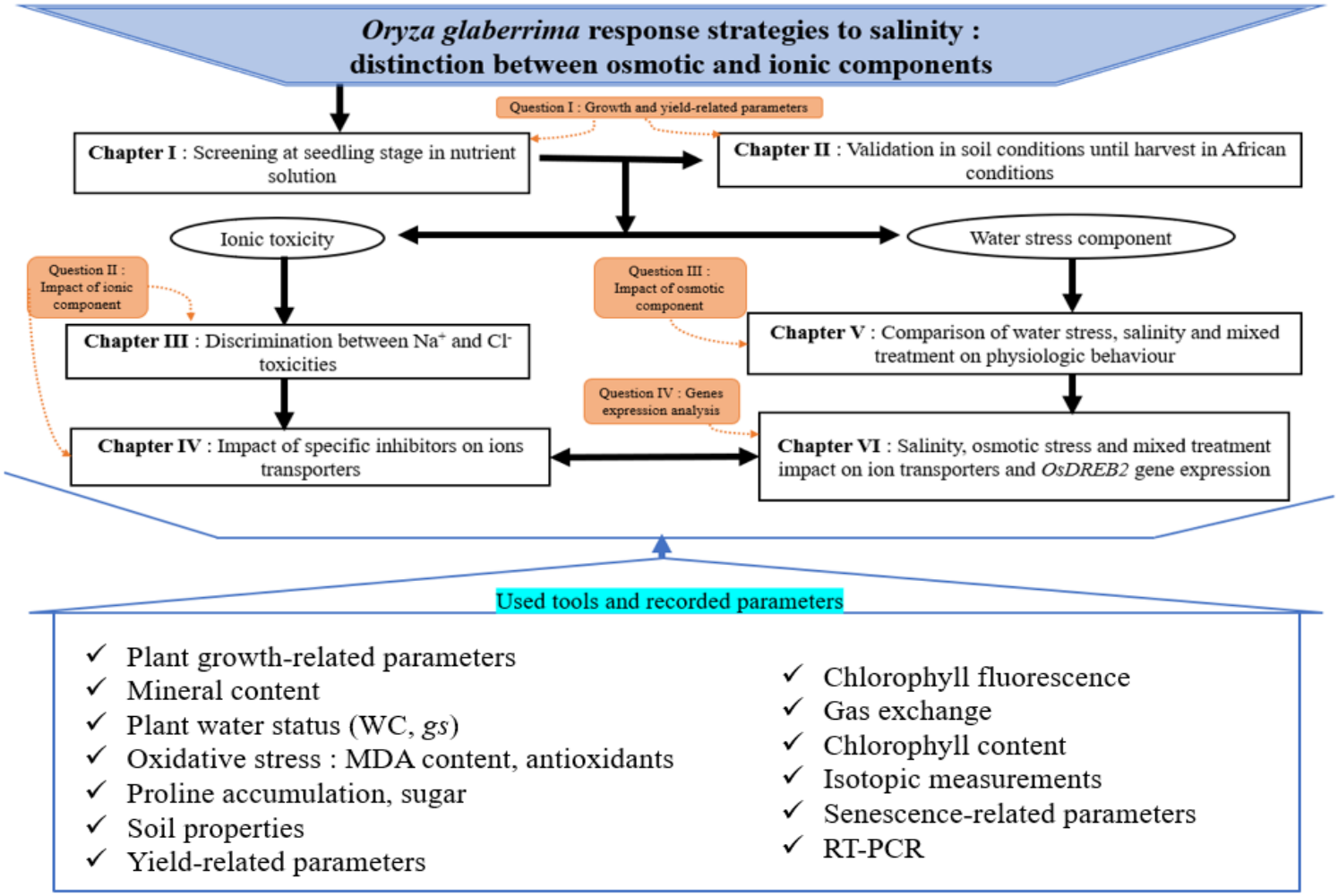


**Figure 1.** Overview of questions addressed during this thesis and their relationships with chapters

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# PART I

# Bibliographic review

# Bibliographic review

## 1.1 Salinity

### 1.1.1 Definition and classification

The progressive accumulation of soluble salts in soil is termed salinization. Pools of several cations and anions are found in saline soil. Involved major cations are sodium ($Na^+$), potassium ($K^+$), calcium ($Ca^{2+}$), and magnesium ($Mg^{2+}$). Anions consist mainly in chloride ($Cl^-$), nitrate ($NO_3^-$), carbonate ($CO_3^{2-}$), bicarbonate ($HCO_3^-$), and sulfate ($SO_4^{2-}$). The combination of cation and anion constitutes the salt present in soils. To a lower extent, some elements like boron ($B^{3+}$), aluminum ($Al^{3+}$), barium ($Ba^{2+}$), molybdenum ($Mo^{2+}$), selenium ($Se^{2-}$), rubidium ($Rb^+$), lithium ($Li^+$), manganese ($Mn^{3+}$), fluoride ($F^-$), and strontium ($Sr^{2+}$) could be found and some of them may accumulate in hyper-saline soils. The concentration of salt in water or soil is measured as total dissolved solutes (TDS) or as total soluble salts (TSS) and expressed in milli mole per liter (mmol $L^{-1}$), or milli gram per liter (mg $L^{-1}$), or parts per million (ppm), or milli equivalents per liter (meq $L^{-1}$).

Salt's ions conduct electric current and the scientific community decided to quantify the presence of salt in soil in terms of electrical conductivity (Rengasamy et al., 2010). Electrical conductivity (EC) is expressed as micro-mhos per centimeter (µmho/cm), or in milli-mhos per centimeter (mmho/cm). For the international system of units, the EC's unit is Siemens per meter (S $m^{-1}$) or deciSiemens per meter (dS/m). The accuracy of EC meter might be checked with 0.01 mole per liter (mol $L^{-1}$) solution of potassium chloride (KCl). The result should be 1.413 dS/m at 25 °C. A soil is considered to be saline, when the EC of its extract from a saturated paste (ECe) equals or surpasses 4 dS/m at 25 °C (Richards, 1954). Depending on the level EC presents, soils are classified in different categories (Table 1): non-saline, slightly, moderately, highly and extremely saline (Abrol et al., 1988).

Table 1: soil classification based on electric conductivity

| Salinity class | Non-saline | Slightly | Moderately | Highly | Extremely |
|---|---|---|---|---|---|
| EC (dS $m^{-1}$) | <2 | 2-4 | 4-8 | 8-16 | >16 |

The corresponding between units can be presented as:

1mmho $cm^{-1}$ = 1 dS $m^{-1}$ = 1 mS $cm^{-1}$ = 1000 micro Siemens per cm (1000 µS $cm^{-1}$)

TDS = 640 * EC (dS/m) (if EC < 5 dS/m)

TDS = 840 * EC (dS/m) (if EC > 5 dS/m)

Sodium chloride is the most common of salts. It represent more than 90% of salt in the world (Dudley, 1994). While some soils are subjected to salinization, others soils are termed "sodic" or alkaline. These designations are based on other criteria that describe the relative amounts of Na present in the soil: the percentage of exchangeable sodium and the sodium adsorption ratio (Chhabra, 2004). The exchangeable sodium percentage (ESP) assesses the amount of exchangeable Na relative to the total amount of major cation (Ca + Mg + K + Na) involved in the exchange capacity of the soil: ESP is therefore expressed as a percentage. The sodium adsorption ratio (SAR) describes the ratio of Na relative to calcium and magnesium (Richards, 1954). According to their EC, pH, ESP and SAR, soils are classified into four distinct classes (Table 2): normal, saline, sodic and saline-sodic soils (Carballar-Hernandez et al., 2013; Richards, 2012). Normal soils have EC below 4 dS $m^{-1}$, an ESP lower than 15%, a pH under 8.5 and an SAR between 0 and 13. Saline soils have EC higher than or equal to 4 dS $m^{-1}$, an ESP below 15%, a pH under 8.5 and an SAR lower than 13. The presence of the low ESP with high EC in saline soils generates aggregated soil particles by flocculation. Saline soils are usually characterized by the presence of a white salt-crust at the ground surface. Sodic soils display an EC lower than 4 dS $m^{-1}$, an ESP above 15%, a pH higher than 8.5 and a SAR up to 13. Sodic soils are also characterized by predominance of sodium ($Na^+$) with bicarbonate and carbonate anions. They have very poor soil structure that dramatically reduces

water infiltration and drainage. Saline-sodic soils presents an EC up to 4 dS $m^{-1}$, an ESP greater than 15%, a pH less than 8.5 and an SAR beyond 13. Due to the high ESP and the EC, the structure of saline-sodic soils is consequently drastically degraded.

Table 2: Classification of salt-affected soils

| Soil class | Electric conductivity of the saturated paste extract (ds m-1 at 25 °C) | Soil pH in water | Exchangeable sodium percent (%) | Sodium Adsorption Ratio |
|---|---|---|---|---|
| Normal | <4 | <8.5 | <15 | <13 |
| Saline | >4 | <8.5 | <15 | <13 |
| Sodic | <4 | >8.5 | >15 | >13 |
| Saline-sodic | >4 | <8.5 | >15 | >13 |

Beside the previous classification, a list of the types of saline or saline prone soil has been established (Table 3). This list takes into account several physical and chemical characteristics of soil in addition to salt contamination. Soil's types are solonetz, solonchak, acrisol, alisol, calcisol, fluvisol, gleysol, luvisol, and vertisol (WRB, 2014).

Table 3: Types of saline or saline prone soils

| Soil types | Main characteristics | Symbol | Saline | Saline prone |
|---|---|---|---|---|
| Solonetz | Subsurface clay accumulation, rich in sodium | SN | X | |
| Solonchak | Strongly saline | SC | X | |

| | | | |
|---|---|---|---|
| Acrisol | Subsurface accumulation of low-activity clays and low base saturation | AC | X |
| Alisol | Subsurface accumulation of high-activity clays, rich in exchangeable aluminum | AL | X |
| Calcisol | Accumulation of secondary calcium carbonates | CL | X |
| Fluvisol | Relatively young in alluvial deposits | FL | X |
| Gleysol | Permanent or temporary wetness near the surface | GL | X |
| Luvisol | Subsurface accumulation of high-activity clays | LV | X |
| Vertisol | Dark-colored cracking and swelling clays | VR | X |

### 1.1.2 Salinity assessment and distribution

In natural conditions, visual indicators of salt-affected soils help for identification. For example, there are: presence of white crust; reduction of seed germination; burning of leaves; stunted plants or their precocious death; occurrence of indicator plants like halophyte plant species (Shahid et al., 2018a). However, for maintaining or improving soil health and crop yield on agricultural soils, accurate assessment of salinity and its distribution is indispensable. The choice of an adequate salinity measurement method must take into account the purpose, the available resources, the accuracy intended, the depth of soil, and the size of the area to be evaluated. There are many tools available to assess soil salinity.

Remote sensing evaluate the Earth's surface health without being in contact with it. Remote sensing may correctly identify the areas where salts are concentrated and properly detect the spatio-temporal changes due to salts accumulation (Zinck, 2002). Different types of sensors for remote sensing are used for salinity assessment: aerial photographs, video imagery, hyperspectral sensors, microwave sensors, satellite- and airborne multispectral sensors, electromagnetic induction meters and airborne geophysics (Metternicht and Zinck, 2003). However, some constraints like the spectral behavior of salt types, the interference of vegetation, and the spectral confusion with other soil surface features, limit the accuracy of remote sensing. To overcome these limits, multi-sensor frameworks coupled with radiative transfer models might be used for salinity measurement with remote sensing (Wen et al., 2020).

Soil salinity can also be measured on georeferenced field sampling coupled with laboratory analysis. Different technics are used according to surface area, clay content and other parameters of soil texture. The classical methods implies the extraction of soil to water suspensions in different rations, and the extraction of saturated paste of soil samples (Rhoades and Loveday, 1990). Then, an EC meter indicates the level of salinity of the extract. Modern equipment, such as electromagnetic induction (EMI-EM38) and salinity electrode probes, are also classically used (Shahid et al., 2009). Beside the soil saturation extract from paste, an alternative method is used for field salinity measurement and it is based on a soil:water suspension using different ratios (1:1, 1:2.5, 1:5).

Reliable establishment of salinity by remote approach allows to obtain precious information regarding the distribution of salt-affected soils on the

Earth and to establish maps. Based on the class and the severity of salinity, Wick et al., (2011) established a map of global salt-affected soils (Figure 2). Mapping of global salt-affected soils shows that salt-affected soils are found in almost all regions of the world, although the extent and severity vary from region to region. Salinization affects more than 1069.3 Mha of surface soils, which account for more than 7% of the world's total land area. Consequently, 20% of world's cultivated lands and 33% of world's irrigated lands are affected by salinity. Based on ground truth data, thermal satellite imagery, and soil property maps, Ivushkin et al., (2019) studied the global mapping of soil salinity change. The authors showed that the total area of salt affected lands increased by more than 100 Mha between 1986 and 2016 (Figure 3). This mean that many of previously unaffected soils are starting to suffer from salinization. Furthermore, every minute a surface of 3 ha of arable land becomes unusable due to salinization, which consist of the loss of 20 million ha of irrigated land every year. Therefore, salt-affected soils are increasing at a rate of 10% annually, and some researches even predict that 50% of the world's arable land will be degraded by salinity by the year 2050 (Butcher et al., 2016; Cuevas et al., 2019). Even if all Earth's continents are threaten by salinization, African continent is the most salt-affected region followed respectively by North and Central Asia, South Asia, and South America (FAO, 2015).

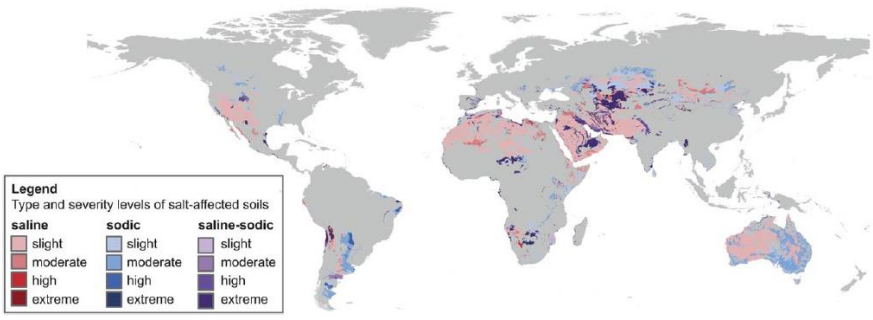


**Figure 2.** Global salt-affected soils, by type and severity (Wicke et al., 2011).

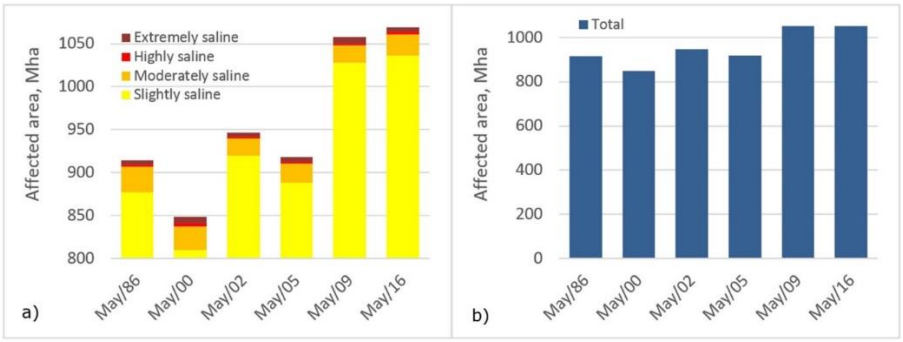


**Figure 3.** Salt affected land area for different years; a) shows division per salinity class, b) shows the affected area (Ivushkin et al., 2019).

### 1.1.3 Causes of salinity

Causes of salinity are considered as "primary" or "secondary" (Figure 4). Primary salinization is due to natural processes which allow the accumulation of salts over long periods of time. Naturally salt-affected soils occur when the weathering of parental rocks releases soluble salts in soil. Climatic conditions and land features might also contribute to primary salinization. For example, the high evapotranspiration rate in tropical region concentrate more salt ions in top soils and thus give rise to salinity (Majeed and Muhammad, 2019). Wind and rain are also primary causes of salinity. Rainwater may be rich in sodium chloride (up to 6-50 mg/L). So every 100 mm of rainfall per year would drop off 10 kg/ha of salt if rain contained 10 mg/L of sodium chloride (Munns and Tester, 2008). Through the phenomenon of sea spray, wind carries salts of seawater and deposit them on lands located near coastal regions. The rise in sea level due to climate change also results in a marine intrusion of seawater into coastal lands well as into the aquifer. In some regions, the flood causes a capillary rise of salts previously buried in the ground either by rain, or by marine intrusion, or by weathering parent rocks (Daliakopoulos et al., 2016; Shrivastava and Kumar, 2015).

Beside primary causes of salinization, soils may be contaminated with salt via secondary causes, also termed "anthropogenic salinity" (Figure 4). Anthropogenic salinity is due to human activity such as massive agricultural activities, use of imbalanced fertilizers, poor irrigation practices, poor drainages, use of industrial effluents and wastewater, mining activities, and

deforestation (Daliakopoulos et al., 2016; Majeed and Muhammad, 2019). Indeed, the use of poor quality fertilizers and over-fertilization cause soil salinization, markedly so in the case of intensive agriculture on compacted soils with low leaching (Moreira Barradas et al., 2014; Eckelmann et al., 2006). Moreover, mining activities and the use of industrial effluents and wastewater enriched with various salt contents can lead to agricultural salinity. Irrigation with saline water or inappropriate irrigation practices often combined with poor drainage conditions also lead to soil salinization (Fan et al., 2012, Trnka et al., 2013). In winter, the excessive use of traditional salt-based de-icing agents contributes to salt accumulation in soil and water (Mateo-Sagasta and Burke, 2011).

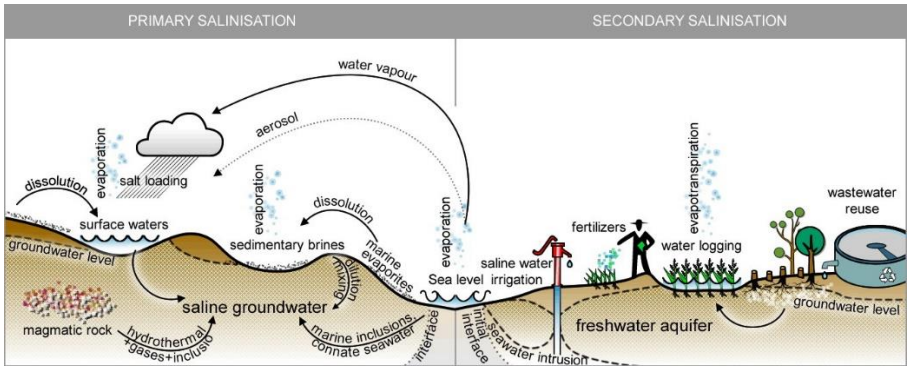


**Figure 4.** Primary and secondary causes of salinization (Daliakopoulos et al., 2016).

### 1.1.4 Consequences of salinity

Consequences of soil salinization are environmental, social, and economic. Salinity induces hazardous alteration or disruption upon biogeochemical cycles (Friedl and Wüest, 2002; Zeman, 2020). Rengasamy et al. (2010) reported that salinization impacts environmental health and agricultural production by the loss of the emerging resources, good and services of soil. Salinity is one of the major factor contributing to desertification. It affects the physical, chemical and biological features of soils. As a consequence of the degradation of soil properties, the fertility of soils decrease. This loss of fertility generates a drastic reduction in crop yields, the disappearance of the natural vegetation, and the changing of the landscape by the appearance of

immense expanses of salty areas. Salinity decreases the soil biodiversity and microorganism activity by having adverse impacts on decomposition of organic matter, respiration of microorganisms, nitrification and denitrification processes (Singh, 2016). Consequently, land value depreciates and production costs increase. Moreover, agricultural production is reduced or even lost and, therefore, food security is negatively affected. There is an increase of poverty due to income loss for local populations. In some regions, salt-affected lands are abandoned and native person move away. They migrate out of rural areas to the cities. This migration leads to the rise of unemployment. Social and economic frameworks are then disturbed. The annual loss of global agricultural production, due to salt-affected soils in irrigated areas could be estimated at US$ 27.3 billion (Qadir et al., 2014). In Europe, the impact of salinity on agriculture production cost around 600 million euro per year (Bosello et al., 2012). Salinity can also damage infrastructure such as buildings, roads and other construction. It causes the corrosion of concrete, brick and asphalt constructions. For example, the durability of road tarmac is significantly reduced when there is salts in the ground. It is estimated that the infrastructure deterioration due to salinity costs US$ 12 million per year (Vargas et al., 2018).

An historical consequence of salinization is the collapse of ancient civilizations in Mesopotamia. Indeed, the "Diyala Basin Archeological Project", jointly conducted by the University of Chicago and the Iraqi Antiquities Department, reveals that the conflict related to the use of the irrigation system and its extension has developed the conditions of soil salinization (El Faiz, 1990; Jacobsen and Adams, 1958). Lands which were suitable for agriculture in 2400 BC, became highly salt-affected and sterile in 1200 AD. Moreover, the soils are so degraded that they have not yet become fertile again until today. Crops yield decreased drastically in time span of almost seven centuries. For example, barley yield was decreased from 2537 kilograms per hectare (Kg/ha) in 2400 BC to 1460 Kg/ha in 2100 BC and 897 Kg/ha in 1700 BC (Tamburrino, 2010). According to El Faiz (1990), the repercussions were so catastrophic that the non-rural classes could only survive thanks to the agricultural surplus released by a minority of farmers. Many Mesopotamian cities have turned into village or fallen into disrepair.

### 1.1.5 Management and reclamation of salt-affected soils

To maintain soil health and to keep getting higher agricultural production, it is indispensable to perform adequate management and efficient reclamation of salt-affected soils. Three main techniques are globally used for salt-affected soils' reclamation: mechanical, chemical and biological.

#### 1.1.5.1 Mechanical method

Mechanical techniques are all physical methods used to limit, to reduce, or to manage the occurrence of salt-affected soil. One of these methods is the installation of anti-salt dikes or dam to prevent marine intrusion into land (Delphine et al., 2013; Wade, 2020). Salt-tolerant trees can also be planted along the coasts to serve as a windbreak for sea spray (Kim, 2010). Another method is soil scraping (Abou-Baker and El-Dardiry, 2015) to remove surface salt crusts, which usually occur in arid and semi-arid areas. It consist of scraping soil's surface by using manual tools or heavy machinery. The advantage of this method is that some populations use scraped soil residues as a raw material to produce cooking salt in an artisanal way. This is the case for the women of Djègbadji, a coastal village in southwestern Benin. Nevertheless soil scraping remain a short-term solution since it only temporarily reduce the salt content. The white salt-crust can reappear at the ground surface with a poor irrigation practices. According to Qadir et al. (1998), flushing is suitable for salt-affected soils which have surface salt crusts. This method flushes the salts from soil surface by using a sufficient volume of clean water. Then, the flushed saline water is drained by an adequate treatment system.

Leaching of saline soils is another technique used to manage salt-affected soils. It is based on the percolation of water through the soil to displace salts under the root zone. A large volume of good quality water is used to leach the salt from surface depending on the soil characteristics and it requires a good internal drainage (Ayars et al., 2012; Qadir et al., 1998). This is accomplished through flooding of water at the surface for salt-affected soils. Leaching required to take in account some exigencies for successful operations: the amount of water needed to get as low as possible the EC of drained water; the timing of leaching; the irrigation system; and the evapotranspiration (Shahid

et al., 2018b). Based on these features, leaching requirement (LR) is a calculated fraction as:

$$LR = \frac{EC_{iw}}{(5EC_e - EC_{iw})} \quad \text{(for surface irrigation);}$$

$$LR = \frac{EC_{iw}}{2(Max\ EC_e)} \quad \text{(for drip irrigation system)}$$

Where, $EC_{iw}$ is the salinity of the irrigation water (dS $m^{-1}$) and $EC_e$ is for a given crop's maximum yield potential.

Unfortunately, both unavailability of clean water and difficulties of management of wastewater issued from leaching limit the efficient use of this technique. Moreover, in presence of sodic or saline-sodic soil, exchangeable sodium hydrolyzes easily when the leaching is applied and this readily increase the soil pH.

#### 1.1.5.2 Chemical methods

Chemical methods are used to reclaim sodic and saline-sodic soils due to their high content in exchangeable sodium percent (ESP > 15). The aim is to reduce the ESP and bring it under the threshold value allowing agricultural practices. To reach this goal, it is necessary to increase the concentration of calcium in the soil. Gypsum ($CaSO_4 \cdot 2H_2O$) is the most common and relatively economical amendment to solve sodicity problem. The following reaction occurs in presence of gypsum:

$$2Na^+\text{-Clay} + CaSO_4.2H_2O \rightarrow Ca^{2+}\text{-Clay} + Na_2SO_4 + 2H_2O$$

Sodic soil Gypsum Normal soil Salts leach out

Indeed, the exchangeable sodium, which was adsorbed onto the clay, is replaced by the calcium from gypsum. Thus, sodic clay changes to calcium clay (normal soil). The $Na_2SO_4$ form is then leached into lower zones (Abrol et al., 1988; Bennett et al., 2014). In order to avoid resodification in sodic or saline-sodic soils, $Na_2SO_4$ should be took away from the root zone using

halophytic species (Abate et al., 2021). In specific cases where soils are sodic (or saline-sodic) and calcareous, HCl and $H_2SO_4$ are also used for soil reclamation. Acids release calcium which act as in gypsum case.

#### 1.1.5.3 Biological methods

Biological methods include all techniques used to improve the fertility of salt-affected soils (saline, sodic and saline-sodic) in order to grow crops and/or trees upon these degraded areas. Biological methods are: organic amendment, agroforestry, breeding salt-resistant cultivars, and genetic engineering.

Salt-affected soils are commonly deficient in organic matter. Organic amendment consist of adding the crop residues and other organic material to improve soil structure by influencing the physical, chemical and biological properties of the soil. For example, by adding 10 tons per hectare of organic wastes to saline soil in dryland conditions near Seville (in Spain), spontaneous vegetation appeared after one year and the plant cover was about 80% after five years (Tejada et al., 2006).

The agroforestry technique is based on the use of the trilogy plant-soil-microorganisms for salt-affected soils reclamation. Salt-tolerant plants and microorganisms are used in association. Salt-tolerant plants or halophytes are often species belonging to the genus *Atriplex*, *Halosarcia* and *Maireana* (Dagar and Minhas, 2016) whereas salt-tolerant microorganisms are mycorrhiza (Porcel et al., 2012) or nitrogen fixing bacteria (Zhu et al., 2016). Using of mycorrhiza and nitrogen fixing bacteria are considered to be crucial bio-ameliorators for saline soils. Roots inoculation with that microorganisms protect the plants from saline stress. Indeed, these microorganisms (i) bring phosphorus and nitrogen to plants, (ii) improve plant-water status, and (iii) build physical barriers throughout the establishment of mycorrhizal mantle or nodules (Diagne et al., 2014; Rabie and Almadini, 2005). As far as halophytes are concerned, they have some hallmark traits, such as succulence, salt glands and bladders where toxic ions may accumulate without impacting plant metabolism. However, the major difference between halophytes and glycophytes relies on the efficiency of halophytes in implementing these traits. Indeed, based on the physiological, anatomical, or genetic traits, there are no fundamental differences between glycophytes and halophytes (Flowers and Colmer, 2008). The latter are suitable for saline agriculture. Saline agriculture

are non-conventional agricultural technologies which consist of using degraded, marginal and saline lands for agriculture by using poor quality water such as seawater and brackish water (Khan and Duke, 2001).

## 1.2 Effect of salt stress on plants

### 1.2.1 Notion of stress: concepts and definitions

In elasticity theory, stress is the amount of force for a given area (Cauchy, 1821). Depending on the force applied, a material bends to a point of failure or no return (Kranner et al., 2010). From this precise concept established in the physics of materials, biologists have published similar definitions on plants; although the nature of stress varies depending on whether one is making a material or a living organism. According to Lichtenthaler (1998), stress is the state of a plant under the condition of a force applied. So the response itself is the stress and the force applied to the plant is termed strain or stressor. Thus, damage is the result of too high stress that can no longer be compensated for (Lichtenthaler, 1998). But all agents can act as stressors, producing both stress and specific action, and there exist stressor-specific responses and non-specific general responses (Selye, 1936). Frequently, there is a confusion between "stress" and "constraint", stress being considered as an environmental factor inducing a potentially harmful effect on a living organism (Levitt, 1980) while, from a fundamental point of view, it is the set of metabolic disturbances caused in the organism by various stressors (Lutts, 1996). According to Lichtenthaler (1996), stress is any unfavorable condition or substance that affects or blocks a plant's metabolism, growth or development. However, plants are able to either resist, tolerate, or adapt to stress, although these three terms are confused in the literature. In fact, resistance is the set of internal properties which can oppose the appearance of a specific constraint, while tolerance happens when adaptation of the systems can allow the plant to overcome the constraint once it has been established (Lutts, 1996). Adaptation, on the other hand, refers to a set of morphological and physiological characters that allow an individual to thrive in a well-defined environment but which would be useless, or even harmful, in another environment (Margulis, 1970). So, stress is a metabolic state in which increasing demands made upon a plant lead to an initial destabilization of functions. Once damage occur, they may be reduced by normalization and improved resistance, and if the limits of tolerance are exceeded and the adaptive capacity is overworked, the result may be permanent damage or even plant death (Larcher, 1987). Nevertheless, the concepts of resistance, tolerance and adaptation, highlight the need to take into account the nature,

intensity (or dose) and duration of stress. Stress applied on plants can be biotic (insects, bacteria, fungi, ...) or abiotic (salinity, drought, flooding, ...). Depending on the intensity or dose, stress can either stimulate (Eu-stress) or harm (dis-stress) the plant. Eu-stress is a stimulating, activating stress and a positive element for plant development, whereas dis-stress is a severe stress that negatively affects the plant and causes damage (Lichtenthaler, 1996). Eu-stress refers to very low intensities of stressors and lead to a physiological processes called "hormesis" which is in fact poorly studied and remained a matter of debate in the literature. However, even for a condition of dis-stress, the intensity or dose may not have harmful effects on the plant if it is not applied for a fixed period of time. So the stress is duration-dependent. In our opinion, stress in plants refers to any biotic or abiotic factor that could adversely affect the growth, development, and productivity of plants.

### 1.2.2 Salt stress: two contrasts in the world' flora

The resistance of plant to salinity is assessed based on the relative plant growth rate (Munns, 2002) and/or the plant survival rate (Munns, 2005) after a treatment with a given concentration of salt during a specific period. In fact, plant species greatly differ in their resistance to salinity. According to their abilities to grow in the presence of different salt concentrations, plants can be distributed among two groups: glycophytes and halophytes. Halophytes (from the Greek *alos*, salt and *phyton*, plant) are plants adapted to live in a saline environment, whether it is a coastal area, a salt marsh or a salt desert. They can tolerate high salt concentrations, and they can complete their whole life cycle at salt concentrations higher than 200 mM NaCl. The halophytes constitute about 1% of the world's flora (Flowers et al., 1986; Flowers and Colmer, 2008). In contrast, glycophytes (from the Greek *glykýs*, sweet and *phyton*, plant) constitute 99% of world's flora. Glycophytes can only thrive in low salt soil (< 4 dS/m). The majority of the terrestrial plants including agricultural crops belong to the glycophyte group. Munns and Tester (2008) reported the diversity in the salt tolerance of various species (Figure 5) such as rice (*Oryza sativa* (Aslam et al., 1993)), durum wheat (*Triticum turgidum ssp durum*), bread wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), tall wheatgrass (*Thinopyrum ponticum*, syn. *Agropyron elongatum*), Arabidopsis (*Arabidopsis thaliana*), alfalfa (*Medicago sativa*), and saltbush (*Atriplex amnicola;* a typical xero-halophyte species). Whether the salt stress is applied

on glycophytes or halophytes, plant responses occur in two distinct phases through time. This highlighted two components of salt stress: osmotic and ionic constraint.

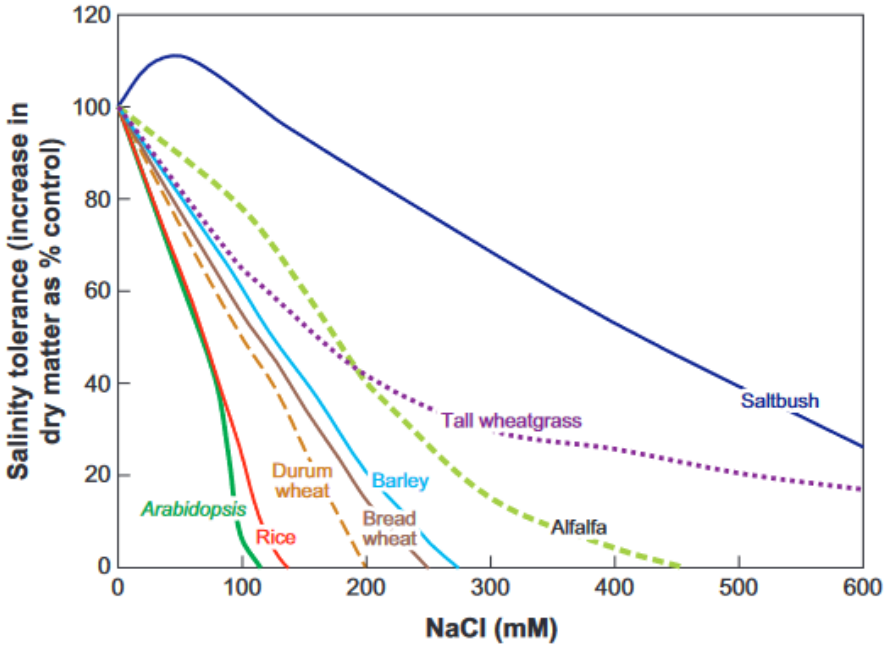


**Figure 5.** Diversity in the salt tolerance of various species, shown as increases in shoot dry matter after growth in solution or sand culture containing NaCl for at least 3 weeks, relative to plant growth in the absence of NaCl (Munns and Tester, 2008).

### 1.2.3 Components of salt stress

Plant nutrition depends on the water flow from the soil through the plant to the atmosphere. The differences in water potential between soils and roots allow the movement of water through the root to xylem vessels in the center of the roots. The nutrient content, the water content and the rate of water movement in soils depend also on the structure, the texture of soil as well as other soil properties. In normal soils, solute concentrations are rather low and the osmotic potential of soil solution is only slightly negative. In contrast, in saline soil, this osmotic potential can reach -0.2 MPa or even lower values because high concentration of NaCl are dissolved in the soil solution. This drastic fall of osmotic potential of soil solution induces a hydric stress in root

zone and plants suffer from dehydration if water absorption is not sufficient to compensate water losses by transpiration: this is the "osmotic component" of salt stress. Beside from this osmotic stress, there is also an ionic component. Indeed, NaCl increases the content of ions $Na^+$ and $Cl^-$ in soil solution; the absorption of these ions and their progressive accumulation trigger an ionic toxicity in plants. In response to these two components of salt stress, the growth of plants in salinity stress takes place into two phases (Figure 6, (Munns and Tester, 2008)). This is the well-established "biphasic model" of plant response to salinity considering the importance of time frame with regard to plant salt stress tolerance.

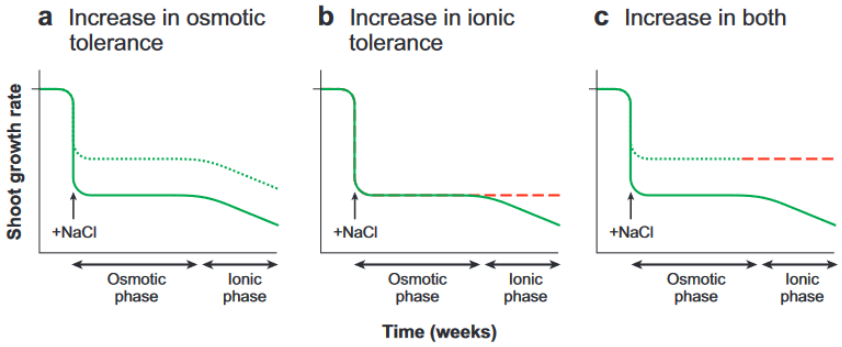


**Figure 6.** The growth response to salinity stress occurs in two phases: a rapid response to the increase in external osmotic pressure (the osmotic phase), and a slower response due to the accumulation of $Na^+$ and $Cl^-$ in photosynthetic tissues (the ionic phase). The solid green line represents the change in the growth rate after the addition of NaCl. (a) The broken green line represents the hypothetical response of a plant with an increased tolerance to the osmotic component of salinity stress. (b) The broken red line represents the response of a plant with an increased tolerance to the ionic component of salinity stress.(c) The green-and-red line represents the response of a plant with increased tolerance to both the osmotic and ionic components of salinity stress (Munns and Tester, 2008).

#### 1.2.3.1 Osmotic component

The excess of sodium and chloride in the root zone causes the osmotic stress, which is commonly considered as the "first" phase of salt stress. This

accentuates the unavailability of water for plants even if this water is present in the soil at field capacity (Rasool et al., 2013). For example, wheat was grown in pots with sandy loam soil and various electrolyte solutions such as NaCl, $CaCl_2$, $Na_2SO_4$ and Hoagland nutrient solution. As EC of soil solution increased in these pot experiments, the unavailable water in the pot soils increased and 89-96% of water was unused when the soil solution EC was higher than 22.6 dS/m. As soon as the EC of the soil solution exceeded 25 dS/m, the growth of wheat was drastically reduced in relation to a strong osmotic effect (Rengasamy, 2010). In fact, the osmotic component of salt stress comes over plants when salt level rises outside the roots, which caused the reduction and the inhibition of water uptake. Moreover, the increasing salt concentration lowers the osmotic potential of the apoplastic water, which is already present in the cortex. Thus, cell expansion and lateral bud development are inhibited, which impair plant growth (Munns and Gilliham, 2015). Pots experiments and field observations confirmed that osmotic component of salinity is an important factor in reducing plant growth and yield under dry land conditions (Kelly and Rengasamy, 2006) and it may therefore be considered that plants exposed to NaCl encounters a "physiological drought". Based on the diagnosis and the management of soil constraints, Kelly and Rengasamy (2006) reported that the increases in effect of the osmotic pressure of the soil solution on plant yield were higher when the pressure raised over 0.7 MPa. They also demonstrated that osmotic effect reduces the plant water uptake, reduces the yield to uneconomic levels when the soil solution osmotic pressure is above 1.0 MPa. Figure 6a displays the effect of osmotic component of salt stress on the rate of increase in shoot dry matter or in leaf area over time, that is, the rate of shoot growth. Indeed, fewer branches or lateral shoots form because lateral buds develop more slowly or remain quiescent. Moreover, new leaves emerge more slowly and the rate at which growing leaves expand is reduced. In monocotyledonous species, the number of tillers is reduced while the number of branches and the size of individual leaves drastically decrease in dicotyledonous species (Munns and Tester, 2008). The dotted line in Figure 6.a shows the effect of increased tolerance to osmotic stress while ionic stress persists.

To cope with osmotic component of salt stress, plant may achieve osmotic adjustment, which imply the accumulation of soluble solutes in plant cells to reduce its own osmotic potential without modification of cell volume. Plant osmotic adjustment pass either by the increasing uptake of inorganic ions or by the production of organic osmolytes (we will develop this point later). The

major ionic contributor to osmotic adjustment is $K^+$ but the excess of $Na^+$ in the soil solution compromises its absorption from the soil solution. The synthesis of organic osmolytes is very expensive and has a cost in terms of C and ATP compare to the use of inorganic ions (Munns et al., 2020; Munns and Gilliham, 2015). Using the inorganic ion for osmotic adjustment seem being the cheaper and faster way. In saline condition, sodium and chloride are the most abundant elements present in the soil at high concentrations but their accumulation in plant tissues induce a wide range of metabolic problems.

#### 1.2.3.2 Ionic component

Theophrastus Bombastus von Hohenheim (Paracelsus), the German-Swiss physician and alchemist (1493-1541), enunciated the following maxim: "*sola dosis facit venenum*" (it is only dose that makes the poison). So each element, sodium or chloride, has a threshold below which it is not toxic. As far as $Na^+$ is concerned, it is considered as an essential micro-element for C4 species but otherwise it has no established biological functions for C3 species. Chloride is an essential element for all plants but is required only in small amounts.

Ionic stress is traditionally considered as "the second phase" of salt stress, which develops over time due to progressive sodium and chloride accumulation in the plants at a threshold where the stressed plants have not the ability to tolerate the presence of these ions in its organs. Beside these theoretical overview, numerous question still persist: What is the duration of each phase? Could these phases overlap? Are these two phases really independent if we consider that the rate of toxic ion accumulation is a function of transpiration flux which is itself influenced by stomatal closure (and thus by the osmotic constraint); on the other hand, accumulated $Na^+$ and $Cl^-$ below their level of toxicity (or rapidly sequestered in vacuoles) may contribute to osmotic adjustment and thus reduce the osmotic constraint? Finally, it is tempting to consider plant as homogeneous organisms. At vegetative stage, plants have numerous leaves and there is more and more evidence that the physiological properties of those leaves strongly vary depending on their age and position. Leaves of different age do not have the same velocity of stomatal closure so that the "osmotic component" may vary within the organs of a same plant. Similarly, toxic concentrations of ions accumulate in the old leaves (Lutts et al., 1996) and they die but at the same time protect younger

photosynthetically-active growing leaves. Figure 6.b shows that an increase in ionic tolerance takes longer to appear. Even if the salt presence persists, plant can develop an increasing tolerance for osmotic and ionic component of salt stress (Figure 6.c). This ability enable the plant to grow throughout its life cycle (Munns and Tester, 2008).

#### 1.2.3.2.1 Sodium

Sodium (*natrium* from Latin) is the eleventh chemical element according to the Mendeleev classification. Earth's crust is constituted of about 2.8% of sodium, which makes sodium the sixth most abundant element on the earth after oxygen, silicon, aluminum, iron, and calcium (Lutgens et al., 2013). Due to the ubiquitous presence of sodium in soils and waters, it is widely taken up by plants. The benefit of sodium for the growth of algae and cyanobacteria is proven (Brownell and Nicholas, 1967; Carrieri et al., 2010; Erdmann and Hagemann, 2001). Its role in higher plants is less obvious. As far as C3 plants are concerned, there is no clear metabolic pathway which is known to specifically require Na. Moreover, it was reported that the application of sodium to the growth medium stimulates the growth and the yield of many species, including asparagus, maize, barley, broccoli, brussels sprout, caraway, carrot, chicory, cotton, flax, millet, oat, pea, rutabaga, tomato, vetch, wheat, cabbage, Ranger alfalfa, celeriac, horseradish, kale, kohlrabi, mustard, radish, rape, celery, sugar beet, red beet, Swiss chard, Cacao tree, and turnip (Kronzucker et al., 2013; Subbarao et al., 2003). The growth stimulation is however observed at very low doses and it is always difficult to attribute the positive effect to Na or to the accompanying ion if Na is added in the form of NaCl or $Na_2SO_4$. Sodium is however required for C4 species. According to Kronzucker et al. (2013), a major role for sodium in C4 species is $Na^+$-coupled pyruvate transport in chloroplasts. Indeed, prior to the Calvin cycle, sodium facilitates the conversion of pyruvate into phosphoenolpyruvate in mesophyll cells. Sodium facilitates also the pyruvate uptake into chloroplasts (Furumoto et al., 2011; Johnston et al., 1988).

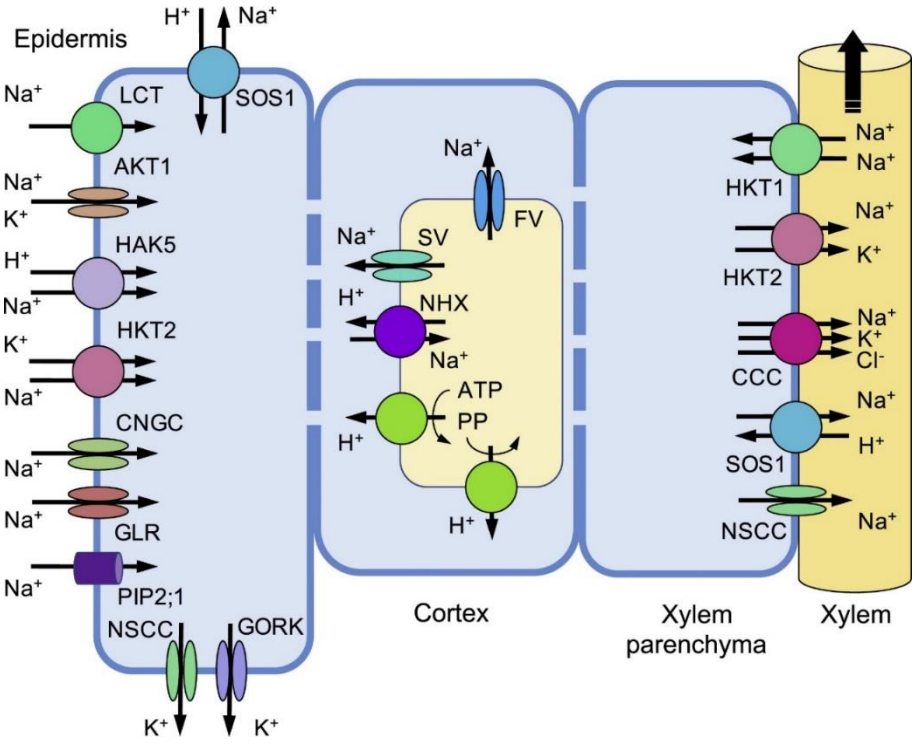


**Figure 7.** Major Transporters Mediating $Na^+$ Homeostasis in Salinized Root Tissues. The major pathways for $Na^+$ uptake in the root epidermis are glutamate receptor-like (GLRs) channels or cyclic nucleotide-gated (CNGCs) non-selective cation channels and HKT2 high-affinity $K^+$ transporters. Other possible pathways for $Na^+$ uptake may involve AKT1 Shaker-type $K^+$ channels, HAK5 high-affinity $K^+$ transporters, the low-affinity cation transporter LCT1, and PIP2;1 aquaporins. The uptake of $Na^+$ is counterbalanced by active $Na^+$ extrusion via SOS1 $Na^+/H^+$ exchangers. Vacuolar $Na^+$ sequestration is conferred by tonoplast-based $Na^+/H^+$ exchangers from the NHX family fueled by either $H^+$-ATPase or $H^+$-PPase pumps. Another component of vacuolar $Na^+$ sequestration is efficient control over tonoplast slow- (SV) and fast- (FV) activating ion channels that may allow $Na^+$ to leak back to the cytosol. Passive $Na^+$ loading into the xylem is mediated by non-selective cation channels (NSCCs), and its active loading requires operation of cotransporters such as SOS1, CCC (cation-chloride cotransporters), and HKT2 ($K^+/Na^+$ symporter). $Na^+$ withdrawal from the xylem is achieved by HKT1 high-affinity $K^+$ transporters. Salinity-induced $K^+$ loss from the root epidermis is mediated by NSCCs and depolarization-activated outward-rectifying GORK $K^+$ channels (Zhao et al., 2020).

Apoplastic and symplastic pathways are both used by sodium to move within the roots to the endodermis. For symplastic pathways, various transporters and

channels are involved in sodium uptake and transport into plants (Figure 7). Non-selective cation channels (NSCC) such as the glutamate receptor-like (GLR) and the cyclic nucleotide-gated (CNGC) are used for the influx of sodium. Cation/$H^+$ exchanger (CHX) and different isoforms of high-affinity $K^+$ transporters (HKTs) are also involved in sodium influx into plant; specifically in long-distance transport of sodium through xylem and phloem. Plasma membrane intrinsic protein (PIP) isoforms are aquaporins which may also facilitate the root sodium uptake. Other channels or transporters which are involved in sodium uptake and/or long-distance transport are: low-affinity cation transporters (LCT1), high-affinity $K^+$ transporters (HAK), shaker-type $K^+$ channels (AKT), and cation-chloride cotransporters (CCC). At certain threshold, sodium is toxic for plant. So the intrusion of sodium is counterbalanced by active sodium extrusion (Figure 7). In plasma membrane, the most prominent element involved in this process is the $Na^+/H^+$ exchanger (SOS1). SOS1 can export back to the rhizosphere sodium taken up by the root (Munns, 2002). Cytosol is the place where the presence of sodium is highly detrimental, mainly because it interferes with numerous enzymatic activities. To avoid sodium toxicity in that cell compartment, the tonoplast-based $Na^+/H^+$ exchangers in the NHX family compartmented sodium in vacuole (Arif et al., 2020; Zhao et al., 2020). It has to be mentioned that each class of transporters comprises several members which can be differently located in the plant and are also differently regulated. This allows the plant to react in a very efficient way to an excess of sodium.

According to Zhao et al (2020), prevention of toxic $Na^+$ accumulation in glycophyte may occur at different steps: sodium exclusion from root uptake; vacuolar sodium sequestration; control of xylem sodium loading; sodium retrieval from the xylem; and sodium recirculation from the shoot via the phloem. However, when sodium reaches toxic level in plants, it hampers photosynthesis, transpiration and, ultimately, growth and yield (Cheeseman, 2013). High sodium concentration interferes with calcium and potassium nutrition of faba bean. It causes the reduction of stomatal conductance, photosynthesis and growth (Tavakkoli et al., 2011, 2010). Sodium significantly decreases the germination rate and biomass production in rice. Sodium disturbs the membrane integrity, which increases the electrolyte leakages. Sodium reduces the leaf chlorophyll content, significantly increases the synthesis of reactive oxygen species (ROS). It induces ionic imbalance, increased malondialdehyde content, hasten senescence and increases the rate of cell death in leaves of rice seedlings. Plant nitrogen content is also affected

by sodium which disturbs nitrate reductase activity. Sodium hampered protein and starch concentration. It significantly reduced number of grains per panicle, weight of grain, percentage of grain filled, and the yield of rice (Khare et al., 2020, 2015; Kumar and Khare, 2016, 2015).

#### 1.2.3.2.2 Chloride

Chloride is the ionic state of chlorine, chemical element with atomic number seventeen. As essential micronutrient for higher plants, chloride is involved in osmoregulation, in turgor regulation, in photosynthesis, and in elongation growth (Chen et al., 2016; Flowers, 1988; Kobayashi et al., 2006; Marschner, 2011). Chloride is consequently actively taken up by higher plants. For example, the abundant uptake and accumulation of chloride assume adaptive functions improving water homeostasis in tobacco plants (Franco-Navarro et al., 2016). Chloride is also involved plant fertilizing in the form of potassium chloride (KCl) because high chloride uptake is positively correlated with high water uptake (Geilfus, 2018; Moya et al., 2003).

In saline conditions, chloride enters in plants by both apoplastic and symplastic pathways. Many different transporters and channels are involved in chloride uptake and long-distance transport. There are : nitrate transporter (NRT), cation chloride cotransporters (CCCs), chloride channel (CLC), aluminum-activated malate transporter (ALMT), anion channel associated homolog 1 (SLASH), nitrate transporter 1/peptide transporter (NPF), $Cl^-/H^+$ co-transporters and $H^+/Cl^-$ symporters (Arif et al., 2020). When the concentration of chloride in cytosol is high, ALMT and CLC are involved in its compartmentation in vacuole (Isayenkov and Maathuis, 2019).

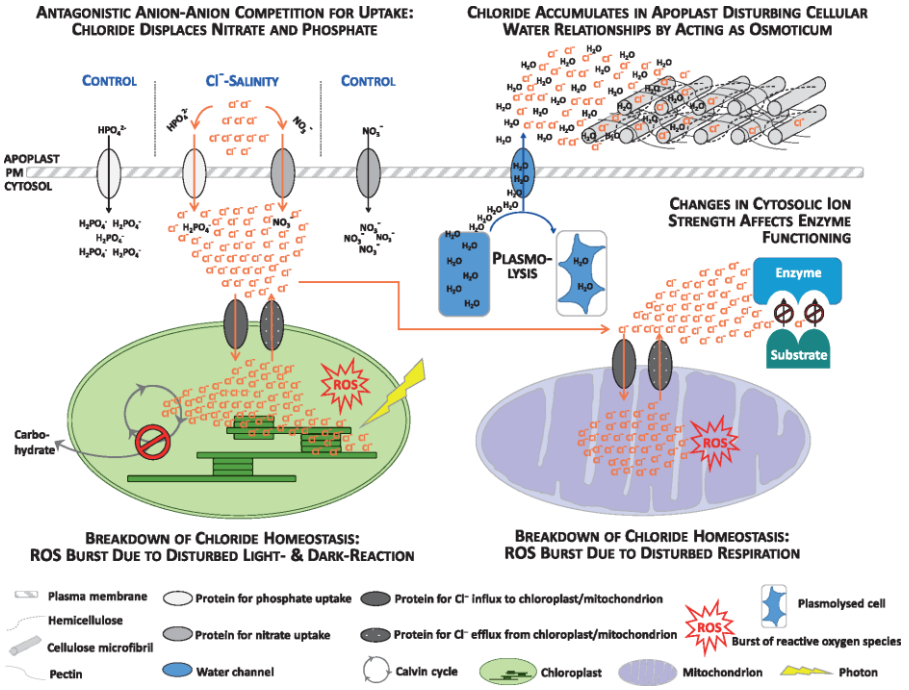


**Figure 8.** Model postulating the reasons why excessive concentrations of Cl− are toxic for the cell. Under conditions of $Cl^-$ salinity, $Cl^-$ accumulates in the apoplast. The increasing concentration lowers the osmotic potential of the apoplastic water film, which disturbs cellular water relationships (upper right corner). Furthermore, apoplastic $Cl^-$ leaks through phosphate and nitrate uptake proteins, preventing these nutrients from being taken up into the cell (upper left corner). Increasing cellular concentrations of $Cl^-$ raise the ion strength in the cytosol, possibly affecting the activity of cytosolic enzymes (right). Under conditions where the $Cl^-$ influx rate into chloroplasts or mitochondria exceeds its efflux, $Cl^-$ homeostatic control cannot be maintained for long. Electron acceptors will be destroyed, triggering the formation of excessive ROS through the reduction of $O_2$ to $O_2^-$. These potent oxidizing agents attack macromolecules causing cellular damage and de-pigmentation (Geilfus, 2018).

However, chloride can have toxic effects on plants at high concentration, especially when that concentration is beyond cell requirements (Figure 8). In saline conditions, high concentrations of chloride may reduce nitrogen or phosphorus uptake. Indeed, there is an antagonistic chloride anion- other anion competition with regard to the uptake of phosphate and nitrate because cellular proteins cannot discriminate efficiently between these three anions (chloride, nitrate, phosphate). This altered selectivity of cellular proteins may

trigger over-accumulation of chloride in cell at toxic levels (Geilfus, 2018). According to Tavakkoli et al. (2010; 2011) high chloride concentration degrades chlorophyll and reduces the actual quantum yield of PSII electron transport. These authors demonstrated that chloride decreases the photosynthetic capacity, reduces growth and yield. Other researches showed that chloride significantly reduces the germination rate, leaf chlorophyll content and biomass production (Kumar and Khare, 2015). Chloride also increases electrolyte leakage and has detrimental effects on membrane stability (Luo et al., 2005). It provokes ionic imbalance, oxidative damage related to excessive ROS generation and cell death (Khare et al., 2015). Chloride increases malondialdehyde content but reduces nitric oxide and nitrate reductase activity (Khare et al., 2020) and yield components (Kumar and Khare, 2016). According to Geilfus (2018), visuals chloride toxicity are chlorotic discolorations, necrotic lesions, and leaf-tip burning.

### 1.2.4 Effect of salinity on plasma membrane and some organelles of cell

Cellular compartments and organelles may be damaged by salt stress. Indeed, numerous ultrastructural changes of organelles were observed due to salt stress (Baranova et al., 2019). The plasma membrane consists of lipids and proteins while the cell wall is composed of pectin, cellulose, hemicellulose and lignin. Cell wall is considered as the "apoplastic compartment", thus plasma membrane is the interface between a cell and its surroundings. The plasma membrane plays a crucial role in cell wall biosynthesis, endocytosis, ion transport, sensing environmental stimuli, and cellular signal transduction (Marmagne et al., 2004). So, plasma membrane integrity is an important factor that determines plant growth in saline conditions (Feng et al., 2018). High salt concentration alkalinizes the apoplast (Geilfus, 2017), affects plasma membrane chemical properties (Shomer et al., 2003), and reduces cell expansion (Proseus and Boyer, 2012). Peroxidation of membrane lipids by salt-induced oxidative stress also has a strong deleterious effect on membrane structure and functions.

Chloroplast is an important organelle which is involved in the photosynthesis and in the biosynthesis of amino acids, fatty acids, lipids, isoprenoids, and vitamins (Chan et al., 2016). So, salt-induced chloroplast stress may have hazardous consequences on the physiological, biochemical, and metabolic

functions of plant cells. By affecting chloroplast, salt stress causes ionic, osmotic, and oxidative stresses which reduce $CO_2$ uptake, degrade the PSII, decreases photosynthetic efficiency, degrade thylakoid membrane, disturb protein synthesis and turnover, disrupt osmotic and ionic homeostasis (Dogra et al., 2019; Suo et al., 2017). Considering the electron-transport chain present in thylakoid membranes, chloroplast is an important site of ROS synthesis in stressed plants.

The mitochondrion in an energy-producing organelle that produces ATP via oxidative phosphorylation. It is the site of several biosynthetic pathway such as nitrogen assimilation, glycine decarboxylation, iron homeostasis, fat metabolism, and vitamins synthesis (Day et al., 2004). As previously reported for chloroplast, mitochondria is a major site for generation of ROS during salt stress (Wang et al., 2018).

The endoplasmic reticulum is the translational and secretory site of proteins (Liu and Howell, 2016). Salt stress may cause the accumulation of misfolded or unfolded proteins in endoplasmic reticulum (Zhao et al., 2020). According to Liu and Howell (2016), salt stress also triggers the degradation of endoplasmic reticulum proteins and the autophagy of that organelle.

Though salt stress has detrimental effects on organelles, cells, tissues, and whole plant, plants can develop different strategies to respond to salinity and overcome that stress.

### 1.2.5 Plant-holistic responses to salt stress

Salt stress has hazardous effects on plant growth and yield. Nevertheless, plants have developed holistic responses to cope with salt stress. These responses are morpho-agronomic, physiological, biochemical, phytohormonal, genomic, transcriptomic, proteomic, and metabolomic (Figure 9; (Arif et al., 2020)). Plant-holistic responses to salt stress vary widely depending on (1) the salt stress intensity; (2) the time of exposure to stress; (3) the species, family, genus, and varieties; and (4) the mean level of salt-resistance (glycophytes or halophytes). Below, accent is put on glycophytes responses.

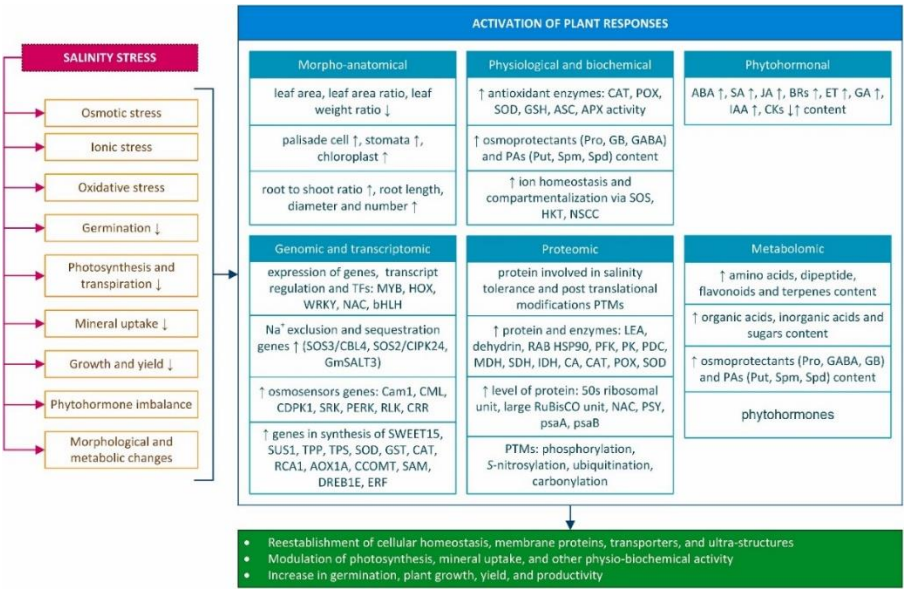


**Figure 9.** Overview of the salt stress impact on plants and their activation of responses in overcoming toxic effects of salinity (Arif et al., 2020).

The plant response to salt stress is extremely complex because salinity acts on almost all aspect of plant physiology and biochemistry. All aspect of plant metabolism may be, to some extent, modified by salinity, but the sequences of modification are not always the same and it is therefore hard to distinguish the causes and the consequences of a given perturbation. Since two decades, scientists are using "holistic approaches" (proteomics, transcriptomics through micro-array or RNAseq, metabolomics) to analyze the complexity of the plant response to abiotic stresses. The advantage of these approaches is that they provide a global overview of the plant behavior. The disadvantage, is that they are very expensive and time-consuming: thus, they are used on a limited number a sample and do not always permit to reconstitute the evolution of symptom appearance on a time-scale basis.

#### 1.2.5.1 Morpho-agronomic adaptations to salinity

The sensitivity of plant to salt also depends on the stage of the development of plant. For example, seedling and reproductive stages are the most salt-

sensitive stages for rice (Lutts et al., 1995). As far as seed germination is concerned, salt stress affects the germination kinetic and the final seed percentage (Parida and Das, 2005; Singh et al., 2012). Different factors may explain the effect of salt on germination: the limitation of the entry of water into the seeds during the first phase of germination; the decrease in the rate of cell divisions; and an inhibition of the mobilization of reserves (Almansouri et al., 2001; Hakim et al., 2010). So, the capacity of seeds to germinate under salt stress conditions could be an adaptive criterium of crops to tolerate salt stress (Bewley and Black, 2012).

Root and shoot morphologies may also influence salt resistance in plants (Acosta-Motos et al., 2017). Root system is an interface between plants and the soil. Its features such as length, diameter, number of root hairs, number and diameter of xylem vessels, suberin deposition in endodermis and exodermis, and width of the root cortex are crucial for water and nutrients uptake (Steudle, 2000). Root system is the first organ which enters in contact with salinity. As consequence, the plasma membranes of root cells of salt-affected plants become unevenly thick and convolute (Acosta-Motos et al., 2017), thus forming a barrier to salt stress. This anatomic changes may increase the replacement rate of plant water lost (Passioura, 1988). According to Acosta-Motos et al. (2017), salt stress facilitates the over-development of Casparian strip in woody tree by promoting the suberisation of the hypodermis and endodermis. This well-developed Casparian strip could constitute a good filter hampering sodium and chloride entries. Other researchers reported that salt stress triggers the suberisation of endodermis and the development of exodermis in many grass roots (Barberon et al., 2016). These morphological barriers prevent the leaking out of water and desirable ions such as potassium. They also restrict the entry of excessive toxic ions (sodium and chloride) into cells or the xylem (Byrt et al., 2018). Despite these root adaptation, toxic level of salinity can have detrimental effects. On one hand, sodium binds to cell wall components and may have deleterious impact on cell wall properties. On other hand, sodium hampers the binding of other ions and the function of pectin during cell growth (Byrt et al., 2018). As consequence, there is a reduction in the length and area of the roots (Munns, 2002). The roots may seem being the most endangered organs because they are directly exposed to salt stress. However, shoots are also affected, with the implementation of adaptation strategies, depending on the case.

Shoot system is the part of the plant that provides photosynthesis and reproduction. Acosta-Motos et al. (2017) reported that salt stress causes a

decrease in shoot fresh weight, shoot dry weight, and the number of leaves. Salt stress also causes a reduction of shoot length and area (Gandonou et al., 2018; Wang and Nii, 2000). Munns and Tester (2008) reported that shoot growth is more sensitive than root growth. According to these authors, salt stress reduce the rate at which growing leaves expand and lateral buds develop. This decreasing in the leaf area and the biomass causes a significant impact on plant yield and its components (Zörb et al., 2019). Nevertheless, slower growth and yield are also an available measure for plant tolerance under salt stress (Zhu, 2001). Different biochemical or physiological processes are the basis of such an adaptation.

At cytological level, the presence of salt is also manifested by multiple symptoms. Biological membranes are very sensitive to the effects of salinity and can lose their selective permeability either due to a reduction in $Ca^{2+}$ levels induced by salt, or by peroxidation of membrane lipids which are preferred targets for reactive oxygen species induced by abiotic stresses (Feng et al., 2018). The presence of salt in high concentration can also modify the size, number and internal structure of many organelles (Zhu, 2016). Salinity can have an impact on the structure and composition of the wall. It generally induces closure of the stomata (total or partial depending on the dose) in order to limit water loss since the water supply is a limiting factor (Shomer et al., 2003). The consequence of this closure may be to make it more difficult to regulate the thermal status of the leaf, but above all to limit gas exchange and therefore photosynthesis. An inhibition of the latter can have negative consequences on plant growth or less effective osmotic adjustment using soluble sugars.

#### 1.2.5.2 Impact of salt stress on photosynthesis

Photosynthesis is one of the most important physico-chemical processes for plant life. By this pathway, plant converts solar energy into chemical energy. However, photosynthesis is highly sensitive to salinity. The unavailability of water in saline condition leads to stomatal closure (total or partial). As consequence, plant can no longer access to needed carbon dioxide. The reduction of stomatal conductance is one of the first effects of salinity on photosynthesis (Parida and Das, 2005).

In a longer term, salinity may also reduce the content of chlorophyll pigments leading to a marked reduction in photosynthesis (Munns and Tester, 2008). Some enzyme activities involved in the Calvin cycle may also be affected by salt stress. Several authors report an effect of salt on Rubisco, phosphoenolpyruvate carboxylase, ribulose-5-phosphate-kinase, glyceraldhedyde-3-phosphate-dehydrogenase and fructose 1,6-biphosphatase activities (Chaves et al., 2009; Sudhir and Murthy, 2004). Chlorophyll fluorescence analysis is a useful and cheap tool to obtain information on the status of photosystem II (PSII) (Maxwell and Johnson, 2000). Under saline conditions, a reduction in parameters associated with photochemical quenching (Fv/Fm, $\phi_{PSII}$, $q_P$) and with electron transport rate (ETR) is generally observed and provide useful indications regarding the precise parameters involved in alteration of light capture by photosystem machinery. On the other hand, there is frequently an increase in the value of the parameters linked to non-photochemical quenching (Acosta-Motos et al., 2017, 2015). Plants resistant to salinity generally exhibit a stable value of the ETR parameter. A reduction in $q_P$ suggests that chloroplasts are altered in relation to the action of ROS produced under stress (Foyer and Harbinson, 2019). It is also well established that stressed plants are more sensitive to photo-oxidation processes (Chow, 1994), which could be linked to an impact of salinity on the synthesis of protective compounds zeaxanbthine / violaxanthin cycle (Zhang et al., 2012).

Photorespiration can be stimulated under conditions of salt stress, which appears to be the consequence of partial closure of the stomata. If photorespiration can be the cause of oxidative stress, it nevertheless allows to limit the accumulation of an excess of reducing power within the tissues and to allow the conversion of 2-phosphoglycolate and 3-phosphoglycerate which can, to some extent, contribute to the regeneration of ribulose-biphosphate (Kangasjärvi et al., 2012).

#### 1.2.5.3 Impact of salt stress on water status, osmotic adjustment and dehydration tolerance

Water is an essential component for plant life. Unfortunately, under salt stress, plant growth is also reduced due to poor water availability. Salinity drastically affects soil water potential. Indeed, although water is present in saline soil, it is retained by the ions sodium and chloride. This unavailability of water makes

its uptake difficult for roots. As consequence, plant water potential ($\Psi w$), osmotic potential ($\Psi s$), and turgor pressure ($\Psi p$) are affected. Turgor pressure tends to decrease which could be responsible for a decrease in cell elongation. The change in the water status in plant drastically affects the water transport and the transpiration (Lobell et al., 2013). Plants may reduce stomatal conductance through stomata closure, but this has a negative impact on photosynthesis, although it contributes to increase the water use efficiency.

To cope with this osmotic stress, plant can synthesize organic osmolytes for osmotic adjustment. Thus, the water potential of the plant becomes negative relative to that of the soil, thereby ensuring the uptake of water (Pérez-López et al., 2009). Osmotic adjustment is crucial for sustaining cell turgor, which maintains growth, productivity, and yield despite of salt presence in soil. Various osmolytes play the role of osmoprotectant in plant: proline, soluble sugars, and quaternary ammonium compounds. These components do not interfere with normal biochemical mechanism. Osmoprotectants do not only regulate the cellular osmotic adjustment, they are also known to stabilize proteins and enzyme, to mitigate damaging risk caused by ROS, and to prevent membrane injury (Muchate et al., 2016; Singh et al., 2015; Zhang and Dai, 2019).

Proline is an amino acid and important signaling molecule that accumulates in plant cytosol. It functions in stabilizing and protecting cell membrane and different proteins. During salt stress, proline contribute to enhance water uptake, and to maintain antioxidant machinery and ROS scavenging (Ismail and Horie, 2017). Soluble sugars include sucrose, glucose, fructose, trehalose, and so on. They may also, to some extent, contribute to osmotic adjustment, although it is sometimes difficult to discriminate between an active osmotic adjustment and a passive accumulation of sugars resulting from plant growth inhibition occurring without impact on photosynthesis. Moreover, it is frequently considered that mainly non-reducing sugar should accumulate since the accumulation of reducing sugar may induce toxic Maillard reaction with amino acid. There are also sugar alcohols such as mannitol, inositol, and sorbitol. All sugars protect protein from aggregation and denaturation. They adjust cell osmoticum and promote plant growth (Adams et al., 1992). Quaternary ammonium compounds such as betaines are found mainly in the chloroplast. Under salt stress conditions, betaines protect chloroplast, thylakoid membrane, and PSII (Ashraf and Harris, 2004). From a metabolic point of view, osmotic adjustement has a cost in terms of C and N

consumption, and ATP. Most osmoprotectant should thus be easy to produce, able to keep solubility even at high concentration, able to protect cellular structure and they should able to be re-used if stress disappear. Some osmoprotectant, such as proline, accumulate in all plant species while others, such as glycinebetaine or polyols accumulate only in a limited number of species, frequently halophyte plants. For example, glycinebetaine which is a very efficient osmoprotectant does not accumulate in the *Oryza* genus.

It is often considered that the aim of osmotic adjustment is to keep the water balance in order to maintain cell turgor. However, plants are able, to a certain extent, to maintain cell elongation despite a decrease in cell turgor if they increase the cell wall extensibility. This could be achieved by a decrease of pH in the apoplasm (auxin stimulation of $H^+$-ATPase) and even by a modification of cell wall composition in growing tissues (Olmos et al., 2017). This strategy is not incompatible with osmotic adjustment, and the two processes may appear concomitantly.

#### 1.2.5.3.1 Desiccation

The above-mentioned strategies intend to maintain water absorption in order to maintain water content. In some cases, however, plants are able to maintain their metabolism despite a more or less pronounced decrease in water content of the tissues: this might be regarded from a conceptual point of view as a "real tolerance" strategy since the constraint (lack of water) is present in the tissue while other strategies aiming to maintain hydration (osmotic adjustment, stomatal closure) are referred as "avoidance" strategy since they allow the plant to maintain the presence of water, and therefore to escape the constraint. Desiccation tolerance commonly implies a large set of precise protecting molecules among which dehydrin, late-embryogenesis protein, or boiling-stable proteins are assuming a function of chaperone, protecting cellular components from dehydration (Challabathula and Bartels, 2013).

### 1.2.5.4 Salt stress and oxidative stress

Besides osmotic and ionic stresses, salinity also induces oxidative stress throughout the generation of ROS. Actually, oxygen is naturally activated in reactive oxygen species during various metabolic pathway such as photosynthetic electron transport, respiration and photorespiration processes

(Foyer and Harbinson, 2019). In that normal condition, the production and elimination of ROS are in a balance state. But salt stress triggers the over production of ROS; hence, oxidative stress is sometimes considered as a "secondary component of salt stress". For example, the alteration of photosynthetic electron transport chain, in saline condition, leads a vast production of ROS since circulating electron may be ejected from the chain and active the nearby dioxygen molecule (Çiçek et al., 2018). ROS consists of singlet oxygen ($^1O_2$), superoxide anion ($O_2^-$), hydroxyl radical ($OH^\cdot$) and hydrogen peroxide ($H_2O_2$). ROS oxidize cellular components, affect organelle integrity, hinder metabolic activities, and oxidize lipids, proteins and nucleic acids (Bose et al., 2014).

The ROS removal system includes ROS-scavenging antioxidant enzymatic and non-enzymatic machinery. Antioxidants are strong quencher and scavenger of ROS.

The antioxidant enzymes mainly include glutathione reductase (GR), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione peroxidase (GPX), guaiacol peroxidase (GOPX), peroxidase (POX), catalase (CAT), glutathione-S-transferase (GST), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). As far as the non-enzymatic antioxidant are concerned, they include glutathione (GSH), ascorbate (ASC) phenolic compounds, tocopherols, alkaloids, non-protein amino acids, alpha- tocopherol and carotenoids (Bose et al., 2014; Singh et al., 2015).

Each class of enzymes contain numerous proteins which may strongly differ for their affinity to the substrate, kinetics properties, cofactor, and cell location. For example, three major types of SOD are identified as Mn-SOD, Zn/Cu-SOD and Fe-SOD. Some of them are present in cytosol while others are located in chloroplasts and mitochondria. The most complex group of anti-oxidant enzyme is probably peroxidase which could be found in almost all cell compartments and even in apoplast where they may indirectly contribute to lignification processes. Situation is by far less complex for catalase which is only found in peroxisome. This implies that the oxidative status of the plant is regulated by a very complex network of genes, some of them exhibiting low constitutive levels, while others are up-regulated in response to specific environmental conditions and in a contrasting way depending on the considered plant organ (Billah et al. 2021). The fact that several enzymes

function simultaneously in the Halliwell-Asada cycle implies that they must be regulated in coordinated manner (Hasanuzzaman et al., 2021).

#### 1.2.5.5 Salt stress and hormonal status

Plant hormones or phytohormones or plant growth regulators are signaling molecules found in small quantities in cells for modulating physiological and molecular responses by acting either at their site of synthesis or transportation to different plant parts to initiate responses against adverse environmental conditions and genetically programmed developmental changes (Ali et al., 2020). They are involved in almost all the pathways in plants including different development stages, different tissues and different cell types. As far as salinity is concerned, plant hormone take part in salt tolerance throughout many processes: maintenance of plant growth and productivity, increase mineral uptake and photosynthesis, normal shape and size of ultrastructure, morpho-anatomical modulation, ROS reduction, osmoprotectant accumulation, production of antioxidant, ion homeostasis and compartmentalization (Arif et al., 2020). Among plant hormones there are abscisic acid, auxins, brassinoteroids, cytokinins, ethylene, gibberellins, jasmonic acid, polyamines and salicylic acid (Figure 10).

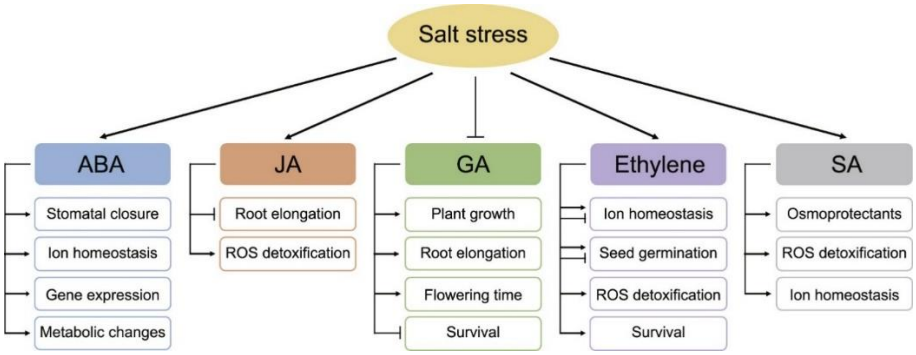


**Figure 10.** The Biological functions of phytohormones in the regulation of salt stress response in plants (Zhao et al., 2020).

ABA is a major hormone involved in the regulation of salt stress response, including the regulation of stomatal closure, ion homeostasis, salt stress-

responsive gene expression, and metabolic changes. JA is required for the inhibition of root elongation and activation of antioxidative enzymes upon exposure to high salinity.

Salt stress reduces the accumulation of endogenous bioactive GAs, leading to inhibition of plant growth and root elongation, delay of flowering, and promotion of survival under high salinity. The effect of ethylene on salt stress tolerance acts in a species- or gene-specific manner. The components involved in ethylene biosynthesis or signaling transduction either positively or negatively regulate ion homeostasis and seed germination, but ethylene induces detoxifying machineries and promotes survival under salt stress (Zhao et al., 2020).

SA participates in the accumulation of osmoprotectants, induction of antioxidative enzymes, and improvement of ion homeostasis under salt stress (Zhao et al., 2020; Liu et al., 2021).

While ethylene is commonly reported to act as a senescing hormone, another class of compound which is not illustrated in Fig. 10 is cytokinins. The compounds are commonly acting as anti-senescing hormone and play major roles in promoting the cell division since they are reported to act as regulators of cell division processes. They may thus be involved in numerous strategies of salinity resistance. They also assume crucial functions in sink-source relationships and therefore have a major influence in yield (Nguyen et al., 2021; Liu et al., 2020).

Polyamines are small aliphatic amines behaving as polycation at cellular pH. The major polyamines are putrescine, spermidine and spermine. They are present in all living organisms and are therefore not specific to plants. However, in higher plants, they assume specific functions, especially in relation to flowering processes and fruit maturation. Putrescine is produced from either arginine or ornithine, and is usually considered to have negative functions in stressed plants and may be considered as indicators of stress. In contrast, spermidine and spermine are produced from reaction between putrescine and the precursor of ethylene S-adenosylmethionine. For this reason, the polyamines and the ethylene pathways are commonly considered as competitive. Spermidine and spermine may assume protective functions, not only as antisenescing compounds, but also in relation to specific interaction between their positive amine groups and negative charges of macromolecules (Alcázar et al., 2021; Pál et al., 2021).

The hormonal status of a plant is a complex property. Indeed, if we except ABA and ethylene, all other classes comprise a huge number of different compounds assuming different functions and exhibiting distinct biochemical properties. Even if phytohormones are frequently studied separately, they interact in numerous way: since different hormones may act on similar cellular targets, the behavior of the plants depends mainly on the ratios or the proportions of the various phytohormone rather than on the absolute concentration of a single compound. This implies the necessity to establish the complete hormonal profile of stressed plants, which is a hard (and expensive!) task (Sasi et al., 2021). It is also important to consider that most hormonal compounds may be conjugated by linking with other compounds: this process frequently impact the strength of the hormone, and the process may be reversible and regulated by a myriad of cell properties (Waadt, 2020). Finally, the impact of phytohormones depends on the presence of specific receptors: for each phytohormone, several receptors exist and the sensitivity of a given tissue to a specific hormones depends on the specificities and the densities of receptors (Hasanuzzaman et al. 2021).

## 1.3 Rice plant

### 1.3.1 Productivity and economic importance in Benin

After wheat and maize, rice is the third most widely grown crop in the world. Rice is the most consumed cereal grain in the world and Khush (2005) estimated that the rice's consumers will reach 5 billion persons by 2030. Rice is a major source of nutrition for about two-thirds of mankind (Vaughan et al., 2003). It provides more than 50% of the calories consumed in several regions of Asia and Africa (Khush, 2005). In 2019, world rice production is 755 million of tons whereas Africa supplies only 5% of this production (Faostat, 2020).

The current Benin rice production is 406 thousand tons (Faostat, 2020). Indeed, with 374,900 ha of irrigable land (141,000 ha of floodplains and 233,900 ha of lowland), Benin has a great potential for rice production. Rice can be grown on five of the country's seven agricultural development poles (Zannou et al., 2018). However, only 21% of this rice potential is used. As consequence, Benin invested nearly 500 billion CFA Francs in 2018 just for the import of rice to meet the demand for 288 thousand tons of rice by consumers. As a consequence, the government of Benin has launched an emergency program to accelerate rice production in order to reach 1 million tons by 2022. The country's goal is to achieve a yield of 7 tons per hectare. Although rice can be grown on five of Benin's seven agricultural development poles (Zannou et al., 2018), it is also crucial to take into account the potential of the saline lands located along Benin's 125 km coastline (from Sèmè-kpodji through Ouidha to Grand-Popo). However, data on the actual level of salinisation of these Beninese soils are practically non-existent. To address this issue, the LAPVESE team (Laboratoire de Physiologie Végétale et d'Etude des Stress Environnementaux) is currently conducting structural studies to assess the level of salinity of the land and water on the Beninese coast.

### 1.3.2 Major Rice Production Systems in Africa

Different rice production systems exist and sometimes, coexist in same region (Figure 11). Rainfed upland rice is usually used on plateau and slopes. Soils are naturally drained without surface water accumulation (Saulter, 2013). It is upland, or dry, rice is grown with minimal water. It is primarily rainfed and highly drought tolerant. Field are steeply or sloping, rarely flooded and aerobic soil. Rice direct seeded on plowed dry soil or dibbled in wet, nonpuddled soil. Lowland rainfed rice is observed in valley bottoms and flood plains. They are slightly sloping bunded fields with non-continuous flooding of variable depth and duration (Zeigler and Puckridge, 1995). Lowland rice mainly subsists on rain or groundwater, but because of its low elevation, it is also flood prone. As far as irrigated rice is concerned, it is used in deltas and flood plains. Rice is grown in bunded fields surrounded by small barriers that keep water in. Irrigated rice is what we traditionally think of when imagining rice cultivation. This rice is planted in paddy field and then, once the seedlings have sprouted, the field is flooded to promote growth. Deepwater and Mangrove swamp rice are generally observed in lagoons and deltas of coastal areas (Defoer et al., 2004). Deep water, or floating, rice grows in conditions of 20 inches of water or more. The fields are slightly sloping or depressed. Mangrove swamp rice (or decrue rice, derived from the French, décrue, or flood recession) is predominantly grown in Africa, where it is planted on river banks and flooded when seasonal rains come.

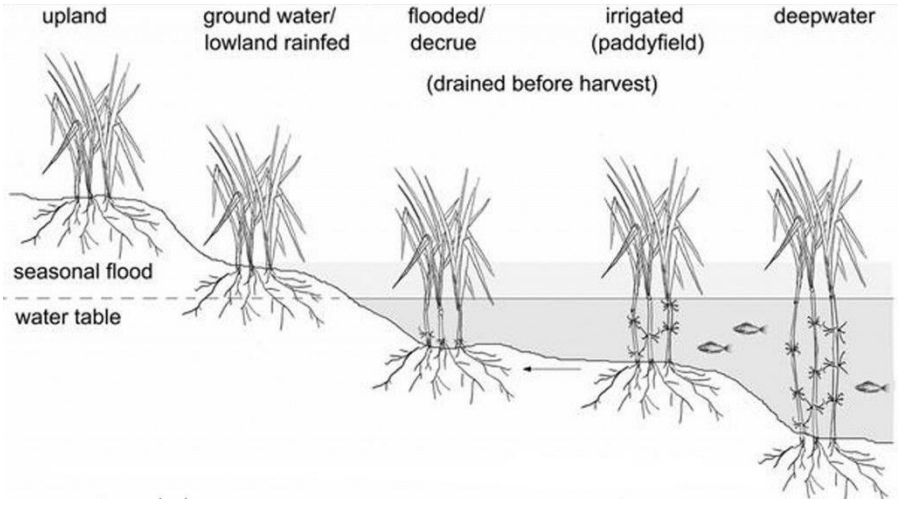


**Figure 11.** Rice production systems (adapted from Saulter, 2013).

### *1.3.3* Genus *Oryza*

The genus *Oryza* includes 24 species (Kellogg, 2009) of which only two are cultivated species*: Oryza sativa* is universally cultivated. *Oryza glaberrima* Steud is mainly cultivated in West Africa. There are also 5 wild species that are present in Africa: *Oryza barthii*, *O. brachyantha*, *O. eichingeri*, *O. longistaminata* and *O. punctata* (Vaughan, 1994). The genus *Oryza* was first described by Linnaeus in 1753 (Smith and Dilday, 2002). Nowadays, taxonomists have classified the genus *Oryza* in the family of *Gramineae* or *Poaceae*, in the subfamily of *Pooideae* or *Oryzoideae*, in the super-tribe of *Oryzanae*, and in the tribe of *Oryzeae* (Duistermaat, 1987; Tzvelev, 1989; Watson et al., 1985). It contains 9 genomes distinguished on the basis of cytological observations and chromosome pairings. It is also remarkable in the diverse ecological adaptations of its species (Table 4; Vaughan et al., 2003).

Table 4 : *Oryza* species: their chromosome number, DNA content, genome group and usual habitat. (Vaughan et al., 2003)

| Section Complex Species | Chromosome number (DNA content [pg/2C]) | Genome group | Usual habitat |
|---|---|---|---|
| ***Oryza*** | | | |
| ***Oryza sativa*** complex | | | |
| *Oryza sativa* L. | 24 (0.91–0.93) | AA | Upland to deepwater; open |
| *O. rufipogon* sensu lato (syn: *O. nivara* for the annual form *O. rufipogon* sensu stricto for the perennial form) | 24 (0.95) | AA | (Annual) Seasonally dry; open (Perennial) Seasonally deepwater and wet year round; open |

| | | | |
|---|---|---|---|
| *O. glaberrima* Steud. | 24 (0.87) | AA | Upland to deepwater; open |
| *O. barthii* A. Chev. | 24 | AA | Seasonally dry; open |
| *O. longistaminata* Chev. et Roehr. | 24 (0.81) | AA | Seasonally dry to deepwater; open |
| *O. meridionalis* Ng | 24 (1.02) | AA | Seasonally dry; open |
| *O. glumaepatula* Steud. | 24 (0.99) | AA | Inundated areas that become seasonally dry; open |
| ***O. officinalis*** complex | | | |
| *O. officinalis* Wall ex Watt | 24 (1.45) | CC | Seasonally dry; open |
| *O. minuta* JS Presl. ex CB Presl. | 48 (2.33) | BBCC | Stream sides; semi shade |
| *O. rhizomatis* Vaughan | 24 | CC | Seasonally dry; open |
| *O. eichingeri* Peter | 24 (1.47) | CC | Stream sides, forest floor; semi shade |
| *O. malapuzhaensis* Krishnaswamy and Chandrasakaran | 48 | BBCC | Seasonally dry forest pools; shade |
| *O. punctata* Kotschy ex Steud. | 24 (1.11) | BB, BBCC | (Diploid) seasonally dry; open |

| | | | |
|---|---|---|---|
| | 48 | | (Tetraploid) forest floor; semi shade |
| *O. latifolia* Desv. | 48 (2.32) | CCDD | Seasonally dry; open |
| *O. alta* Swallen | 48 | CCDD | Seasonally inundated; open |
| *O. grandiglumis* (Doell.) Prod. | 48 (1.99) | CCDD | Seasonally inundated; open |
| *O. australiensis* Domin | 24 (1.96) | EE | Seasonally dry; open |
| Ridleyanae Tateoka | | | |
| *O. schlechteri* Pilger | 48 | Unknown | River banks; open |
| ***O. ridleyi*** complex | | | |
| *O. ridleyi* Hook. | 48 (1.31–1.93) | HHJJ | Seasonally inundated forest floor; shade |
| *O. longiglumis* Jansen | 48 | HHJJ | Seasonally inundated forest floor; shade |
| Granulata Roschev. | | | |
| ***O. granulata*** complex | | | |
| *O. granulata* Nees et Arn ex Watt | 24 | GG | Forest floor; shade |
| *O. meyeriana* (Zoll. et Mor. ex Steud.) Baill. | 24 | GG | Forest floor; shade |
| *O. brachyantha* Chev. Et Roehr. | 24 (0.72) | FF | Rock pools; open |

### 1.3.4 *Oryza sativa* L.

*Oryza sativa* L. is a cereal of Asian origin cultivated worldwide. It was domesticated from the wild rice *Oryza rufipogon* in China between 8,200 and 13,500 years ago (Londo et al., 2006). Today it is cultivated on every continent save Antarctica. The genetic resources of *Oryza sativa* L. are very diverse as the species has undergone numerous domestications and introgressions from the wild. Its dispersal area is very large, which allows it to be cultivated in many different ecosystems. There are currently more than 100,000 different varieties in collection (Garris et al., 2005; Lapitan et al., 2007). *Oryza sativa* L. has pendulous panicles. Its ligule is triangular and pointed. Its lemmas are pubescent or glabrous. The spikelet is carried by branches from the 4th or 5th of the panicle. The grain is awned or not, ridges up to 7 cm. *Oryza sativa* L. groups together two subspecies: *Indica* and *Japonica*. The *Indica* subspecies has long, thin grains. The blade is pale green. The panicle leaf-stem angle is acute. The tillering is high. It has a poor response to nitrogen. Its rooting is generally shallow and it is sensitive to drought. In contrast, the *Japonica* subspecies has short and broad grains. Its blade is dark green. The panicle leaf-stem angle is obtuse. The tillering is weak. The response to nitrogen is moderate. Its rooting is usually deep and it is drought tolerant (Wang and Li, 2005; Zhang et al., 1992).

### 1.3.5 *Oryza glaberrima* Steud.

#### 1.3.5.1 Origin and distribution

*Oryza glaberrima* Steud., commonly known as African rice, was first described in 1855 by Steudel. It is domesticated from the wild rice *Oryza barthii* in West Africa more than 3,500 years ago over an area of 50,000 $km^2$ in the inland delta of the Upper Niger river, in what is now Mali (Li et al., 2011; Portères, 1956; Wang, 2014). Then, *Oryza glaberrima* Steud. conquered several African countries. Figure 12 shows that African rice spread out from the delta of the river Senegal to Lake Chad (Linares, 2002; Ndjiondjop et al., 2018). *Oryza glaberrima* Steud. is also found in America continent because enslaved West African rice farmers brought it to the Brazil,

Carribbean, Carolina, El Salvador, Guyana, Panama, and Suriname (Nayar, 2012; Vaughan, 1994; Van Andel et al., 2016).

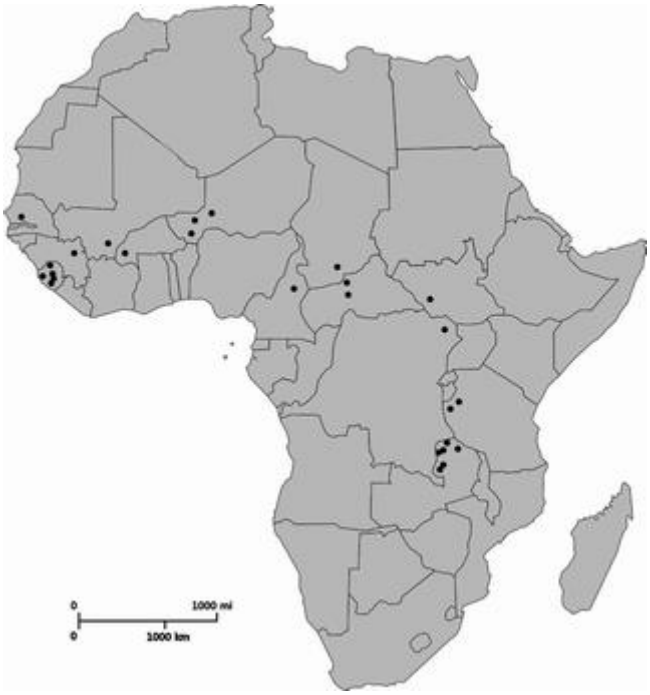


**Figure 12.** Map of *O. glaberrima* distribution. The dots indicate countries of origin of *O. glaberrima* (Ndjiondjop et al., 2018).

### 1.3.5.2 Description

*Oryza glaberrima* Steud. is an annual grass. It is up to 120 cm tall in upland or irrigated conditions (Figure 13). In some floating condition, it is up to 5m (Agnoun et al., 2012). It has fibrous root systems, which can be radicle, mesocotyl, or nodal. Its stems are without ramification, except sometimes in floating conditions. Leaves are alternate and simple. Leaves are composed of the sheath, the blade, the ligule, and the auricles. Leaf sheath is ribbon-shaped, elongated, and attached to the stem. Leaf blade is flat, linear, and narrow. The ligule is short, white, truncate and membranous. The auricles look like a pair of hairy and are located at the junction of blade and sheath. The presence of

auricles is often a convenient guide for differentiating African rice from barnyard grasses. The inflorescence is an ellipsoid, compact, stiff and terminal panicle. At maturity, the inflorescence is erected with ascendant racemose branches. The flower is a spikelet. It is an upper flower bisexual by a stipe consists of an ovary extended by two feathery stigmas, and is surrounded by six stamens. Spikelet carried by third order branches of the panicle. The fruit is a grain, commonly called seed. It is a laterally compressed caryopsis tightly enveloped by lemma and palea. Lemma and palea are respectively the glume inferior and the glume superior of the seed. Seed is often terminated by a beak or a short ridge. When seed is sowing, *Oryza glaberrima* emerge in 4 days or more if dormancy is higher. Then the vegetative is very rapid, and followed by reproductive repining phases. *Oryza glaberrima* grows on a wide range of soils (from sea-level to 1700 m altitude). It is a short-day plant, but some cultivars are photosensitive or not. Its ideal temperature is 30 °C. Above 35 °C, spikelet fertility is affected and yield is reduced. Below 25 °C, growth and yield decreased (Agnoun et al., 2012; Yoshida, 1981).

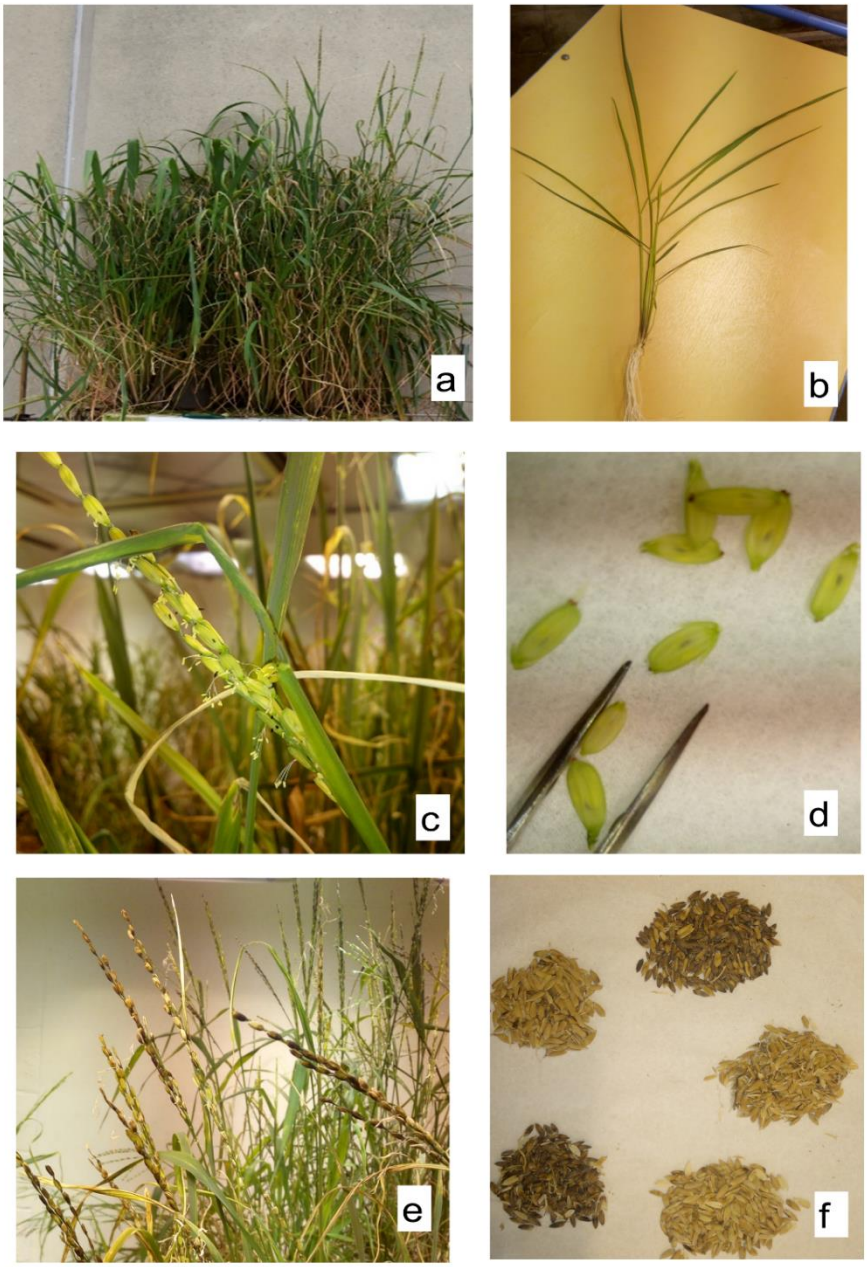


**Figure 13.** *O. glaberrima* in term of plant **(a-b)**, opened spikelets **(c)**, closed spikelets **(d)**, panicle **(e)**, and polymorphism of grains **(f)**.

#### 1.3.5.3 Resistance to biotic and abiotic stresses

Linares (2002) reported that *Oryza glaberrima* has a significant ginning, a red pericarpus and fragile grains. African rice tends also to break easily during milling, thus making rice less attractive when polished. Despite these disadvantages, many researchers have found various resistance traits of African rice to diverse biotic and abiotic stresses (Table 5). As far as biotic stresses are concerned, *Oryza glaberrima* is proofed to resist to African gall midge (Nwilene et al., 2002; Williams et al., 1999). While bacterial leaf blight was recognized as one of the most disease on Asian rice (Mizukami and Wakimoto, 1969), there was not damage on yield of African rice (Djedatin et al., 2011). *Oryza glaberrima* resists also to green rice leafhopper (Fujita et al., 2010) and to rice blast disease (Bidaux, 1978; Silue, 1991). Rice yellow mottle virus (RYMV) is the causal pathogen of yellow mottle of rice. Affected plants show a yellow or orange discoloration of the leaves, reduced tillering and stunting of the plants, and sterility of the flowers (Bakker, 1974). However, *Oryza glaberrima* is proofed to resist to rice yellow mottle virus (Albar et al., 2003; Ghesquière, 2003; Ndjiondjop et al., 1999; Thiémélé et al., 2010; Thottappilly and Rossel, 1993), to nematodes (Cabasan et al., 2015; Plowright et al., 1999; Reversat and Destombes, 1998), and to weed competition (Fofana and Rauber, 2000; Johnston et al., 1988; Jones et al., 1997; Moukoumbi et al., 2011).

Abiotic stresses are one of major constraints of rice production in Africa. The resistance of *Oryza glaberrima* to those stresses is considered to be one of its good agronomic characteristics. African rice resists to grain shattering (Futakuchi et al., 2012; Montcho et al., 2013), to lodging (Futakuchi et al., 2008), to soil acidity (Sano et al., 1984), to iron toxicity (Sahrawat and Sika, 2002; Sikirou et al., 2018), and to submergence (Aliyu et al., 2015; Futakuchi et al., 2001; Sakagami et al., 2013; Watarai and Inouye, 1998). Climate warming and sea level raising placed drought and salinity are the first of limit of rice production. Despite the complexity of these stresses, there are evidences of resistance of *Oryza glaberrima* to salinity (Akbar et al., 1986; Linares, 2002; Platten et al., 2013) and to drought (Efisue and Derera, 2012; Maji, 2001; Mohapatra, 2009; Ndjiondjop et al., 2012; Shaibu et al., 2018).

Table 5: Resistance of *Oryza glaberrima* to biotic and abiotic stresses

| Stresses | | References |
|---|---|---|
| **Biotic** | African gall midge | Nwilene et al., (2002) ; Williams et al. (1999) |
| | Bacterial leaf blight | Djedatin et al. (2011) |
| | Blast | Bidaux (1978) ; Silue (1991) |
| | Green rice leafhopper (*Nephotettix cincticeps* Uhler) | Fujita et al. (2010) |
| | Nematodes | Cabasan et al., (2015) ; Plowright et al., (1999) ; Reversat and Destombes (1998) |
| | Rice yellow mottle virus | Albar et al., (2003) ; Ghesquière (2003) ; Ndjiondjop et al., (1999) ; Thiémélé et al., (2010) ; Thottappilly and Rossel (1993) |
| | Weed competition | Fofana and Rauber (2000), Johnson et al., (1998) ; Jones et al., (1997) ; Moukoumbi et al., (2011) |
| **Abiotic** | Grain shattering | Futakuchi et al., (2012) ; Montcho et al., (2013) |
| | Drought | Efisue and Derera (2012) ; Maji (2001) ; Mohapatra (2009) ; Ndjiondjop et al., (2012) ; Shaibu et al., (2018) |
| | Iron toxicity | Sahrawat and Sika (2002) ; Sikirou et al., (2018) |
| | Lodging | Futakuchi et al., (2008) |
| | Salinity | Akbar et al., (1986) ; Linares (2002); Mondal et al., (2018) ; Platten et al., (2013) |
| | Soil acidity | Sano et al., (1984) |
| | Submergence | Aliyu et al., (2015) ; Futakuchi et al., (2001) ; Sakagami et al., (2013) ; Watarai and Inouye (1998) |

#### 1.3.5.4 Cultural and nutritional importance

Asian rice is the most cultivated rice in Africa. Nevertheless, the work on the perceptions of producers and consumers on African rice in Danyi (Togo) within 30 households has shown that African rice remains a perennial agricultural heritage. Although its production is mainly family-owned, 67% of households have adopted it and 30% cultivate both species with a preference for African rice (Gnacadja et al., 2017). Indeed, *Oryza glaberrima* is closely associated with specific social practices. Linares (2002) pointed out some ritual contexts in which African rice survived since its domestication. The author reported that in communities south of the Casamance River, the majority of the inhabitants are practitioners of the traditional *awasena* religion. Their traditions relate that the supreme deity, the rain ‘‘god’’ known as Emitai, gave ‘‘Diola rice’’ (*O. glaberrima*) to the ancestors. This rice carried a life-giving power that explained the ultimate origins of the land that Emitai had best owed upon the inhabitants. For this reason, some varieties of O. *glaberrima* should always be planted, to preserve the link to the ancestors, and to Emitai, who sends rain.

O. *glaberrima* also has specific organoleptic qualities. Researches carried out on grain protein content of African rice and Asian rice varieties have showed that varieties of *Oryza glaberrima* have, on average, twice as much protein content as those of *Oryza sativa* species (Watanabe, 2004). Compared to Asian rice, *Oryza glaberrima* has also higher gelatinization temperature and amylose content (Wang et al., 2015). Its high amylose content may explain why *Oryza glaberrima* is digested slowly, then providing a great post-meal satiety (Cai et al., 2015; Chung et al., 2011; Nuijten et al., 2013). Ndjiondjop et al., (2018) reported that analysis of data on grain quality of the African rice collection conserved at the AfricaRice Genetic Resources Unit displayed that *Oryza glaberrima* has good characteristics such as firmer texture and unique pasting proterties (low breakdown, low peak, and higher setback viscosities).

#### 1.3.5.5 Interest of *Oryza glaberrima* for improving rice

In the 1950s, collection and conservation *Oryza glaberrima* germplasm began. Different national and international research institutes proceeded to this collection: Office de la Recherche Scientifique et Technique d'Outre-Mer (ORSTOM), Institut de Recherches Agronomiques Tropicales (IRAT), and International Institute for Tropical Agriculture (IITA). Nowadays, the great *Oryza glaberrima* collection is available at AfricaRice genebank. Breeders of this institute argue that *Oryza glaberrima* can produce yield of about 5-6 tons per hectare (Ndjiondjop et al., 2018).

African rice genome has been sequenced (Monat et al., 2017; Wang et al., 2014). Table 6 shows a comparison between both cultivated rice species. Genes for resistance to biotic stress have been identified in *O. glaberrima*: this is the case of the RYM3 gene which gives the Tog5307 variety of *O. glaberrima* resistance to "rice yellow mottle virus". This disease is a major constraint on rice cultivation. Crosses were made between Tog5681 and the susceptible variety of *O. sativa* IR64: these crosses confirmed the monogenic and dominant character of the resistance and the gene was localized over a possible interval of 15 kb (Pidon et al., 2017). Likewise, Cabasan et al. (2015) identified resistance strategies in *O. glaberrima* to nematodes (and in particular *Meloidgyne graminicola*) that could be transferred to hybrid lines obtained by crossing with *O. sativa*. Several studies have shown that the starch produced in the albumen of *O. glaberrima* exhibits properties quite distinct from those observed in *O. sativa*. Gayin et al. (2016) compared the molecular and thermal properties of starches from the two cultivated species and their hybrids. The specific properties of *O. glaberrima* and, to a lesser extent of hybrids, would be closely related to a higher amylose content and longer chains. Differences between viscosity directly condition the cooking behavior (Gayin et al., 2016). The aromas of the grain are also criteria that differentiate the two species. Cho et al. (2014) point out that among the 41 volatile compounds that are responsible for the aroma, several are specific to *O. glaberrima* and are not found in *O. sativa*. This is particularly the case for 4-ethylphenol and (E, E) -2,4-heptadienal (Cho et al., 2014). Finally, the grain's mineral content is also different. Rice is a food poor in certain essential elements, such as Fe or Zn. The grain of *O. glaberrima* is much richer in these elements and that these properties can be transferred to hybrid lines (Ohmori et al., 2016). *Oryza glaberrima* therefore constitutes an interesting material for aspects of human health.

Table 6: Gene feature statistics of *Oryza glaberrima* and *Oryza sativa* genomes (Ndjiondjop et al., 2018)

| Feature | *O. glaberrima* | *O. sativa* sp. Japonica (MSU V. 6.1) |
|---|---|---|
| Gene count | 33,164 | 41,620 |
| Exon count | 142,095 | 171,163 |
| Intron count | 108,931 | 129,543 |
| Gene size (bp) | 2,393 | 2,306 |
| Exon size (bp) | 255 | 254 |

Interesting properties in *O. glaberrima* are highlighted in relation to the problem of iron toxicity. This constraint appears in flooded areas, when conditions of anoxia and acidic soils lead to a massive reduction of $Fe^{3+}$ to $Fe^{2+}$. As iron is then absorbed in excess, its accumulation is responsible for multiple metabolic disorders related to an overproduction of free radicals and oxidative stress. The synthesis of ferritin on the one hand, the management of oxidative stress and Fe sequestration in the sheath on the other hand are involved in the resistance of African rice to this stress (Majerus et al., 2007b). Stimulation of ferritin synthesis in *O. glaberrima*, however, differs from what is observed in most plant species in that ABA does not intervene to stimulate the transcription of the corresponding genes (Majerus et al., 2009). Other compounds involved in the regulation of the water status of the plant by osmotic adjustment (proline, soluble sugars, etc.) would also be involved in the establishment of resistance strategies to iron toxicity and polyamines are also present in higher concentrations in the most resistant lines (Majerus et al., 2007a). Silicon can contribute to the resistance of rice to iron toxicity, but the capacities for upgrading Si under these conditions depend on the genotype and are not identical for *O. sativa* and *O. glaberrima* (Mbonankira et al., 2015; Dufey et al., 2014). As consequence, *O. glaberrima* constitutes a particularly interesting source of new alleles which could be used in improvement programs by exploiting a strategy based on a QTL approach (Dufey et al., 2015). Several QTL mapping studies were undertook to assess indices for plant rice survival, development, and reproduction under normal vis-a-vis salt stress conditions. Various molecular markers in the form of isozymes and

DNA markers were designed and used : AFLP, CAPS, RAPD, RFLP, RAD-Seq, SSR, VNTRs (Singh et al., 2021). These QTL mapping studies in rice for salinity tolerance were focused on traits such as those governing germination rate, seedling survival, $Na^+$ accumulation in roots and shoots, $Na^+/K^+$ ratio, total biomass, leaf bronzing, chlorophyll content, $Cl^-$ ion concentration in leaves, shoot dry weight at maturity, and yield-related parameters (Dhanayaka et al., 2018 ; Gregorio, 1997 ; Hossain et al., 2015 ; Pandit et al., 2010 ; Sabouri and Sabouri, 2008 ; Wang et al., 2011)

It is undoubtedly for the management of water stress and drought resistance that crosses between the two species are often considered. Bocco et al. (2012) analyzed the drought resistance of introgression lines derived from crosses between the IR64 variety of *O. sativa* and the TOG5681 variety of *O. glaberrima*. The plants were subjected to water stress by stopping irrigation 45 days after sowing. Data obtained during two consecutive seasons confirm a negative effect of water stress on plant height, spikelet fertility, grain yield, and total leaf area. The leaf temperature, the "leaf rolling index", the time from sowing to anthesis and the maturity of the grain are increased. On the other hand, the chlorophyll contents estimated on the basis of a SPAD counter and the average number of tillers per plant are not affected. The authors identify introgression lines that provide significantly higher yield values than the best NERICA varieties (Bocco et al., 2012).

Lysimeter experiments carried out on several varieties of *O. sativa*, *O. glaberrima* and recombinant lines made it possible to underline the interest of the African material, and to put in direct relation a certain number of morphological properties of the root system with the maintenance water absorption capacities in partially desiccated soil (Kijoji et al., 2013). According to Ndjiondjop et al. (2012), the Tog5691, Tog6679 and Tog5591 lines of *O. glaberrima* are particularly resistant to drought. These authors reported that on these lines water stress has only a very limited effect on plant height and grain yield. While flowering and grain ripening are delayed in susceptible lines, these resistant lines show little change at this level. The authors underline an important significant interaction between the environment and the genotype for parameters such as the height of the plants, the date of flowering, the percentage of green leaves, the duration of maturation but, in general, these parameters appear better correlated with yield in *O. glaberrima* than in *O. sativa* (Ndjiondjop et al., 2012). Other parameters taken into consideration are leaf temperature and aerial part biomass (Kijoji et al., 2014). The portions of the genome responsible for resistance to drought in

*O. glaberrima* are gradually identified and notable progress is obtained in the context of crossing between the two species (Bimpong et al., 2011).

### *1.3.6 NERICA: An introgression of Oryza glaberrima Steud. into Oryza sativa L.*

Although African rice *Oryza glaberrima* Steud. is known to withstand various abiotic and biotic stresses on the African continent, it is generally characterized by a wide range of undesirable agronomic traits (Linares 2002; Vikal et al., 2007). To address this weakness, interspecific breeding programs have been initiated by AfricaRice to combine the economically important traits of Asian and African rice. These include the high yield of the Asian parent and the ability of the African parent to thrive in harsh environments. For example, a team of rice breeders developed a stable and fertile progeny from a cross between Asian rice, *O. sativa* L. and African rice, *O. glaberrima* Steud. (Jones et al., 1997; Jones et al., 1999a). *O. sativa* L. was used as the female parent and *O. glaberrima* Steud. as the male parent. The resulting interspecific progeny was named New Rice for Africa (NERICA). NERICA is produced by conventional crossing and is therefore not a genetically modified rice. Fully fertile, genetically stable, and morphologically diverse, NERICA was developed either by the refined method of conventional breeding or by using anther culture and double-haploidization techniques specifically developed to overcome sterility and speed up the breeding process. Crosses were made and embryo rescue was used to remove fertilized embryos and grow them in an artificial medium. Anther culture allowed rapid fixation and helped to maintain interspecific lines combining the desirable characteristics of both parents (*O. sativa* L. and *O. glaberrima* Steud.). Obtaining the interspecific hybrid, NERICA, is a real scientific breakthrough. Previous conventional crossing efforts elsewhere in the world to develop interspecific rice hydrides had failed, yielding only infertile offspring of the two species used for the cross. This breakthrough established AfricaRice's leading role in interspecific hybridization and anther culture for rice (Jones, 1999b; Somado et al., 2008).

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# PART II

# Experimental section

This doctoral project is supported by several scientific experiments whose major results are presented here as published articles, submitted or to be submitted to a journal. The choice of this form of presentation could generate some redundancies in the sections "introduction" and "material and methods".

# To get started…

## What do we want to know ?

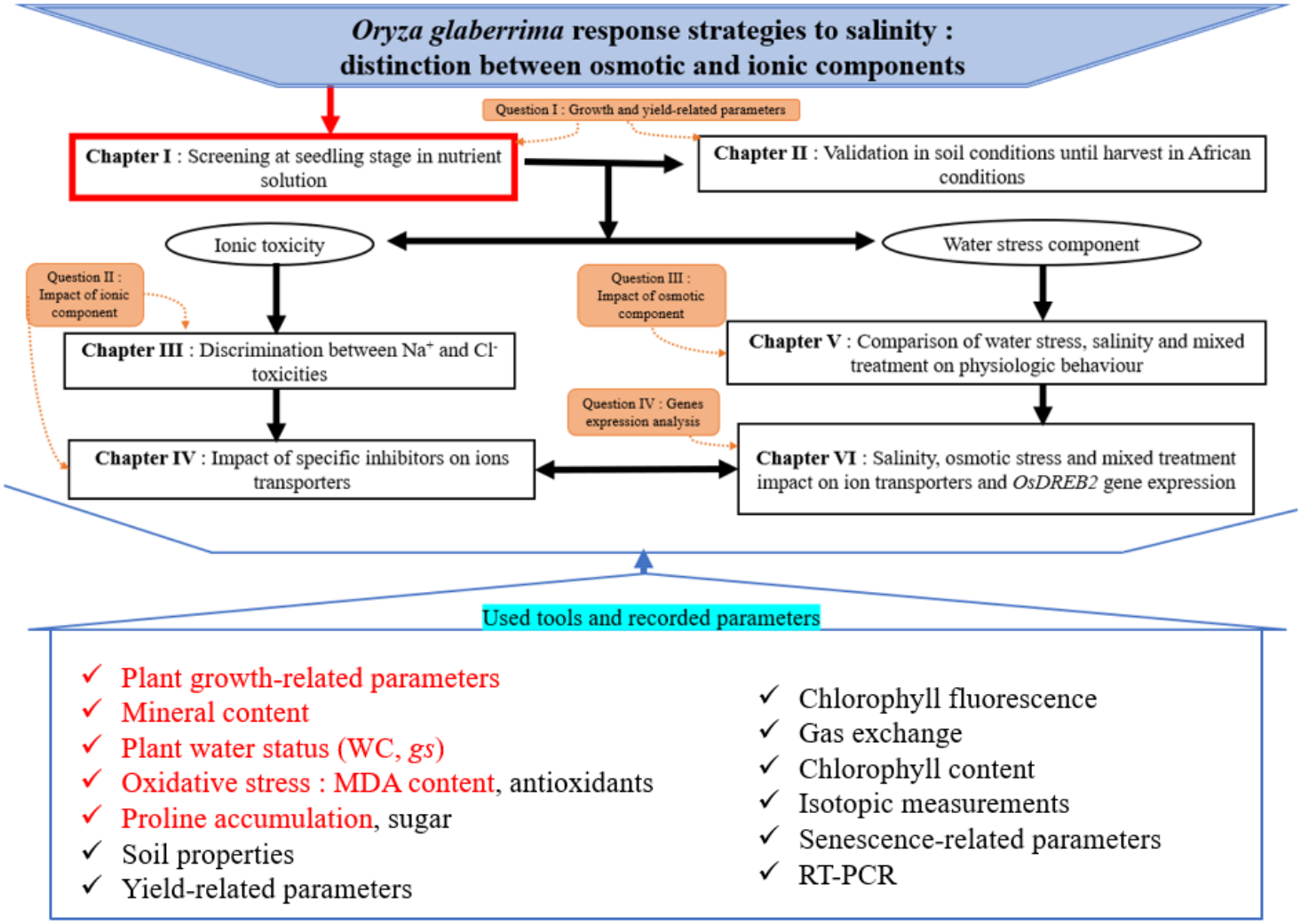
The aim of the first Chapter will be to identify two cultivars of *Oryza glaberrima* exhibiting contrasting levels of resistance to salinity (NaCl) : one sensitive cultivar and one resistant cultivar. This material will be used throughout the thesis for subsequent analysis.

## What is our strategy ?

A screening will be performed using a set of 25 cultivars of *Oryza glaberrima* provided by Africa Rice. This set of cultivars was chosen in the framework of a collaboration between our laboratory and Africa Rice on the basis of their putative interest for cultivation in Benin since they are well adapted to local climatic and phytosanitary conditions.

The screening will be performed under controlled conditions in phytotrons to ensure that no interferring parameters will influence the plant behaviour. Plants will be cultivated in nutrient solution and screening will be performed for two weeks at the seedling stage (33 days old-plant) at a NaCl dose of 60 mM. It is well known that seedling stage before tillering is one of the most sensitive stage in rice. Although no data are available for African rice, the dose of 60 mM is frequently considered as a sub-lethal dose inducing more than 50% mortality until 15 days in *Oryza sativa*. A similar procedure will thus be used with our material. The cultivar I Kong Pao from *Oryza sativa* will be included in the screening as a reference : this « moderately salt-resistant » cultivar is well characterized for its response to salinity and frequently used under our laboratory conditions.

Beside the rate of survival, we will consider plant growing parameters, ion content, osmotic adjustment, photosynthesis-related parameters, malondialdehyde content (as an estimation of secondary oxidative stress) and accumulation of the osmoprotectant proline.




**Overview of questions addressed during this thesis and their relationships with chapters.**

# Chapter I. Screening for salinity tolerance at the young seedling stage in *Oryza glaberrima* Steud.: a morphophysiological approach

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## Abstract:

Salinity is an important environmental constraint which strongly reduces crop yield in several areas in the world. Rice (*Oryza sativa*) is considered as a salt-sensitive species and improvement of salt resistance is a major goal for plant breeders. Some species in the *Oryza genus* may constitute an interesting source of gene for rice improvement. The African rice *Oryza glaberrima* is poorly described for its response to salt stress. The present study considers 25 accessions of *O. glaberrima* exposed during two weeks to 0 or 60 mM NaCl in nutrient solution. Morphological (fresh and dry weight, shoot and root length, number of leaves, number of tillers,…) and physiological (ion concentration, osmotic adjustment, proline and malondialdehyde content, stomatal conductance, net photosynthesis and instantaneous transpiration) were recorded and used to perform principal component analysis allowing us to consider three contrasting groups (salt-resistant, medium, and salt-sensitive). Most of the tested lines appeared more salt-sensitive than the moderately salt-resistant cultivar I Kong Pao from *Oryza sativa*. Salt-sensitivity index was higher for roots than for shoots and *Oryza glaberrima* was poorly efficient for regulation of $Na^+$ translocation from the root to the shoot. Some accessions such as TOG5307 however were able to maintain a high net photosynthesis under salt conditions and exhibited a high level of tolerance to accumulated $Na^+$ ions and a high capacity for osmotic adjustment. It is concluded that these salt-tolerant accessions constitute a promising material for rice improvement through inter-specific crosses with *O. sativa*.

**Key words**: African rice, NaCl, *Oryza glaberrima*, salinity, salt stress.

# Introduction

Rice is an important staple food for more than half of the human population. It provides 50% of the calories consumed in several areas of Asia and Africa (Kush, 2005). In numerous African countries, however, rice production is still not sufficient and the estimated rice import in Africa accounts for several millions of tons each year which represent more than one-fourth of its requirements (Nhamo et al., 2014). There is consequently an urgent need to increase rice production, especially considering that the world's population is predicted to reach around 10 billion people by 2050 (Hoang et al., 2016). Because of a very limited potential for future expansion of arable lands, such a goal implies to extend rice culture to marginal lands which are not used at the moment for rice culture.

Numerous environmental constraints are limiting rice production. Among them, drought and soil salinity are probably the most prevalent abiotic stresses hampering plant growth and development. Salinity affects more than 830 $10^6$ ha in the world. Of the 230 $10^6$ ha of the world's irrigated lands, 45 $10^6$ ha (20%) have already been affected by salt, and the problem is increasing due to sea level rise and to erratic irrigation (Munns, 2005). Salinity imposes a double constraint to plants: an osmotic stress and an ionic toxicity. Osmotic stress is related to the presence of a high external salt concentration which decreases the external water potential and thus compromises water uptake by the plant. The ionic component of salt stress is due to progressive accumulation of toxic ions such as $Na^+$, excess of $Cl^-$ and salt-induced decrease in essential elements, mainly $K^+$ (Acosta-Motos et al., 2017).

Rice is very sensitive to salt stress (Hoang et al., 2016) and a NaCl dose as low as 50 mM NaCl in nutrient solution is considered to be lethal for salt-sensitive cultivars (Yeo and Flowers, 1986; Zhu et al., 2001). Salt-sensitivity in rice varies depending on the phenological stage with young seedlings and flowering stages being considered as the most sensitive ones (Lutts et al., 1995; Hakim et al., 2010). Despite a high number of available cultivars, *Oryza sativa* still performs poorly under salt stress conditions (Singh and Sengar 2014). The most salt-tolerant genotypes are tall indica landraces which suffer from major agronomic drawback under West African conditions. Numerous evidences are now available regarding the loss of genetic diversity encountered by *O. sativa* since its domestication (Caicedo et al., 2007).

Some biotechnological tools may be used to improve salt-tolerance of existing cultivar (Lutts et al., 1999; Singh and Sengar, 2014) but both *in vitro* selection and transgenic approaches suffer from technical and/or social limitations. A promising alternative is to use other species of the *Oryza* genus for breeding purposes in order to improve abiotic stress resistance in *Oryza sativa* (Atwell et al., 2014). The cultivated African rice *Oryza glaberrima* is receiving a considerable attention since several years. This species was domesticated 3000 years ago. Although it was progressively replaced by the high-yielding Asian rice *O. sativa*, this hardy species has qualities that make it superior to Asian rice as a subsistence crop (Linares, 2002). This species suffers from easy shedding of grains, resistance to milling, greater breaking of grains, red pericarp and lower yield (Nayar, 2010). However, it also presents a greater resistance to various biotic and abiotic stresses. Resistance to yellow mottle virus (Pidon et al., 2017) and to the nematode *Meloidgyne graminicola* (Casaban et al., 2015) has been identified in this species. *Oryza glaberrima* produces extra-tillers allowing it to efficiently compete with weeds (Sarla and Mallikarjuna Swamy, 2005). It displays interesting properties for resistance to iron toxicity (Majerus et al., 1999; Dufey et al., 2015) and to submergence (Sakagami et al., 2009). It also possesses promising characters for drought resistance (Bimpong et al., 2011; Bocco et al., 2012; Ndjiondjop et al., 2012a; Kijoji et al., 2013).

Numerous strategies, such as a near-isogenic line (NIL)-*S37*, have been efficiently used by breeder to overcome hybrid sterility between *O. sativa* and *O. glaberrima* (Shen et al., 2015). Several varieties issued from selected recombined plants obtained after interspecific crosses between the two species are now available and known as NERICA (New Rice for Africa) varieties. Most of them resemble *O. glaberrima* during early growth displaying weed competitive ability, and to *O. sativa* at the reproductive stage allowing high yielding capacities (Jones et al., 1997; Sarla and Mallikarjuma Swamy, 2005). Despite the large set of data available for water stress resistance in *O. glaberrima*, information regarding salinity resistance in this species remains poorly documented. Awala et al. (2010) reported that *O. glaberrima* CG14 appeared rather salt-sensitive but that hybrids obtained after crossing this line with the *O. sativa* WAB56-104 cultivar produced hybrids exhibiting a high level of salinity resistance. Platten et al. (2013) quantified $Na^+$ accumulation in several salt-exposed lines of *O. glaberrima* and identified a specific gene (*OgHKT1;5*) which partly contribute to regulate $Na^+$ absorption and translocation.

Screening for salinity resistance in *O. glaberrima* is still required in order to identify the most promising material to integrate in interspecific crosses with *O. sativa*. The present study therefore screened 25 lines of *O. glaberrima* exposed to salinity at the seedling stage, and analyzed their overall behavior in terms of growth in relation to physiological properties influencing salt-stress resistance.

## Material And Methods

### Plant material and growing conditions

Twenty-five accessions of *Oryza glaberrima* and one genotype of *Oryza sativa* were used in the present study. Seeds of *Oryza glaberrima* were obtained from Africa Rice (Abomey-Calavi, Benin) and are listed in Table 1. The cultivar I Kong Pao (IKP) of *Oryza sativa* was used as a reference since this genotype exhibited a medium level of salt resistance and is well adapted to environmental conditions where *O. glaberrima* usually occurs (Lutts et al., 1995; 1999; Zhu et al., 2001). Seeds were germinated in glass vessels on two layers of Whatman (85 mm, Grade 1) filter paper moistened with 10 ml of deionized water. They were placed in a growth chamber at 25-21 °C (day/night) under a 16h daylight period (150-220 µmole $m^{-2}$ $s^{-1}$). Illumination was provided by SYLVANIA fluorescent tubes (F36W/840-T8, cool white). For each genotype, 25 seeds were place in each glass vessel.

Eleven-day old seedlings were transferred in phytotrons maintained at 24°C/21°C (day/night). They were fixed on polystyrene plates floating on Yoshida et al. (1976) nutritive solution. For each genotype, seedlings were distributed among tanks containing 1.5 l of nutrient solution. Illumination was provided by PHILIPS metal iodide lamp (HPIT/400W) for 16 h $d^{-1}$ at a photon flux density (PAR) of 180-200 µmoles $m^{-2}$ $s^{-1}$. Daytime humidity was 65%. The nutrient solution was renewed every week and tanks were randomly rearranged in the phytotron. Salt stress was applied when plants were 33 days-old: NaCl was added to nutrient solution in order to reach a final concentration of 60 mM to one half of the tanks, the other half being used as unstressed controls. Salt stress was applied for two weeks.

**Morphological and physiological analysis**

The length of the longest leaf (LHL), the number of tillers (NT) and the number of leaves (NL) were measured. The stomatal conductance ($g_s$) was estimated using porometer (type AP4-UM-3) on 6 plants per treatment. The net photosynthesis (*A;* net carbon assimilation rate was estimated under constant photosynthetic photon flux (500 µmol. $m^{-2}$ $s^{-1}$), the instantaneous transpiration (*E*) and the intercellular $CO_2$ content (*C*i) were measured on the youngest fully expanded leaf of 6 plants per treatment using a water vapor analyzer (LCA 2 8.7, ADC, Great Amwell, England) and an air supply unit (ASU 10.87, ADC) mounted in series in an open system. All these measurements were performed at the time of stress imposition and after 2 weeks of treatment.

Plants were then harvested at the end of stress exposure. Roots and shoots were separated, and roots were quickly rinsed during 30 s in deionized water to remove ions from the root surface and the free spaces. Shoots and roots were weighed for fresh weight determination, then incubated in an oven at 72 °C during 48h until constant dry weight were reached. Water content ($mL.g^{-1}$ WC) was estimated using the equation: WC = (FW – DW)/DW.

The sensitivity index (SI) that means the difference between dry matter production of salt-treated plants and the control, expressed in percent of the matter, was calculated according to the following expression:

$$SI = (100 \times (DWcontrol - DWtreatment)) / DWcontrol$$

The mean tolerance index ($TI_{Na}$) to endogenous $Na^+$ was estimated for each physiological parameter as the ratio between the relative value of this parameter recorded in stress conditions expressed as a percentage of the mean value recorded in control conditions divided by the concentration of accumulated $Na^+$ in the considered organ:

$$TI_{Na} = ((X_{NaCl}/X_{control}) \times 100)/Na^+ \text{ content}$$

**Ions measurement**

For ion content determination, *c.a.* 100 mg DW were weighed. Samples were placed in flask of 10 ml and digested with nitric acid (68%) at 80 °C. After

complete evaporation, residues were dissolved with $HNO_3$ (68%) + $HCl_{cc}$ (1:3, v/v). Solution was filtered using a layer of Whatman (85 mm, Grade 1). The filtrate was used to determine the cations concentration (K, Na, Mg, Ca and Fe) by flame emission using Atomic Absorption Spectrophotometer (Thermo scientific S series model AAS4). The analysis was performed on three plants per treatment and each sample was analyzed in triplicate

**Osmotic potential measurement**

For osmotic potential ($\Psi s$) measurement, roots and leaves of three plants per treatment were frozen in liquid nitrogen at harvest. After 3 cycles of frozen/thaw, samples were centrifuged at 15,000 g during 15 min at 4 °C. The supernatant corresponding to the extracted sap was used to measure the osmolarity (c) using a Wescor 5500 vapour pressure osmometer as previously detailed (Lutts et al. 1999). The $\Psi s$ was then calculated according to:

$\Psi s$ (MPa) = - c (mosmoles.$Kg^{-1}$) $\times 2.58 \times 10^{-3}$ according to the Van't Hoff equation.

**Malondialdehyde and proline concentrations**

Malondialdehyde (MDA) is a common indicator of oxidative stress. It was quantified on roots and leaves of 3 plants per treatment using the method of Heath and Packer (1968). Frozen 250 mg were homogenized in prechilled mortar with a solution of 0.5% TBA in 20% trichloroacetic acid (TCA) and were heated to 95 °C for 30 min and then samples were cooled at room temperature. After centrifugation at 3000 rpm for 5 min the absorbance of supernatant was read at 532 nm, and the values of the non-specific absorbance were taken at 600 nm and subtracted from the original (532 nm). The MDA concentrations were calculated using the molar extinction coefficient of 155 mM $cm^{-1}$.

Proline (a soluble osmoprotectant) content was measured as described by Bates et al. (1973). Frozen tissue (0.5 g) were homogenized in 10 mL of 3% sulfosalicylic acid and then centrifuged at 10,000 × g. The supernatant (0.5 mL) was mixed with 1 mL of glacial acetic acid and 1 mL of 2.5% acid ninhydrin (2.5 g of ninhydrin dissolved in a mixture of 60 mL glacial acetic acid and 40 mL 6 M phosphoric acid). The mixture was incubated for 1 h at

100◦C and then the reaction was terminated by cooling in an ice bath. The reaction mixture was extracted with 2 mL of toluene, mixed vigorously with the test tubes stirrer for 15 s. The chromophore-containing toluene was warmed to room temperature and absorbance was read at 520 nm using toluene as a blank. Proline concentration was estimated on the basis of a standard curve.

**Statistical analysis**

The statistical analyses were performed with the "JMP Pro 12" soft-ware. Mean values and standard error (SE) were obtained from at least 3 replicates for genotypes. A *P-valu*e of <0.05 was considered to be statistically significant. A two-way ANOVA was performed to detect cultivar, treatment, and interaction effects. Screenings among accessions and between treatments were displayed using principal component analysis (PCA) with R 3.3.2 Statistics software ('FactoMineR' package). Pearson correlation between analyzed parameters were also performed for three contrasting groups (salt-resistant, medium, salt-sensitive) using the 'corrplot' package in R 3.3.2 Statistics software.

## Results

In the absence of salt, TOG5685 had the highest shoot and total biomass and difference was significant when compared with IKP (Fig1A). In the presence of 60 mM NaCl, TOG5307, TOG5385 and TOG5588 exhibited the highest total biomass while TOG5885, TOG5949 and TOG5672 presented the lowest values. The mean sensitivity index was estimated for roots, shoots and whole plants (Table 1). Mean sensitivity remained low for TOG5307 and TOG5775, suggesting that these accessions displayed a similar level of tolerance comparatively to IKP. In contrast, SI values were especially high for TOG5949 and TOG5685. The mean leaf water content was similar in all genotypes under control conditions with a mean value of 83.7% in shoots and 89.4% in roots. Although the mean shoot WC decreased in response to salinity (78.4%), no significant difference was recorded between the considered accessions. The number of leaves was reduced in response to 60 mM NaCl

and was the highest in CG17 and the lowest in TOG5949 and TOG5672 (Fig. 1B).

**Table 1**: List of accessions from *Oryza glaberrima* used on the current study and their corresponding salt-sensitivity index estimated for roots. shoots and a whole plant basis. I Kong Pao (IKP) is a moderately salt-resistant variety from *Oryza sativa*.

| Variety | Provenance | SI Root | SI Shoot | SI Total |
|---|---|---|---|---|
| IKP | Thailand | 67.1 | 34.8 | 38.8 |
| CG17 | Senegal | 73.0 | 50.0 | 54.1 |
| CG20 | Senegal | 77.7 | 49.6 | 55.7 |
| TOG5293 | Nigeria | 84.9 | 47.9 | 55.1 |
| TOG5307 | Nigeria | 47.0 | 9.7 | 16.1 |
| TOG5385 | Nigeria | 74.6 | 44.2 | 49.6 |
| TOG5390 | Nigeria | 85.7 | 56.0 | 62.0 |
| TOG5420 | Nigeria | 75.7 | 48.6 | 53.2 |
| TOG5440 | Nigeria | 82.0 | 42.1 | 49.3 |
| TOG5442 | Nigeria | 72.6 | 52.4 | 55.7 |
| TOG5456 | Nigeria | 78.8 | 50.1 | 55.2 |
| TOG5479 | Nigeria | 86.1 | 52.1 | 58.3 |
| TOG5482 | Nigeria | 65.3 | 46.1 | 49.8 |
| TOG5500 | Nigeria | 72.3 | 43.2 | 46.3 |
| TOG5566 | Nigeria | 76.3 | 60.1 | 62.8 |
| TOG5588 | Ghana | 77.1 | 38.6 | 46.2 |

| | | | | |
|---|---|---|---|---|
| TOG5641 | Nigeria | 73.6 | 48.5 | 51.2 |
| TOG5666 | Nigeria | 88.5 | 56.2 | 63.7 |
| TOG5672 | Nigeria | 70.8 | 57.6 | 59.3 |
| TOG5681 | Nigeria | 69.7 | 45.9 | 48.7 |
| TOG5685 | Nigeria | 82.3 | 68.2 | 70.7 |
| TOG5775 | Libéria | 35.6 | 25.4 | 26.9 |
| TOG5885 | Liberia | 77.1 | 64.3 | 66.5 |
| TOG5949 | Nigeria | 91.3 | 79.9 | 82.3 |
| TOG5969 | Nigeria | 82.7 | 54.3 | 60.1 |
| TOG5979 | Nigeria | 75.4 | 62.6 | 64.8 |

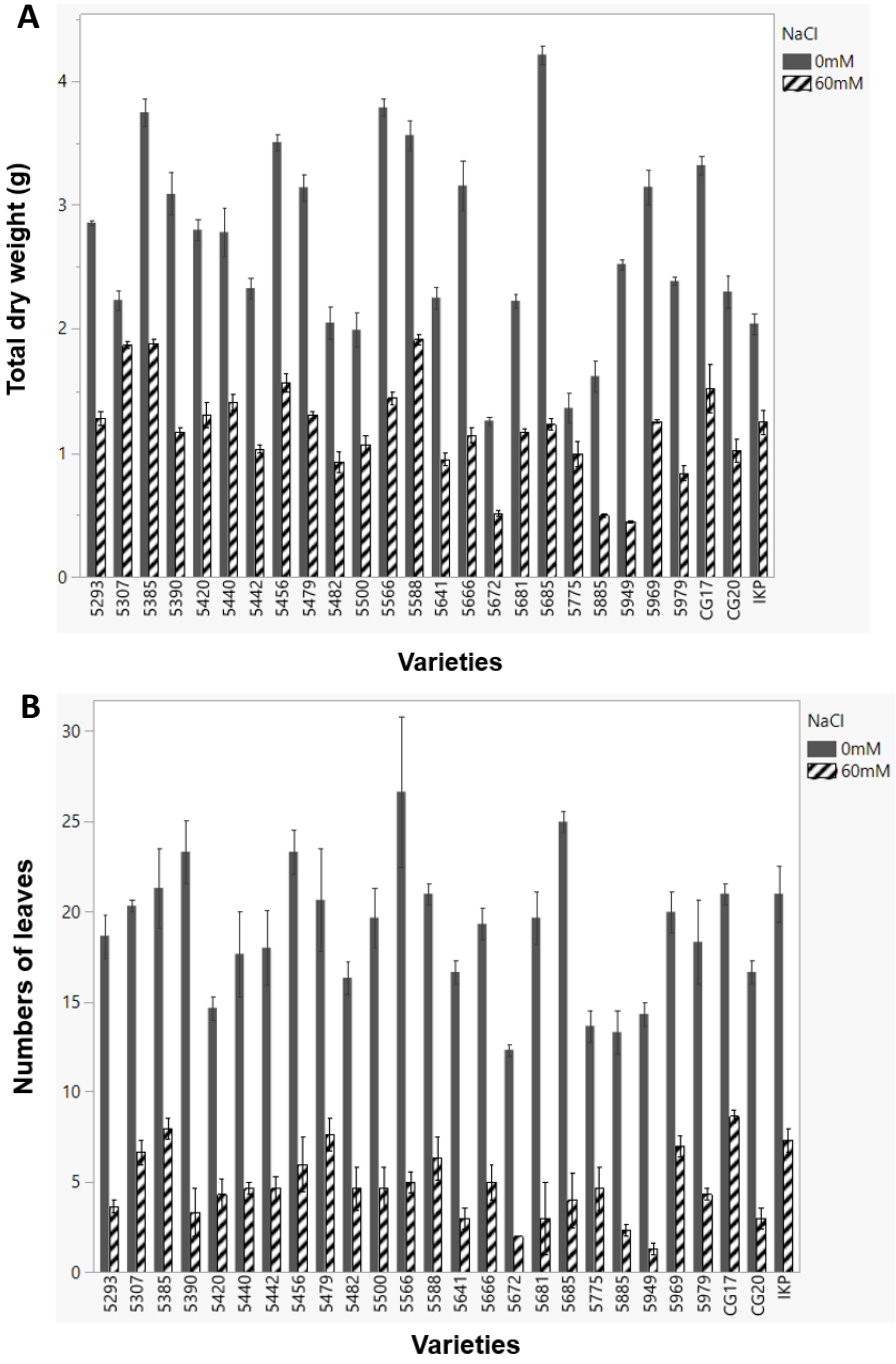


**Fig. 1**. Total dry weight (A) and mean number of leaves (B) in 25 accessions of *Oryza glaberrima* and one moderately salt-tolerant cultivar of *Oryza sativa* (IKP). Seedlings

were exposed for two weeks to 0 (control) or 60 mM NaCl (stress) in hydroponic culture under controlled environmental conditions. Each value is the mean of 3 replicates and vertical bars are standard errors.

Stomatal conductance in numerous varieties of *O. glaberrima* cultivated under control conditions was clearly higher than in IKP (Fig. 2A). Salinity decreased stomatal conductance except in one single variety of *O. glaberrima* (TOG5979) where stomatal conductance remained unaffected by NaCl. In the absence of salt, instantaneous transpiration (*E;* Fig 2B) was higher in TOG5775 and TOG5500 than in other genotypes. Salinity reduced *E* values which however remained higher in TOG5420, TOG5775, TOG5307 and TOG5588 than in other genotypes.

Net photosynthesis (*A*; Fig. 2C) slightly varied among genotypes under control conditions. From a relative point of view, differences among accessions appeared higher in NaCl-treated plants than in control conditions: while *A* values recorded in TOG5307 remained low affected by NaCl, salinity almost completely inhibited photosynthesis in TG5949 and strongly decreased it in CG20 and TOG5390. In the absence of salt, *C*i values was higher in numerous *O. glaberrima* varieties than in IKP (detailed data not shown). Salinity only had a limited impact on *C*i values, the highest concentration being recorded for TOG5442, TOG5482, TOG5775 and TOG5979.

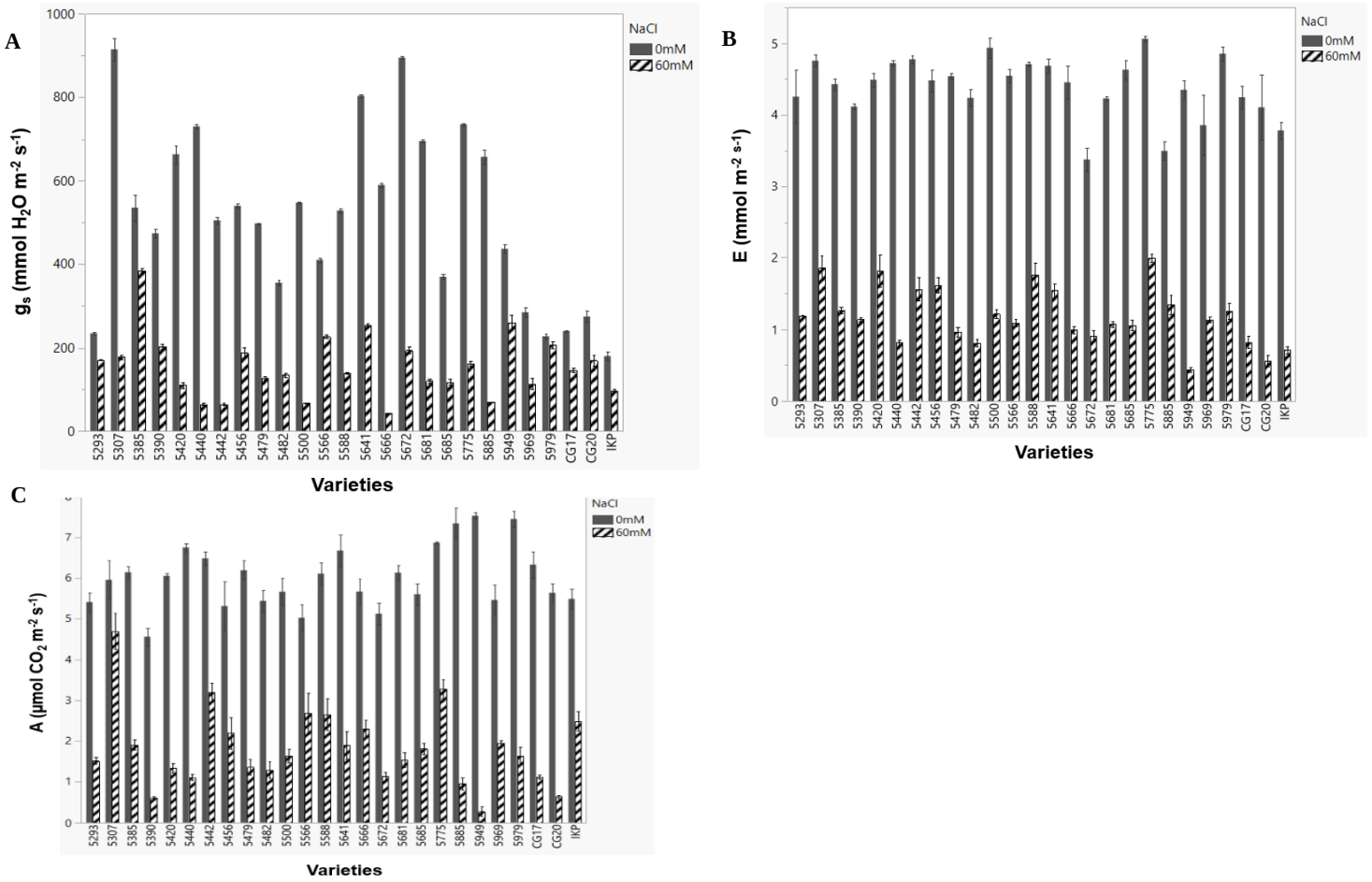


**Fig. 2**. Stomatal conductance (A), instantaneous transpiration (B) and net photosynthesis (C) in 25 accessions of *Oryza glaberrima* and one moderately salt-tolerant cultivar of *Oryza sativa* (IKP). Seedlings were exposed for two weeks to 0 (control) or 60 mM NaCl (stress) in hydroponic culture under controlled environmental conditions. Each value is the mean of 3 replicates and vertical bars are standard errors.

The root osmotic potential (Fig. 3A) was statistically similar in all accessions in the absence of salt, but salinity decreased root $\Psi$s, mainly in TOG5775, TOG5969, CG17, TOG5979, TOG5456, TOG5672, and TOG5500. In the presence of NaCl, these accessions displayed significant lower root $\Psi$s values than IKP. An important decrease in the shoot water potential was also observed (Fig. 3B), recorded values being minimal for TOG5456, TOG5500 and GC17.

Numerous accessions of *O. glaberrima* accumulated more $Na^+$ in roots and shoots than IKP (Fig. 3C and 3D). Some of them, such as TOG5307 presented high concentration in the roots but was able to restrict $Na^+$ accumulation in the shoot, at least to some extent. In contrast, TOG5681 exhibited high $Na^+$ concentration in the roots and in the shoots, suggesting that both $Na^+$ absorption and translocation were not efficiently regulated in this accession. Salt stress reduced the $K^+$ concentrations in all plant organs (Fig. 3C and 3D). The mean root $K^+$ concentration was especially low for TOG5390, TOG5420, TOG5949, CG20 and TOG5385 in plants exposed to NaCl (detailed data not shown). For the main morphological parameter and net photosynthesis, we estimated the mean tolerance index to accumulated $Na^+$ (Table 2). The $TI_{Na}$ values were globally higher for roots (root DW and root length) and varied depending on the cultivar. However, for almost all considered parameters the $TI_{Na}$ value was higher for IKP than for *O. glaberrima*, whatever the accession. Some accessions of *O. glaberrima*, however presented high $TI_{Na}$ values for some parameter, as it was the case for TO5307 (shoot DW, root DW and net photosynthesis), TOG5566 (net photosynthesis) and TOG5775 (shoot and root DW).

The shoot MDA content was similar in all accessions for control plants (Fig. 4A) but salt stress obviously increased MDA in all tested accessions, indicating the occurrence of a secondary oxidative stress. However, MDA remained low in TOG5440 and high in TOG5672. Proline (Fig. 4B) also accumulated in shoots as a result of salt exposure. While IKP, TOG5666 and TOG5420 exhibited the highest concentration of proline, TOG5307, TOG5385 and TOG5479 presented the lowest concentration in salt-treated shoots.

**Table 2**: Tolerance index (TI) of accessions from *Oryza glaberrima* estimated for shoot dry weight (TI_Shoot DW), roots dry weight (TI_Root DW), plant height (TI_plant height), number of tillers (TI_Tillers), number of leaves (TI_Leaves), net photosynthesis (TI_Net.photo) and root length (TI_Root.length). I Kong Pao (IKP) is a moderately salt-resistant variety from *Oryza sativa*.

| Variety | TI_Shoot DW | TI_Root DW | TI_Plant height | TI_Tillers | TI_Leaves | TI_Net.photo | TI_Root.length |
|---|---|---|---|---|---|---|---|
| **IKP** | **1.68** | **2.23** | **1.91** | **1.49** | **0.90** | **1.17** | **4.96** |
| | | | | | | | |
| CG17 | 0.95 | 1.66 | 1.15 | 1.00 | 0.79 | 0.34 | 4.81 |
| CG20 | 0.71 | 1.33 | 0.54 | 0.35 | 0.26 | 0.16 | 4.85 |
| TOG5293 | 0.73 | 0.94 | 0.83 | 0.50 | 0.28 | 0.39 | 5.16 |
| TOG5307 | **1.59** | 2.09 | 1.30 | 0.78 | 0.58 | **1.39** | 3.19 |
| TOG5385 | 0.76 | 1.63 | 0.78 | 0.85 | 0.51 | 0.42 | 4.03 |
| TOG5390 | 0.62 | 1.20 | 0.65 | 0.31 | 0.20 | 0.19 | 6.76 |
| TOG5420 | 0.86 | 1.34 | 1.10 | 0.84 | 0.50 | 0.37 | 4.11 |
| TOG5440 | 1.10 | 0.77 | 1.03 | 1.00 | 0.50 | 0.31 | 2.24 |
| TOG5442 | 0.89 | 1.19 | 1.02 | 0.87 | 0.48 | 0.92 | 2.80 |
| TOG5456 | 0.83 | 1.21 | 1.19 | 0.67 | 0.43 | 0.69 | 4.65 |
| TOG5479 | 0.80 | 1.20 | 1.27 | 0.98 | 0.62 | 0.37 | 5.86 |
| TOG5482 | 0.66 | 1.69 | 1.01 | 0.58 | 0.40 | 0.33 | 4.81 |
| TOG5500 | 0.94 | 1.23 | 0.84 | 0.52 | 0.39 | 0.48 | 3.57 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| TOG5566 | 0.80 | 1.16 | 1.21 | 0.51 | 0.36 | **1.04** | 4.23 |
| TOG5588 | 1.05 | 1.29 | 0.98 | 0.82 | 0.52 | 0.75 | 3.77 |
| TOG5641 | 0.64 | 1.15 | 0.97 | 0.58 | 0.26 | 0.41 | 3.22 |
| TOG5666 | 0.82 | 0.74 | 1.11 | 0.69 | 0.49 | 0.76 | 4.33 |
| TOG5672 | 0.86 | 1.20 | 1.16 | 0.78 | 0.33 | 0.45 | 2.87 |
| TOG5681 | 0.73 | 1.35 | 0.86 | 0.56 | 0.20 | 0.33 | 3.26 |
| TOG5685 | 0.58 | 1.11 | 0.87 | 0.58 | 0.29 | 0.59 | 4.55 |
| TOG5775 | **1.41** | **3.62** | 1.03 | 0.86 | 0.65 | 0.90 | 5.11 |
| TOG5885 | 0.60 | 1.19 | 1.01 | 0.40 | 0.33 | 0.24 | 4.39 |
| TOG5949 | 0.41 | 0.61 | 1.09 | 0.47 | 0.19 | 0.07 | 5.85 |
| TOG5969 | 0.67 | 0.90 | 0.88 | 0.78 | 0.51 | 0.52 | 3.88 |
| TOG5979 | 0.60 | 1.33 | 0.91 | 0.61 | 0.38 | 0.36 | 4.43 |
| | | | | | | | |
| **Mean** | **0.86** | **1.36** | **1.03** | **0.71** | **0.44** | **0.54** | **4.30** |

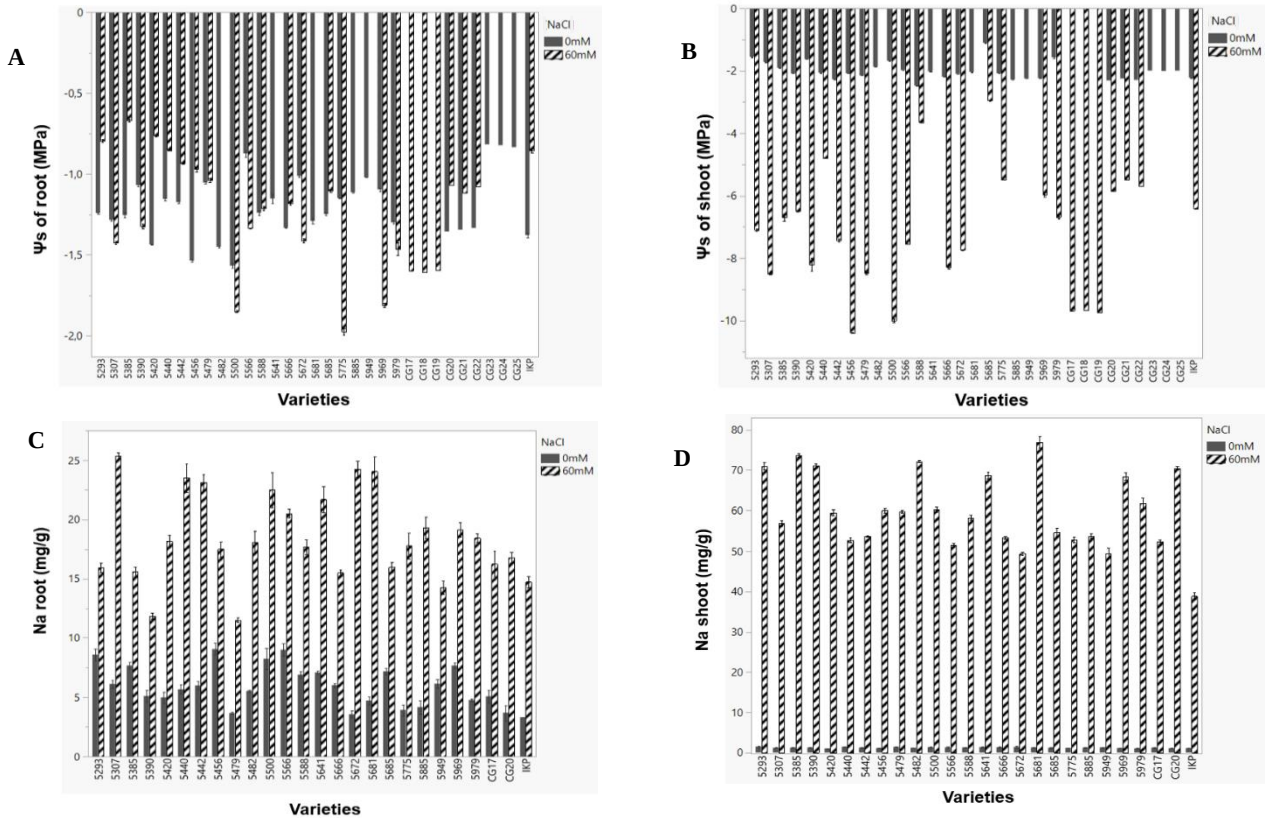


**Fig. 3**. Root osmotic potential (A), shoot osmotic potential (B), root $Na^+$ concentration (C) and shoot $Na^+$ concentration (D) in 25 accessions of *Oryza glaberrima* and one moderately salt-tolerant cultivar of *Oryza sativa* (IKP). Seedlings were exposed for two weeks to 0 (control) or 60 mM NaCl (stress) in hydroponic culture under controlled environmental conditions. Each value is the mean of 3 replicates and vertical bars are standard errors. Pay attention that no values are available for shoot *Ψs* in the case of salt stressed plants of TOG5482, TOG5885 and TOG5949, and for root *Ψs* in the case of salt stressed plants of TOG5482, TOG5641, TOG5681, TOG885 and TOG5949 as a consequence of a lack of available material.

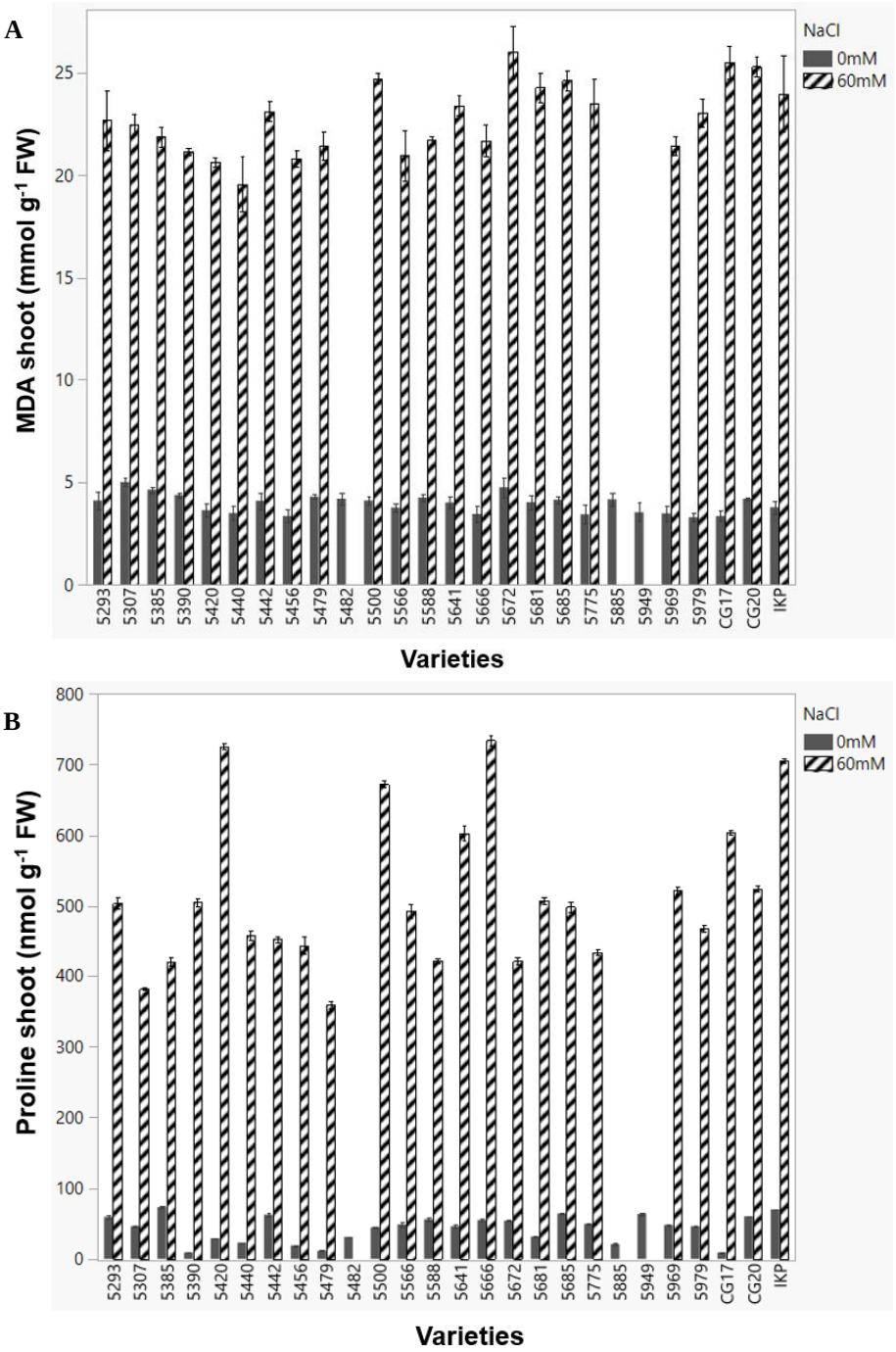


**Fig. 4**. Shoot malondialdehyde (A) and shoot proline concentration (B) in 25 accessions of *Oryza glaberrima* and one moderately salt-tolerant cultivar of *Oryza sativa* (IKP). Seedlings were exposed for two weeks to 0 (control) or 60 mM NaCl

(stress) in hydroponic culture under controlled environmental conditions. Each value is the mean of 3 replicates and vertical bars are standard errors. . Pay attention that no values are available for salt stressed plants of TOG5482, TOG5885 and TOG5949 as a consequence of a lack of available material.

A first principal component analysis (PCA) was performed in order to reveal the global impact of NaCl on the whole tested material in relation to the set of analyzed parameters (except proline and MDA since data were not available for three accessions). PCA revealed that 77.49% of variance was explained by the principal component 1 (Dim 1) and the principal component 2 (Dim 2) (Fig. 5; Fig S.A). Dim1 alone displayed 68.65% of variance. Parameters that have the highest value factor coordinate for the Dim 1, with the highest variable contribution, based on correlations, were, at left, toxic ion ($Na^+$) and sub-stomatal cavity $CO_2$ concentration (*C*i). At right, they were potassium content, stomatal conductance ($g_s$), water content, instantaneous transpiration (*E*), net photosynthesis (*A*), height of plant, number of leaves, number of tillers, plant dry weight, root length, root dry weight and shoot dry weight.

The second plot showed the discrimination of seedlings in response to salt treatment in multivariate space of the first PCA (Fig. 5B). Dim1 displayed a clear opposition between the two groups: at the left, the salt-stressed seedlings and the control seedlings at right. Salt-stressed seedlings showed positive correlation along the left side of Dim 1 which is link to toxic ion ($Na^+$) and sub-stomatal cavity $CO_2$ concentration (*C*i). They had also displayed negative correlation along the right side of Dim 1 which is link to growth parameters. So the left side Dim 1 revealed the seedlings whom were severely affected by salt stress. Despite the high Ci content, salt-stressed seedlings showed low content of net photosynthesis (A), of stomatal conductance ($g_s$) and of transpiration (E). This indicates that their whole photosynthetic machinery was severely affected by salt stress. The control plants, on the other hand, are positively correlated with photosynthesis and growth parameters. But they are negatively correlated to sodium toxicity (Dim 1). However, Dim 2 revealed a great agro-morphological diversity within the control group. While some control plants are very large, others are very small and have high photosynthetic activity. This confirms that our study has taken into account the varietal diversity within *Oryza glaberrima*.

In order to discriminate salt-tolerant and salt-sensitive accessions, we performed a second PCA on salt-stressed seedlings. This PCA showed that 26.85%, 15.18%, 13.36, and 10.46% of variance were respectively explained by the principal component 1 (Dim 1), the principal component 2 (Dim 2), the

principal component 3 (Dim 3), and the principal component 4 (Dim 4) (Fig. 6 ; Fig S.B). Here we focused on the 42.03% of variability explained by Dim 1-2. Because the analysis of Dim 1-3 (40.21%) or Dim 1-4 (37.31%) gave very similar results to what is observed in Dim 1-2. Furthermore, although Dim 1-2 only explained 42.03% of the variability in the data, the remaining 57.97% of variability carried by the other 14 principal components seemed to provide quite subtle information that confirmed the results observed in Dim 1-2 (data not shown).

Parameters that have the highest value factor coordinate for the Dim 1, with the highest variable contribution, based on correlations, were potassium content, water content, instantaneous transpiration (*E*), net photosynthesis (*A*), height of plant, number of leaves, number of tillers, plant dry weight, root dry weight and shoot dry weight. The Dim 2 had high positive loading for root sodium concentration, root potassium concentration, instantaneous transpiration, net photosynthesis, height of plant, water content and high negative loading for plant dry weight, root dry weight, shoot dry weight, stomatal conductance ($g_s$), root length and number of leaves (Fig. 6A). The Fig. 6B shows the position of all tested accessions under salt stress in the multivariate space of the Fig. 6A. Dim1 highlighted on the opposition between three groups species under salt stress which could be related to salt-tolerant, salt-sensitive and “medium”.

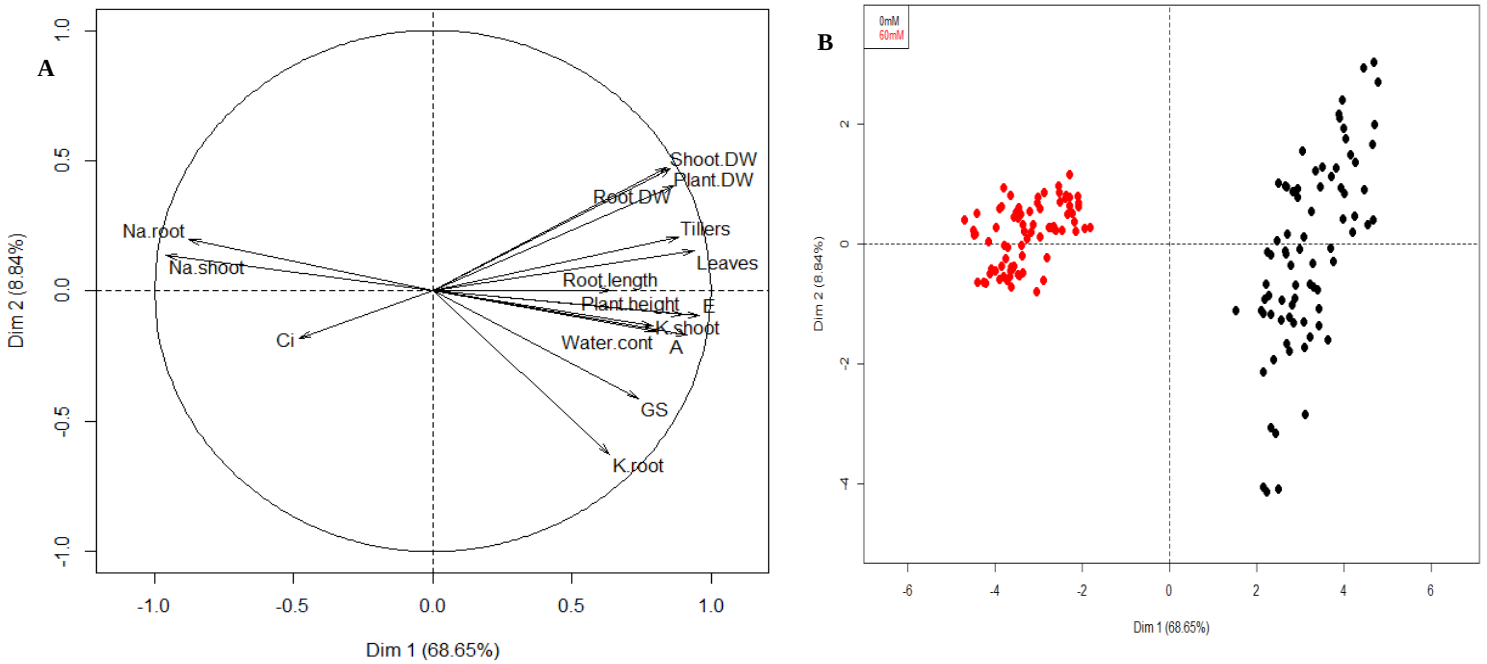


**Fig. 5**. Principal Component Analysis (A and B) of growth and physiological parameters in the seedlings rice (25 accessions of *O. glaberrima* and one moderately salt-resistant cv of *O. sativa*) cultivated for 2 weeks in the presence of 0 (control) or 60 mM NaCl (stress). (A) Variable graph and (B) individual graph of PCA showing the control and salt-stressed seedlings groups. Only significant parameters were shown ($P < 0.05$) in (A). Plant.DW, plant dry weight; Shoot.DW, shoot dry weight; Root.DW, root dry weight; Water.cont, total water content; GS, stomatal conductance; K.shoot, shoot potassium concentration; K.root, root potassium concentration; Na.shoot, shoot sodium concentration; Na.root, root sodium concentration; *C*i, sub-stomatal cavity $CO_2$ concentration; *E*, instantaneous transpiration; A, net photosynthesis; Plant.height, height of plant; Leaves, number of leaves; Tillers, number of tillers; Root.length, root length.

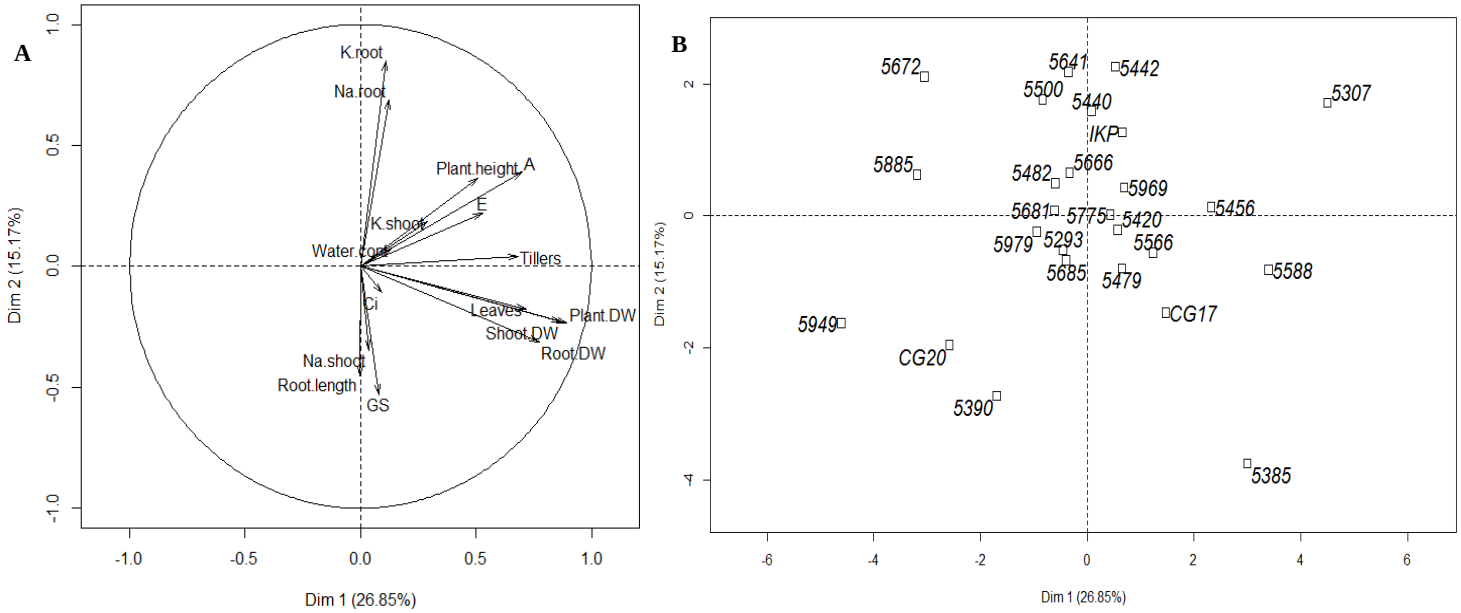


**Fig. 6**. Principal Component Analysis (A and B) of growth and physiological parameters in salt-stressed seedlings of rice (25 accessions of *O. glaberrima* and one moderately salt-resistant cv of *O. sativa* (IKP)) cultivated for 2 weeks in 60 mM NaCl. (A) Variable graph and (B) individual graph of PCA showing the impact of salt stress in seedlings. Only significant parameters were shown ($P < 0.05$) in (A). Plant.DW, plant dry weight; Shoot.DW, shoot dry weight; Root.DW, root dry weight; Water.cont, total water content; GS, stomatal conductance; K.shoot, shoot potassium concentration; K.root, root potassium concentration; Na.shoot, shoot sodium concentration; Na.root, root sodium concentration; Ci, sub-stomatal cavity $CO_2$ concentration; E, instantaneous transpiration; A, net photosynthesis; Plant.height, height of plant; Leaves, number of leaves; Tillers, number of tillers; Root.length, root length.

Under salt stress the salt-tolerant lines showed a strong positive correlation along the Dim 1 and this part of the plot was characterized by potassium content, stomatal conductance ($g_s$), water content, instantaneous transpiration (*E*), net photosynthesis (*A*), height of plant, number of leaves, number of tillers, plant dry weight, root dry weight and shoot dry weight. Lines TOG5307, TOG5456, TOG5588, TOG5385 and CG17 belong to the salt-tolerant group. Indeed, they managed to maintain high photosynthetic activity and growth despite the presence of NaCl. In contrast, the salt-sensitive species displayed a strong negative correlation along the Dim 1. Species TOG5885, TOG5672, TOG5949, TOG5390 and CG20 belong to salt-sensitive group. The "center-reaction" group contains the other lines which have a weak correlation with analyzed parameters by PCA.

## Discussion

The present works confirms that *Oryza glaberrima* displays high variability in terms of salinity resistance at the seedling stage. When compared to the moderate salt-resistant *cv*. from *Oryza sativa* I Kong Pao, most accessions of *O. glaberrima* appear at first sight rather salt sensitive. In the absence of salt, numerous accessions of *O. glaberrima* displayed a high vegetative growth leading to a high total plant biomass. Such a property might be, at least partly, related to a high net photosynthesis (Fig. 2). Since the mean *C*i value was usually high in *O. glaberrima*, it could be postulated that such a high photosynthesis may be linked to a high level of gas exchange which is confirmed by the high values recorded for stomatal conductance (Fig. 2). We found indeed a positive correlation between *A* and $g_s$ values under control conditions. The fast vegetative growth of *O. glaberrima* at the seedling stage is frequently considered as an advantage in terms of weed competition (Sarla and Mallikarjuna Swamy, 2005; Nayar, 2010).

Salt stress induces both an osmotic and an ionic constraint in stressed plants (Acosta-Motos et al., 2017; Munns, 2005). Salinity resistance is considered to rely on avoidance mechanisms, allowing the plant to limit Na and Cl absorption and accumulation, and tolerance mechanisms allowing the plant to maintain efficient metabolism despite toxic ion accumulation. Under our experimental conditions, our tested material appeared able to efficiently manage with physiological drought since no obvious decrease was recorded

for the leaf water content. In contrast, salt-treated plants accumulated high Na concentration, suggesting that the ionic constraint is the major problem for *O. glaberrima*. Although the osmotic component is frequently considered as the first component acting on salt-treated plants (Munns, 2005) it has been previously demonstrated that sodium may reach high toxic concentration in a short term basis in rice and could be toxic even before modification of the plant water status (Lefèvre et al., 2001). The high transpiration rate recorded in some accessions of *O. glaberrima* (Fig. 2) should probably contribute to increase $Na^+$ concentration on a short term basis. Total $Na^+$ concentration was indeed higher in the shoots than in the roots: although roots are commonly acting as a barrier sequestering toxic ions and avoiding their accumulation in photosynthetic tissues, our results suggest that this accumulation was not efficient in *O. glaberrima* which could be related to the fact that at the young seedling stage, endoderm is not completely differentiated in the young seedling rice plant (Yeo and Flowers, 1986; Zhu et al., 2001). Despite the lower accumulation of $Na^+$ in the root system, it is noteworthy that under our experimental conditions, the mean sensitivity index was higher for root than for shoots (Table 1), suggesting that root metabolism could be highly sensitive to salinity in *O. glaberrima*.

Beside restriction of $Na^+$ absorption and translocation, tolerance of photosynthetic tissues to the accumulated toxic ions is an important component of salinity resistance in plants (Roshandal and Flowers, 2009). It implies that biochemical protecting compounds have to be synthesized and/or that compartmentation processes leading to $Na^+$ accumulation in apoplasm or vacuoles must be operating to limit the deleterious impact of toxic ion on cytoplasm where the major steps of cell metabolism occur. In our study, we found a highly significant negative correlation between $TI_{Na}$ estimated, for each accession as a mean value of individual $TI_{Na}$ provided in Table 2 for each parameter on the one hand, and the overall plant sensitivity index ($r=-0.88$; $P < 0.01$), thus confirming that in *O. glaberrima*, tolerance mechanisms to accumulated ions is of paramount importance for the overall plant performance.

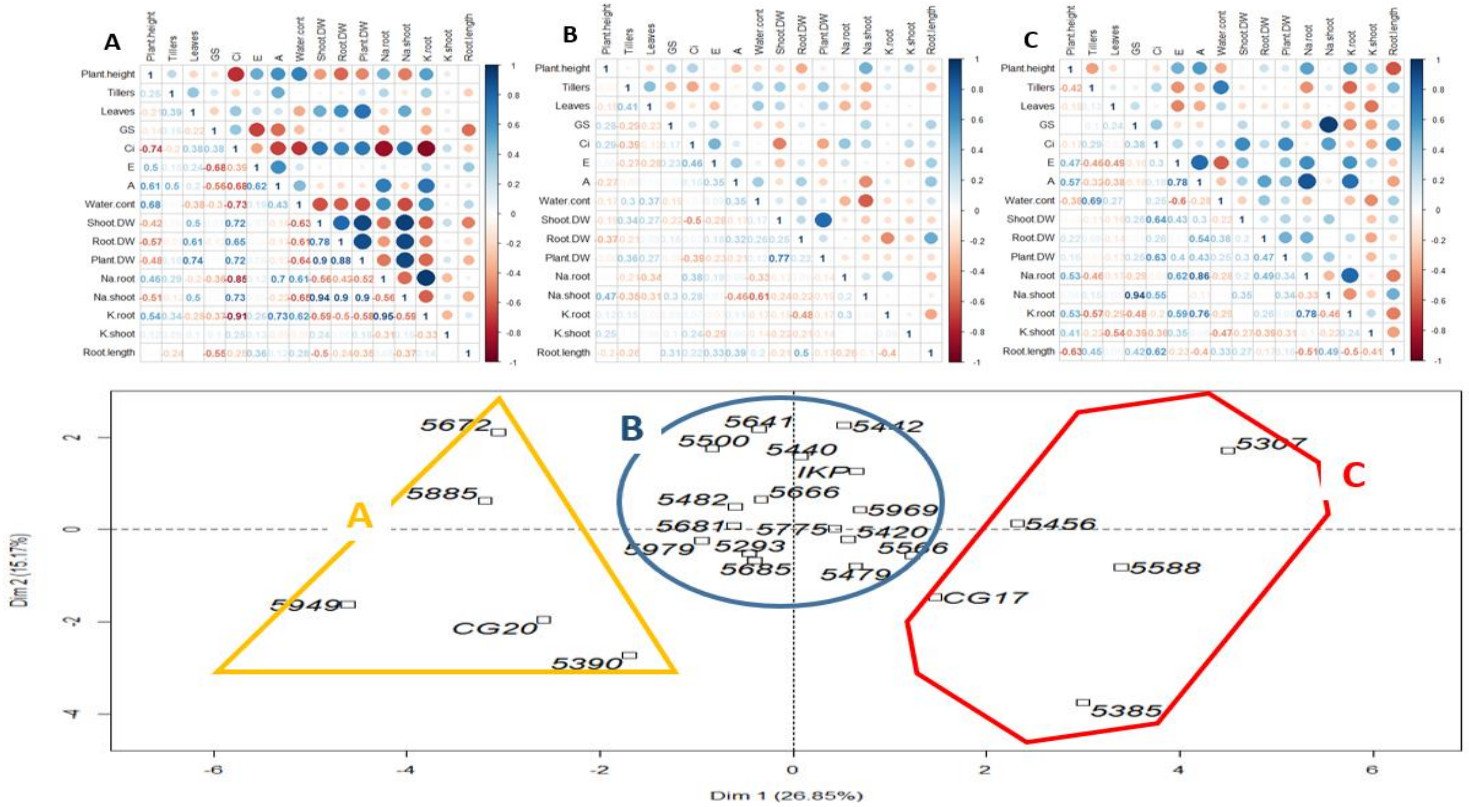


**Fig. 7**. Correlative analysis performed separately for three distinct groups of accessions exhibiting a salt-sensitive type (yellow triangle), medium-range reaction (blue circle) and resistant type (red hexagone). For each group, correlations among morpho-physiological parameters were estimated for stressed plants, a blue circle indicating a positive correlation and a brown circle indicating a negative one. The diameter of the circle and the intensity of the colours directly relevant of the importance and the nature of correlation.

Because salinity resistance is a highly complex property, it poses serious challenge to plant breeders (Flowers and Flowers, 2005). The ability of *Oryza glaberrima* to display tolerance mechanisms may be a promising aspect for further breeding schemes which confirms the putative interest of the African rice for crop improvement after inter-specific crosses with *Oryza sativa* (Adedze et al., 2016). Proline has often been regarded as an osmoprotecting compound positively involved in salinity resistance. Proline is thought to be involved in osmotic adjustment but it may also directly act to protect cellular structures and enzymes or scavenge reactive oxygen species (see Amansour and Ali 2017 for recent review). It is noteworthy, however, that in *O. glaberrima*, the most salt-resistant accessions such as TOG5307, TOG5588 and TOG5456 accumulated lower proline concentrations than salt-sensitive one. This suggests that proline does not assume key functions in salinity resistance in this species or that the signaling pathway leading to proline oversynthesis is still not triggered in these salt-resistant accessions. A similar situation was reported in *O. sativa* where salt-resistant cultivars accumulated lower proline concentrations than salt-sensitive ones (Lutts et al., 1996). According to Lutts et al. (1999), proline accumulation in this species might be due to overaccumulation of putatively toxic ammonium which induces oversynthesis of glutamine through activation of the GS/GOGAT cycle. Independently of proline synthesis, some accessions of *O. glaberrima* exhibited a fascinating ability to perform osmotic adjustment at the shoot level (Fig. 3) and the identification of compounds involved in this process could be extremely useful for further improvement of salinity resistance in rice.

Principal component analysis allowed us to discriminate three groups among the tested accessions i) a salt-resistant group which comprises TOG5307, TOG5456, TOG5588, TOG5385 and CG17 ii) a salt sensitive group includes TOG5949, TOG5390, CG20, TOG5885 and TOG5672 iii) all other accessions were classified as « medium range » for salinity resistance. A correlative analysis was performed among tested parameters in salt-stressed material within each group. Proline and MDA, however, were not included since data are not available for some accessions. Figure 7 demonstrated that the correlation profile is clearly different in each group for stressed plants. While A was negatively correlated with $g_s$ and Ci in the salt-sensitive group, this was not anymore the case in the salt-resistant one. Similarly, in the salt-sensitive group, the shoot DW was positively correlated with the root DW but this correlation disappeared in the salt-resistant accession, suggesting that root and shoot behaviour were not so directly linked in this material. This

hypothesis is supported by the fact that under our experimental conditions, salt-sensitivity index was frequently higher for roots than for shoots (Table 1). These observations, however, are based on biomass production but maintenance of metabolic processes in stressed conditions are not always devoted to growing processes. Root metabolism may be involved in root-to-shoot signaling, mainly in relation to hormonal translocation and play a key role in salinity resistance (Ghanem et al., 2011). While the salt-resistant and the salt-sensitive group differed for correlation profile (Fig. 7), the « medium-range » cultivars exhibited an intermediate behaviour characterized by a rather poor level of correlations among recorded parameters.

It is concluded from the present study that *Oryza glaberrima* exhibit some variability for salinity resistance and that some accessions, such as TOG5307, exhibits interesting properties such as a high capacity of osmotic adjustment, maintenance of photosynthesis and high level of tolerance to accumulated $Na^+$ ions.

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**Conflict of interests**

There is not any conflict of interests between the authors.

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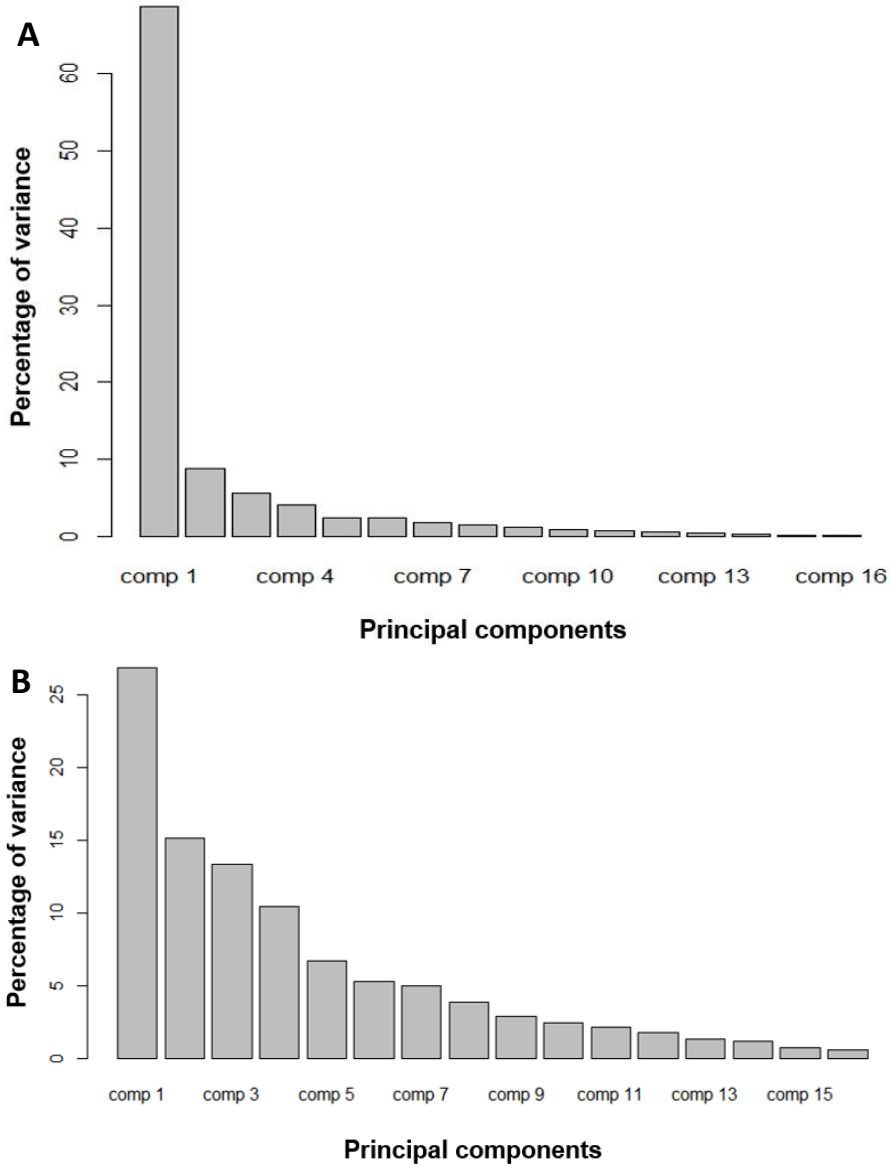



**Supplemental data (Figure S):** Percentage variance of principal components analysis performed on (A) all seedlings or (B) on only salt-stressed seedlings.

# Interchapter Chapter 1 – Chapter 2

## What do we already know ?

In Chapter I, we screened 25 cultivars of *O. glaberrima* in fully-controlled environmental conditions at the seedling stage (see Figure A below) : based on several morphophysiological parameters related to growth, mineral nutrition, plant water status and oxidative stress, we were able to gather the cultivars in three distinct groups : sensitive, medium and resistant. We calculated the sensitivity index for each cultivar and concluded that the African rice species *Oryza glaberrima* displays a high level of variability for plant response to salinity stress. Considering that tolerance to accumulated $Na^+$ at the leaf level appears as a paramount criteria for salinity resistance at the whole plant level, we retained TOG5307 as the resistant cultivar for subsequent characterization. Based on its poor ability to cope with salinity, TOG5949 was chosen as the salt-sensitive cultivar.

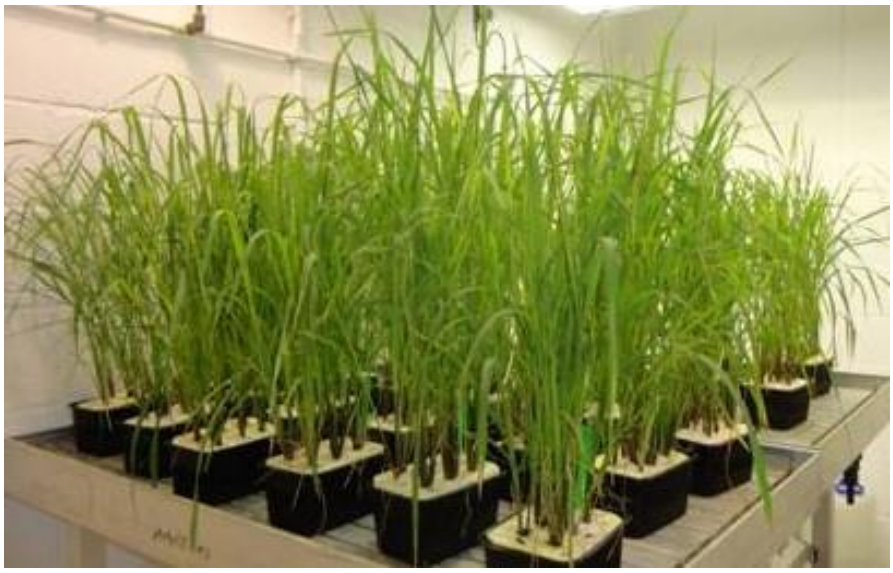


**Figure A** : African rice seedlings (*Oryza glaberrima* Steud.) cultivated in hydroponic conditions.

## What do we want to know ?

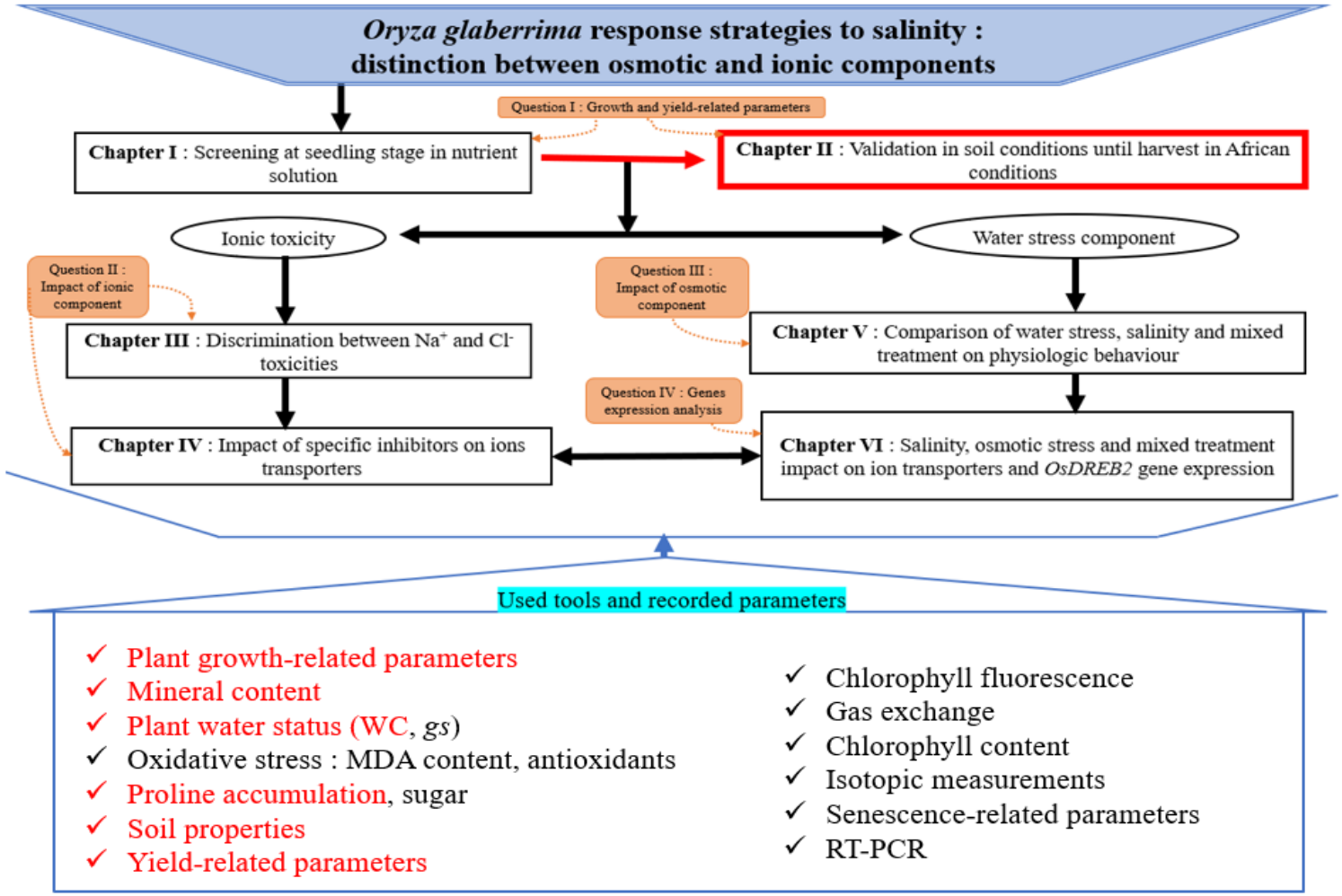
The screening procedure carried out during Chapter 1 was conducted in quite artificial conditions. Indeed, the material was screened in nutrient solution and not in soil, and only the vegetative stage was considered. Moreover, plant culture was performed in a growth chamber (phytotron) under fully-controlled environmental conditions.

Beside vegetative stage, the flowering stage is also very sensitive to salinity in rice. It has been determined that salinity resistance relies on different mechanisms controlled by distinct genes at the vegetative and reproductive phases of development. Since yield-related parameters are directly influenced by the efficiency of the flowering processes, it appears of tremendous importance to check the level of resistance of the two selected cultivars during the reproductive phase of development. Moreover, the root behaviour may be quite different in nutrient solution on the one hand and on a soil substrate on the other hand, considering that soil structure may drastically impact on the plant response to salinity.

The purposes of chapter II are therefore i) to determine the correspondence of the salinity resistance level exhibited at the seedling stage (Chap 1) and at the reproductive phase and ii) to quantify the impact of salinity on yield-related parameters in both cultivars.

## What is our strategy ?

A pot experiment will be conducted in Cotonou (Benin) using the two considered cultivars. We decide to use artificially salt spiked soil, in order to avoid the well-known heterogeneity of salinity occurring in field conditions. Soil will be sampled at Djègbadji. Plants will be exposed to salinity from the seedling stage until harvest : as a consequence, the level of salinity will be reduced from 60 mM NaCl (Chap 1) to 30 mM NaCl (Chap 2). Stressed plants will be regularly irrigated with this solution (EC 3 $dS.m^{-1}$, corresponding to a moderate salinity) while control plants will be irrigated with tap water. Soil properties will be characterized. Yield-related parameters will be considered after final harvest considering the main stem and the time required to observe 50% heading will also be determined. Mineral concentrations ($Na^+$ and $K^+$) will be determined in the leaves, in the grain and in the hull while the osmoprotectant proline will be quantified in the leaves.




**Overview of questions addressed during this thesis and their relationships with chapters.**

# Chapter II. Impact of salinity on yield-related parameters of *Oryza glaberrima*

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## Abstract

*Aims* Salinity is one of the major environmental stresses limiting growth and yield of rice. The objective of the present study was to analyze the impact of NaCl on yield-related parameters of *Oryza glaberrima*.

*Methods* Two contrasted cultivars of *Oryza glaberrima* previously tested for salt resistance at the vegetative stage [salt-resistant (TOG5307) and salt-sensitive (TOG5949)] were subjected to saline irrigation treatments by using the saline solution equivalent to 30 mM NaCl (EC: 3 $dS.m^{-1}$ NaCl). After 6 months of treatments, mineral nutrient, proline content and yield-related parameters were assessed.

*Results* NaCl treatment affected most yield-related parameters :panicle length, panicle leaf dry weight, number of branches per panicle, panicle leaf length, days to 50% heading, straw fresh weight per plant, grain yield per plant, number of grain per panicle, number of grain filled per panicle, and weight of 1000 grains. The salt-resistant cultivar TOG5307 was less affected than the salt-sensitive TOG5949 and accumulated lower amounts of $Na^+$ in the grains

*Conclusion* The cultivar exhibiting the highest salt-resistance at the vegetative stage also exhibited the highest resistance at the reproductive one. The opportunity to use the seedling behavior as predicting criteria of yield efficiency is an opportunity for further discussions and studies, in view of eventual implementations.


**Keywords**: NaCl, *Oryza glaberrima,* rice, salinity, yield-related parameters.

# Introduction

Salinity is one of the major environmental stresses limiting growth and yield of rice. More than 5% of agricultural land and 20% of irrigated cultivated areas are affected by an excess of soluble salts (Zörb et al. 2019). In salt stress condition, plants are exposed to i) osmotic stress due to the reduction of the osmotic potential of the root medium and ii) ionic stress caused by the progressive accumulation of large amounts of toxic ions into plant tissues (Castillo et al., 2007). These major constraints compromise water uptake, nutrient acquisition causing nutritional imbalances and alter a wide range of physiological functions (Zhao et al. 2020). Rice is considered as one of the most salt-sensitive crop. Mohammadi et al. (2013) reported that above a threshold of 3 dS/m soil electrical conductivity (EC), there is a 12% yield reduction per dS/m. Indeed, salinity affects most seriously seedling and reproductive stages of rice (Lutts et al. 1995; Sudharani et al., 2012). Surekha Rao et al. (2013) argued that screening for salinity tolerance potential at reproductive stage to identify tolerant rice varieties is crucial. Salt stress negatively impacts the number of tillers per plant, number of grains per panicle, 1000-grain weight and grain yield (Hosamuzzaman et al. 2009 ; Mohammadi-Nejad et al. 2010 ; Uddin et al. 2007). Kumar and Khare (2016) reported that salt stress not only reduces the rice yield substantially but also adversely affects the grain quality. Besides the well-known Asian rice species *Oryza sativa*, the African rice species, *Oryza glaberrima* is the second cultivated rice species. While *Oryza sativa* is cultivated in most continents, *Oryza glaberrima* is only cultivated in Africa (Linares, 2002). However, it is consider as able to tolerate various biotic and abiotic stresses (Futakuchi et al 2012; Wambugu et al. 2019) but its capacity to cope with salinity remains poorly documented. Prodjinoto et al. (2018) screened 25 varieties at seedling stage for salinity tolerance. They identified three groups for salinity resistance among the tested varieties: a salt-resistant group, a salt sensitive group, and « medium range ». Moreover, some varieties such as TOG5307 are able to maintain a high net photosynthesis under salt conditions and exhibited a high level of tolerance to accumulated $Na^+$ ions and is able to efficiently regulate chloride accumulation in aerial part in contrast to TOG5949 which was classified as a salt-sensitive cultivar.

The information available for these two contrasting cultivars are issued from experiment performed at the vegetative stage, for plants cultivated in nutrient solutions under controlled environment conditions. It is now clearly

established that the mechanisms involved in salinity resistance may vary depending on the plant phenological stage (Liu et al., 2021). To the best of our knowledge, no data are available on the impact of salinity on yield-related parameters of *Oryza glaberrima*. The objective of the present study was therefore to analyze the impact of NaCl on yield-related parameters of two contrasting varieties of *Oryza glaberrima* steud (TOG5307 and TOG5949) cultivated in soil.

## Material and Methods

### *Soil characterization*

Soil samples were randomly collected from a surface layer (0.20 m depth) in a farmer's field of Djègbadji (2°4'36"E, 6°19'27"N) (Republic of Benin; Figure 1). Soil electrical conductivity (EC, mS $cm^{-1}$) and $pH\text{-}_{H2O}$ were determined in a 1:5 soil/water suspension ratio with a shaking time of about 5 min using glass electrodes. Cation exchangeable capacity (CEC) was assessed using percolation columns and KCl 1.0 M pH 3.0. Exchangeable base concentrations ($Mg^{2+,}$ $Ca^{2+}$, $Na^{+}$ and $K^{+}$) were measured using the same percolation columns and ammonium acetate 1.0 M pH 7.0 (Page et al., 1982) and inductive-coupled plasma atomic emission spectrometry (ICP-AES; Thermo Jarrell Ash Iris Advantage). Total N and organic C were determined according to Kjeldahl and Walkley–Black methods. The content of the soil solution in soluble cations was determined in 1:2 soil/water suspension ratio. Cations concentration ($Mg^{2+}$, $Ca^{2+}$, $Na^{+}$ and $K^{+}$) were quantified by the inductive-coupled plasma atomic emission spectrometry (ICP-AES; Thermo Jarrell Ash Iris Advantage) (Page et al., 1982). Soil analyses were carried out at Soil Science Laboratory of Earth and Life Institute in Belgium.

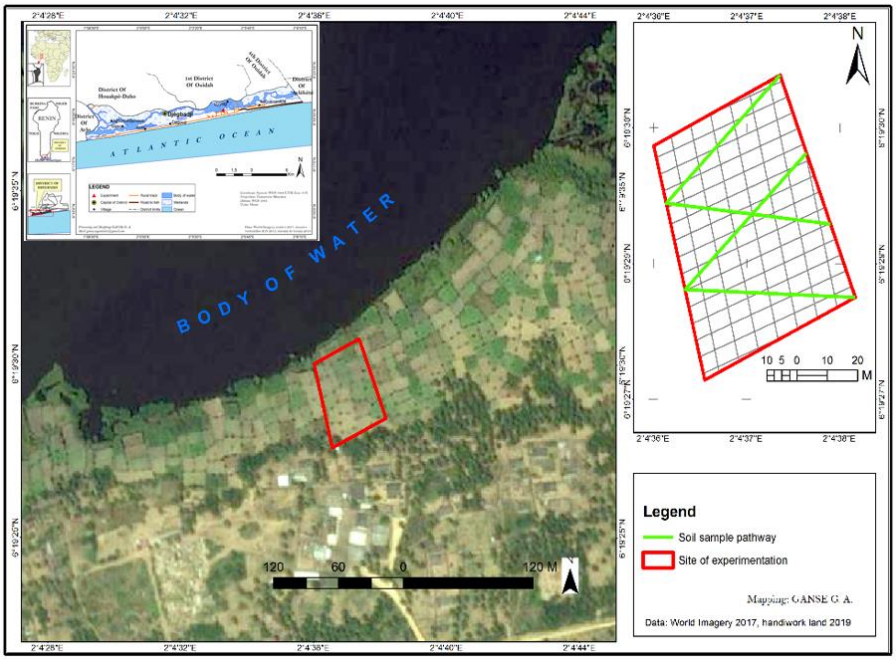


**Figure 1** : Geographical location of Djègbadji and soil sampling plan.

*Experimental setup*

This study was carried out in the greenhouses of The IITA Benin Republic station. The temperature in the greenhouse with natural light varied between 25-30 °C (day time) and 20-25 °C (night time). The soil, brought from Djégbadji, was sterilized at 180 ° C for 72 hours in order to avoid any microbial contamination and nematodes. The pots were fertilized according to the protocol of Agnoun et al., (2012). Fertilizers were used to provide the equivalent of NPK 15-15-15 at the rate of 100 kg $ha^{-1}$ and were uniformly mixed in soil before filling the pots. In each pot, 4 kg of soil was poured. In order to determine the water-holding capacity of the soil, three "test" pots were watered until saturation (we will consider that there is saturation when a light layer of water will stagnate on the surface of the soil). 20 pots per cultivar and per treatment were randomly arranged during this study and randomly re-distributed each week.

*Plant material, growth conditions and stress application*

Two contrasted cultivars of *Oryza glaberrima* [salt-tolerant (TOG5307; Accession number WAB0021855) and salt-sensitive (TOG5949; Accession number WAB0020144)], as described by Prodjinoto et al. (2018) were used. Seeds were obtained from AfricaRice (Bouaké, Ivoiry coast). For each cultivar, non-dehusked seeds were surface-sterilized and germinated on two layers of filter papers (Whatman 85 mm, Grade 1) moistened with sterile deionized water in glass vessels at 25 °C and under 12 h daylight period. After 10 days, equally grown seedlings were transferred into in pots containing sterilized and fertilized soil. Four seedlings were transplanted into each pot and afterwards thinned to two plants per pot. After two weeks, half of the rice seedlings were subjected to saline irrigation treatments by using a saline solution equivalent to 30 mM NaCl (EC: 3 $dS.m^{-1}$ NaCl). The salinity was applied after dissolving NaCl in tap water. 1500 ml of the saline solution or salt-free tap water were added to the soil depending on the treatment provided (control or NaCl stress). Electrical conductivity of soils after NaCl treatment was 3.4 dS/m. Each treatment was replicated for three times in a randomized complete block design. Thereafter, irrigation was applied manually to pot water-holding capacity using the tap water.

*Yield parameters estimation*

At the end of the experiment, the following growth and yield-related parameters were measured considering the main stem: plant height at maturity, panicle length, panicle leaf dry weight, panicle leaf length, panicle leaf width, number of branches per panicle. We also quantified the mean number of tillers per plant, fertile tillers per plant, days to 50% heading, straw fresh weight per plant, grain weight per plant, number of grain per panicle, number of filled grains per panicle and 1000 grains weight.

Plant height was assessed at maturity as the distance from the ground to the tip of the tallest panicle of five plants randomly selected per cultivar and per treatment. Days to 50% heading was considered as days required to observe

50% of plants with panicle emergence. Weight of grains were recorded from filled grain collected from random plants. All other yield-related parameter were assessed at maturity stage (6 months experiment).

*Estimation of ion and proline concentrations*

Ion determination was performed as described by Gouveitcha et al. (2021). Dried mater (leaf, grain, or grain hull) were individually ground in a mortar, and the powder was dried for 24 h. To determine the concentrations of $Na^+$ and $K^+$, *c.a* 20 mg of the obtained powders were placed in 10 ml jars and digested in nitric acid (68%) at room temperature. The solutions were filtered through Whatman paper (85 mm, Grade 1). The filtrate was used for the determination of cations ($Na^+$ and $K^+$) using a flame spectrophotometer (Sherwood Model 360).

Proline concentration was determined spectrophotometrically using the method of Bates et al. (1973) and results were expressed as µmol $g^{-1}$ FW (Fresh Weight).

*Statistical analysis*

All the data in this research were calculated with JMP Pro 15 software. The analysis of the main effects of cultivars and stresses was based on the variance analysis. At $P < 0.05$ a post-hoc test Tukey's HSD all-pairwise comparisons were used to compare means.

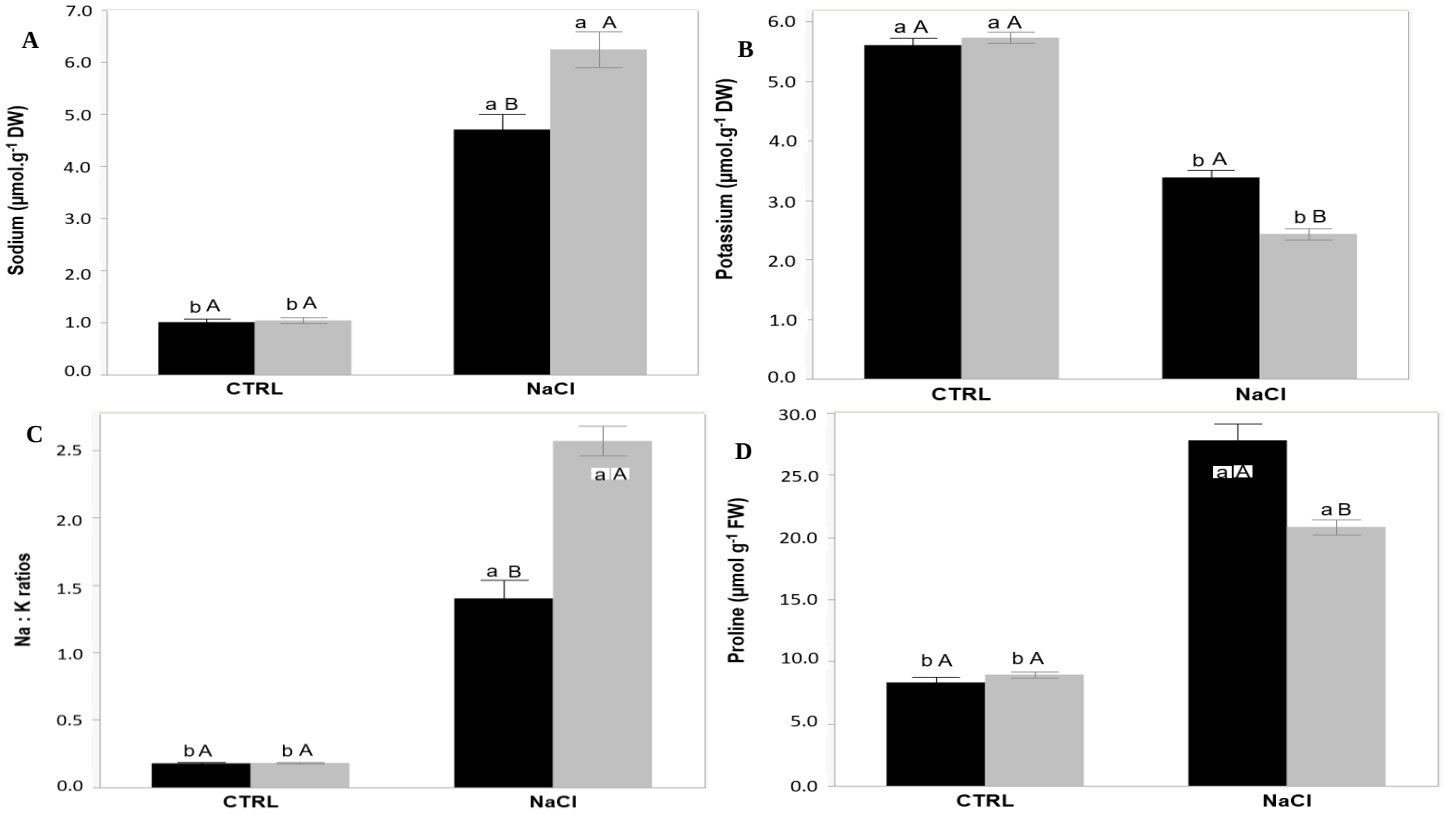


**Figure 2**. (A) sodium, (B) potassium, (C) Na/K ratios and (D) proline concentration in shoots of African rice (*Oryza glaberrima* Steud.) cultivars TOG5307 and TOG5949 cultivated during 6 months in control (CTRL) conditions or in the presence of 30 mM NaCl. Treatments followed by the same lowercase latter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase latter in a particular condition do not differ statistically.

# Results

*Soil characteristics*

We had used the Djègbadji soil (from municipality of Ouidah, Atlantic department, Republic of Benin) to carry out this work. Djègbadji has a sandy type soil (Capo-Chichi and Guidibi, 2006). However, our analyses of chemical properties showed that Djègbadji has a neutral soil with low organic carbon and total nitrogen content (Table 1). The soil we used had a very low EC (0.268 dS/m), a medium CEC (13.59 meq /100 g soil) with a majority of $Ca^{2+}$ as exchangeable bases. Consequently, the $Ca^{2+}$ / $Mg^{2+}$ ratio is 5.05. Djègbadji therefore has a calci-magnesic soil (Table 1). This might explain why local people till crops in the area where we sampled soil. After soil salinization, the EC value rose to 3.4 dS/m, which should be considered as a moderate salinity for rice plants (Singh et al., 2021).

**Table 1.** Djègbadji soil chemical properties

| | | |
|---|---|---|
| | $pH\text{-}_{H2O}$ | 6.81 ± 0.10 |
| | $pH\text{-}_{KCl}$ | 6.23 ± 0.03 |
| | EC (dS/m) | 0.268 ± 0.002 |
| | CEC (meq/100 g soil) | 13.59 ± 0.27 |
| | Total N [%] | 0.04 ± 0.0008 |
| | Organic C [%] | 0.43 ± 0.006 |
| | C/N ratio | 12.17 ± 0.14 |
| | $NH_4$ (mg/kg) | 520.6 ± 10.87 |
| **Exchangeable Cations (meq/100g Soil)** | $Mg^{2+}$ | 0.20 ± 001 |
| | $Ca^{2+}$ | 1.01 ± 0.1 |
| | $Na^{+}$ | 0.4 ± 0.01 |
| | $K^{+}$ | 0.22 ± 0.03 |
| **Water Soluble Ions (mmol/L)** | Mg | 2.81 ± 0.16 |
| | Ca | 8.31 ± 0.08 |
| | Na | 3.1 ± 0.06 |
| | K | 1.46 ± 0.14 |

*Physiological parameters*

All plants remained alive and set seeds during the time-course of our experiment. In control condition, plants had similar sodium content. Salt stress increased sodium content in shoots of both studied varieties of *Oryza glaberrima*. Salt-sensitive variety TOG5949 accumulated about 1.5 time more $Na^+$ in the shoots than TOG5307 (Figure 1.A). An opposite trend was observed for $K^+$ as indicated in Figure 1.B. Indeed, potassium content decreased almost by half when salt stress was applied but TOG5307 had significantly higher content of $K^+$ in the shoots than TOG5949 in the presence of NaCl. $Na^+/K^+$ ratio was low in control condition than in salt-treated plants. In plants exposed to NaCl however, $Na^+/K^+$ ratio was significantly higher (1.8 time greater) in TOG5949 than in TOG5307 (Figure 1.C).

Proline content did not change between both studied varieties in control condition. However, proline concentration increased in response to NaCl. The salt-resistant cultivar TOG5307 had significantly higher content of proline than TOG5949 (Figure 1.D).

Figure 2 shows the accumulation of sodium and potassium contents in grain and grain hull. Under control conditions, the sodium content in the grain and in the grain hull was negligible in both cultivars. However, in the presence of salt stress, the sodium content increased significantly and higher sodium content was measured in grain hull than in grain. As far as grain is concerned, $Na^+$ accumulation was obviously higher in TOG5949 than in TOG5307. Even if grain hull had higher content of $Na^+$, variety TOG5949 still had higher content of $Na^+$ than TOG5307 during salt stress.

As far as potassium content is concerned, grain had significantly lower content than grain hull whatever cultivars and conditions. However, plants exposed to control condition had higher potassium contents than those exposed to NaCl. As far grain is concerned, TOG5307 significantly accumulated more potassium than TOG5949 whatever the applied treatment. When salt stress was applied, potassium content in grain decreased by 36% and 75% respectively for TOG5307 and TOG5949. As far as grain hull is concerned, no difference between varieties was recorded for potassium concentration. However, during salt stress, potassium content in grain hull decreased of 40% and 53% for TOG5307 and TOG5949, respectively (Figure 2).

$Na^+/K^+$ ratio for grain and grain hull were closed to zero for both varieties in control condition. In TOG5307, $Na^+/K^+$ was higher in grain hull than in grain during salt stress. An opposite trend was recorded for TOG5949. The $Na^+/K^+$ was extremely high in grains of TOG5949 exposed to salinity.

*Reproductive development under salt stress*

Table 2 presented yield-related parameters of *Oryza glaberrima* Steud. cultivars TOG5307 and TOG5949 cultivated in control and saline conditions. As far as control condition is concerned, plant height at maturity, panicle leaf dry weight, panicle leaf width, number of tillers per plant, number of fertile tillers per plant, number of grain per panicle, and weight of 1,000 grains were similar for both cultivars. However, some contrasting properties were recorded: TOG5307 showed a higher panicle length, branches per panicle, panicle leaf length, straw fresh weight per plant, and grain yield per plant than TOG5949 while an opposite trend was observed for the number of filled grains per panicle and days to 50% heading. NaCl treatment affected most yield-related parameters. Nevertheless, the deleterious impacts of salt stress were significantly lower in TOG5307 than TOG5949 for plant height at maturity, panicle length, panicle leaf dry weight, number of branches per panicle, panicle leaf length, days to 50% heading, straw fresh weight per plant, grain yield per plant, number of grain per panicle, number of grain filled per panicle, and weight of 1000 grains. This means that TOG5307 was more salt tolerant than TOG5949. Salt stress delayed panicle emergence (flowering) and reduced filled grain by 15% and 36% for TOG5307 and TOG5949, respectively.

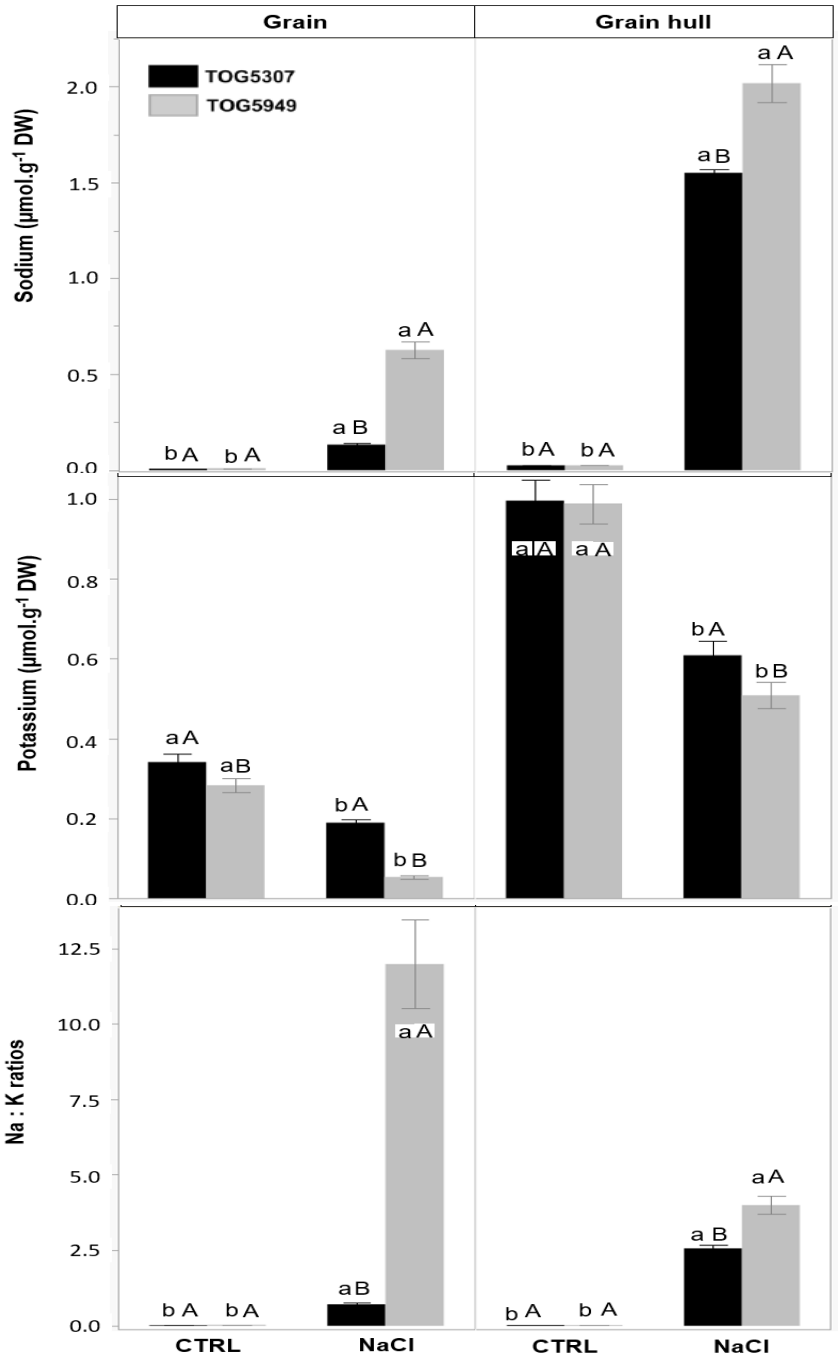


**Figure 3**. Mineral nutrients concentration in grain and grain hull of African rice (*Oryza glaberrima* Steud.) cultivars TOG5307 and TOG5949 cultivated during 6 months in control (CTRL) conditions or in the presence of 30 mM NaCl. Treatments followed by the same lowercase latter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase latter in a particular condition do not differ statistically.

**Table 2.** Yield parameters of African rice (*Oryza glaberrima* Steud.) cultivars TOG5307 and TOG5949 cultivated during 6 months in control (CTRL) conditions or in the presence of 30 mM NaCl. 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.

| | TOG5307 | | TOG5949 | |
|---|---|---|---|---|
| Yield Parameters Measured | CTRL | NaCl | CTRL | NaCl |
| Plant height at maturity (cm) | 176 ± 4.2 aA | 172 ± 2.6 bA | 178 ± 3.4 aA | 166 ± 3.2 bB |
| Panicle length (cm) | 30 ± 1.3 aA | 28 ± 2.1 aA | 26 ± 2.2 aB | 23 ± 3.1 aB |
| Panicle leaf dry weight (g) | 0.12 ± 0.004 aA | 0.09 ± 0.003 bA | 0.12 ± 0.006 aA | 0.07 ± 0.003 bB |
| Branches /panicle | 7.47 ± 0.04 aA | 6.34 ± 0.04 bA | 7.32 ± 0.03 aB | 6.27 ± 0.03 bB |
| Panicle leaf length | 44 ± 0.1 aA | 39 ± 0.4 bA | 42.7 ± 0.5 aB | 38 ± 0.2 bB |
| Panicle leaf width | 1.5 ± 0.2 aA | 1.4 ± 0.1 bA | 1.5 ± 0.1 aA | 1.4 ± 0.1 bA |
| Tillers /plant | 32 ± 0.5 aA | 27 ± 1.4 bA | 34 ± 1.3 aA | 26 ± 0.8 bA |
| Fertile tilles /plant | 32 ± 0.5 aA | 23 ± 1.4 bA | 34 ± 1.3 aA | 22 ± 0.8 bA |
| Days to 50% heading | 114 | 123 | 117 | 133 |
| Straw fresh weight (g)/plant | 92 ± 1.4 aA | 78 ± 2.1 bA | 81 ± 1.6 aB | 75 ± 1.3 bB |
| Grain yield/plant (g) | 29 ± 0.5 aA | 23 ± 0.6 bA | 28 ± 0.4 aB | 21 ± 0.4 bB |
| Grain /panicle | 130 ± 2 aA | 120 ± 1 bA | 131 ± 1 aA | 115 ± 2 bB |
| filled grain / panicle | 96 ± 1 aB | 81 ± 2 bA | 98 ± 2 aA | 63 ± 2 bB |
| Weight of 1000 grains (g) | 24 ± 0.5 aA | 23.5 ± 0.8 bA | 25 ± 0.4 aA | 21 ± 0.5 bB |

## Discussion

In this research, we studied the impact of salt stress on the yield parameters of two contrasting varieties of *Oryza glaberrima*: TOG5307 and TOG5949. We found that applied saline stress significantly affected mineral nutrients and yield-related parameters of *Oryza glaberrima* cultivars. Even if all plants remained alive and set seeds during the time-course of the experiment, we found that TOG5307 was more salt-tolerant than TOG5949. This complies with the report of Prodjinoto et al., (2018) who initiated the demonstration according which TOG5307 and TOG5949 displayed contrasting levels of salt-resistance. Compared to Asian rice species *Oryza sativa*, the African rice species *Oryza glaberrima* is considered as low yielding due to lodging, grain shattering, and low spikelet number (Linares, 2002 ; Montcho, 2013). In this study, we recorded that both studied varieties produced over 50% of filled grains during salt stress.

Reduction in growth due to salinity have been implicated as causative factors for yield losses (Zeng and Shannon 2000). In our set experiment, NaCl reduced plant height, panicle length, panicle leaf dry weight, number of branches per panicle, panicle leaf length, straw fresh weight per plant. In fact salinity stress caused reduction in overall vigor especially in the number of filled grain per panicle and yield per plant (Mohammadi-Nejad et al. 2010). Similarly, Uddin et al. (2007) and Hosamuzzaman et al. (2009) reported that salinity reduced the number of effective tillers per plant, number of grains panicle, 1000-grain weight and grain yield. As far as African rice is concerned, we found that 30 mM of NaCl significantly reduced grain yield per plant, number of grain per panicle, number of grain filled per panicle, and weight of 1000 grains. To set the impact of NaCl on flowering, we recorded days when 50% of plants bloomed. We observed that NaCl delayed flowering of African rice. Even if TOG5307 bloomed earlier than TOG5949 during salt stress. Delayed flowering was also reported for Asian rice species *Oryza sativa* (Lutts et al. 1995; Khatun et al. 1995).

Salt stress induces a dehydration of plant cells, a nutritional imbalance, and an ion toxicity. According to Negrão et al. (2017), sodium appears to accumulate to toxic levels before chloride in most species. We focus here on Na+ in our study and we did not quantify chloride content because of a lack of equipment. Our results revealed that, plants of the both cultivars accumulated high

amounts of $Na^+$ in shoots, grains and grains hulls when exposed to NaCl. Moreover, the salt-resistant TOG5307 accumulated less $Na^+$ than the salt-sensitive TOG5949. This was already reported for plants at the vegetative stage (Prodjinoto et al. 2018). It implies that TOG5307 displayed an avoidance strategy and reduces $Na^+$ absorption at this stage of development. Beside $Na^+$ accumulation, a decrease in $K^+$ concentration is a major deleterious effect of salt stress. According to Lee et al. (2003), $Na^+/K^+$ ratio in leaves of crops can be used as an important indicator of salinity tolerance. Once again, the trend previously observed for plants exposed to NaCl at the vegetative stage is still valid at the reproductive one, and the higher ability of TOG5307 to maintain $K^+$ homeostasis clearly appeared at the grain level with a very low $Na^+/K^+$ ratio comparatively to salt-sensitive TOG5949.

In the present work, salinity was applied already at the vegetative stage. This strategy is relevant to what occurs in field conditions. However, it does not allow us to specifically discriminate the impact of salinity at the reproductive stage. Indeed, it might be argued that the salt-induced decrease in vigor occurring at the vegetative stage has an effect on the capacity of the plant to efficiently conduct a reproductive phase. In that sense, salinity impact at the seedling stage has "a delayed" effect on the reproductive phase. Hence, the fact that TOG5949 had a lower yield than TOG5307 is not an absolute proof that it is more sensitive than TOG5307 at the reproductive stage, but could be the consequence that it was weaker when it initiated the reproduction phase. Applying salinity at the heading stage may provide useful information in that respect (Lutts et al., 1995). It has been demonstrated that NaCl in flower organs may drastically influence reproductive behavior and that a decrease in pollen viability may be responsible for a decrease in spikelets fertility, which is a major parameter determining yield in rice (Zhu et al., 2004).

Plants synthesis compatibles solutes and osmoprotectants such as proline in order to survive in salt-affected soils (Gupta and Huang, 2014). We demonstrated that proline content significantly increased in salt-tolerant variety than in salt-sensitive one. However, it is important to insist on the fact that in the present study, proline was quantified at harvest. This implies that the plant already encountered at programmed monocarpic senescence process, and that dehydration occurring during maturation did not result from a stress factor, but from a naturally occurring process in an annual plant. To the best of our knowledge, no study provided information regarding the fate of leaf proline during maturation in rice, but it is hard to understand why the plant

accumulated a protecting compound in an organ encountering programmed cell death. It might be argued that accumulated proline measured in the present experiment previously accumulated during salt stress at the vegetative stage, and then remained in the leaves. This hypothesis needs to be tested in further works since it is well established that proline may be rapidly metabolized through proline dehydrogenase activation (Mansour and Ali 2017).

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**Conflict of interest**

The authors declare no conflict of interest.

**Availability of the data**

The full data are available from the corresponding author after justified request.

**Author's contribution**

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

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# Interchapter : chapter 2 – Chapter 3

## What do we already know ?

Two cultivars of *O. glaberrima* were selected on the basis of their mean level of salinity resistance at the seedling stage (Chap 1) : TOG5307 is salt-resistant and TOG5949 is salt-sensitive. We confirmed that the cultivar exhibiting the higher salt-resistance at the seedling stage also exhibited the highest level of resistance at the reproductive one. TOG5307 was indeed less affected that TOG5949 for panicle length and dry weight, grain yield per plant, number of grain per panicle and it also accumulated lower amounts of $Na^+$ in the grains. It could therefore be considered that, as far as these two cultivars are concerned, the use of the hydroponic system is a convenient tool to study plant response to salinity and that the behaviour at the seedling stage is relevant from the behaviour at subsequent reproductive stage.

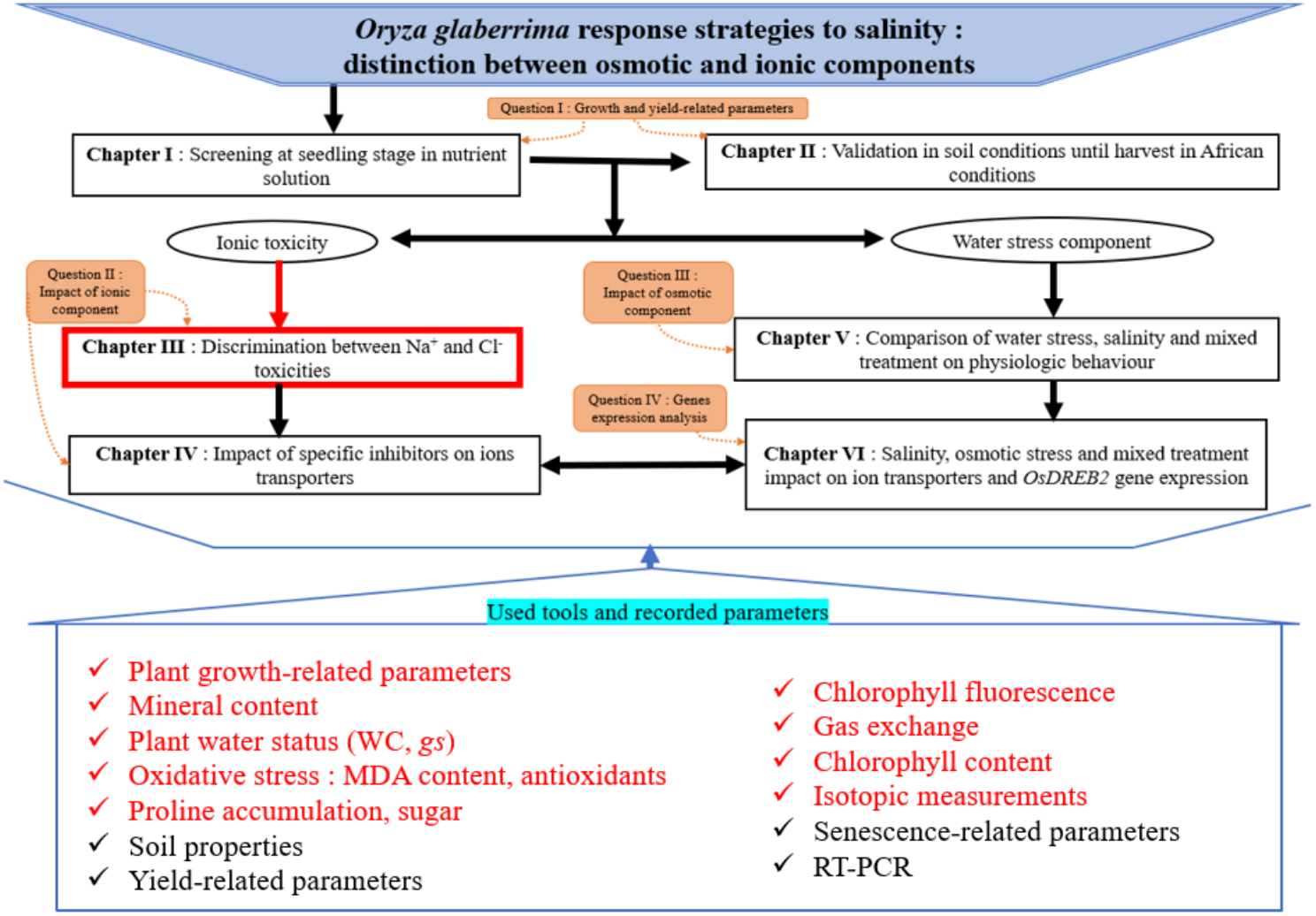
## What do we want to know ?

Salinity is a complex environmental constraint inasmuch as it induces an osmotic stress to the plant (due to the decrease in the external osmotic potential) and an ionic stress (due to the accumulation of toxic ion and the salt-induced decrease in essential elements). Independently of the osmotic stress component, the ionic component itself is a complex constraint since it implies two distinct ions : $Na^+$ and $Cl^-$. Until recently, most studies dealing with salinity mainly focused on $Na^+$ accumulation and neglected the $Cl^-$ toxicity. There is a paucity of information regarding chloride toxicity and it is not clear if chloride act on the same targets as $Na^+$ and if this is the case, whether the two accumulated ions could interact in their impact (in a synergistic or antagonist way) or act in an additive manner. It is also unknown if the interaction could be different in a sensitive and in a resistant cultivar.

The aim of Chapter III is therefore to compare the impact of $Na^+$ and $Cl^-$ applied separately or simultaneously on TOG5307 and TOG5949 in relation to mineral nutrition, plant water status and photosynthetic properties.

## What is our strategy ?

In the following Chapter, we will prepare three types of stressing solution : one contains 50 mM NaCl, a second one contains 50 mM $Na^+$ added as salts with non-toxic counteranion ($Na^+$-dominant) and the last one contains 50 mM $Cl^-$ added as salt with non-toxic countercation. All solution will exhibit similar osmotic potential and electrical conductivity. This procedure will allow us to discriminate the toxic effects of each ion ($Na^+$-dominant versus $Cl^-$-dominant) and to determine the possible interaction among ions when present together in excess within the plant tissues (NaCl). Plants will be exposed at the seedling stage for two weeks, as it was the case in Chapter 1 and similar parameters will be quantified. However, in order to enlarge the panel of analyzed physiological properties, we will also consider antioxidant content (flavonoids and phenolics), soluble sugar content, chlorophyll-fluorescence related parameters as well as carbon and nitrogen isotopic measurements. An « ion toxicity index » will be defined to quantify, for each parameter, the impact of each treatment comparatively to control for the two cultivars.




**Overview of questions addressed during this thesis and their relationships with chapters.**

# Chapter III. 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. Most recorded physiological parameters were affected by both $Na^+$ and $Cl^-$. TOG5307 exhibited a higher level of resistance than TOG5949 in terms of growth and photosynthesis maintenance and control of $Cl^-$ and $Na^+$ accumulation. 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. It is concluded that cultivars exhibiting a contrasting level of salinity resistance are available in *Oryza glaberrima* and salt-tolerant genotypes may constitute a valuable source of gene for classical rice improvement.


**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 interesting properties for resistance to high temperatures (Li et al. 2015), drought (Bimpong et al. 2010; Shaibu et al. 2018; Kang and Futakuchi 2019; Kartika et al. 2020), iron toxicity (Majerus et al. 2007; Sikirou et al. 2010; 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, such as photosynthesis, 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 (Accession number WAB0021855) is salt-tolerant while TOG5949 (Accession number 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 at 25 °C under a 16 h daylight period (150 µmole $m^{-2}$ $s^{-1}$). 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 µmoles $m^{-2}$ $s^{-1}$. 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 $MgCl_2.6H_2O$, 20 mM KCl)
- NaCl (YNS plus 50 mM NaCl)

The control nutrient solution had an osmotic potential ($\Psi s$) 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 $\Psi s$ of -0.31 ± 0.002 MPa while both $Na^+$-dominant and $Cl^-$-dominant exhibited a similar $\Psi s$ 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 $\Psi s$, 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 mosmoles $Kg^{-1}$ to MPa using the formula:

$\Psi s$ (MPa) = - c (mosmoles.$kg^{-1}$) x 2.58 x $10^{-3}$ according to the Van't Hoff equation.

*Ion content*

For shoots and roots of each cultivar per treatment, *c.a.* 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 quantification*

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 1,000 *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 (*c.a*. 300 mg FW) were mixed with 7 mL of ethanol 70% (w/v) for 5 min on ice and centrifuged at 8,000 *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 6,700 *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 $g^{-1}$ of 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 ($\Phi_{PSII}$) 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 $moles^{-1}$, *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, chlorophyll 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 1,000 *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 according to Lichtenthaler (1987).

*Carbon and nitrogen isotopic measurements*

Dry matter of roots and shoots were ground to homogenous powder and *c.a.* 2 mg were weighed and placed in tin capsules (3 x 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:

$$\delta X = [(R\ sample/R\ standard) - 1]x\ 1000$$

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:

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

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, $Na^+$ and $Cl^-$ toxicity act in a strict additive way (i.e. $ITI_{NaCl} = ITI_{Na} + ITI_{Cl}$). Divergence from this hypothesis could indicate synergetic negative effect (i.e. $ITI_{NaCl} > ITI_{Na} + ITI_{Cl}$) or compensatory effect ($ITI_{NaCl} < ITI_{Na} + ITI_{Cl}$).

*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. Pearson chi-squared test using Yate's correction for continuity was used to 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 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 treatments. Chloride-dominant treatment had no impact on root length in TOG5307 but reduced root elongation in TOG5949. The ratio root/shoot DW decreased in $Na^+$- dominant and NaCl treatments for TOG5307 but increased only in NaCl treatment for TOG5949 (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 $\Psi$s 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 $\Psi_s$ of TOG5949 was significantly lower than the values recorded for TOG5307. A similar difference was also observed for the root $\Psi$s (Table 2).

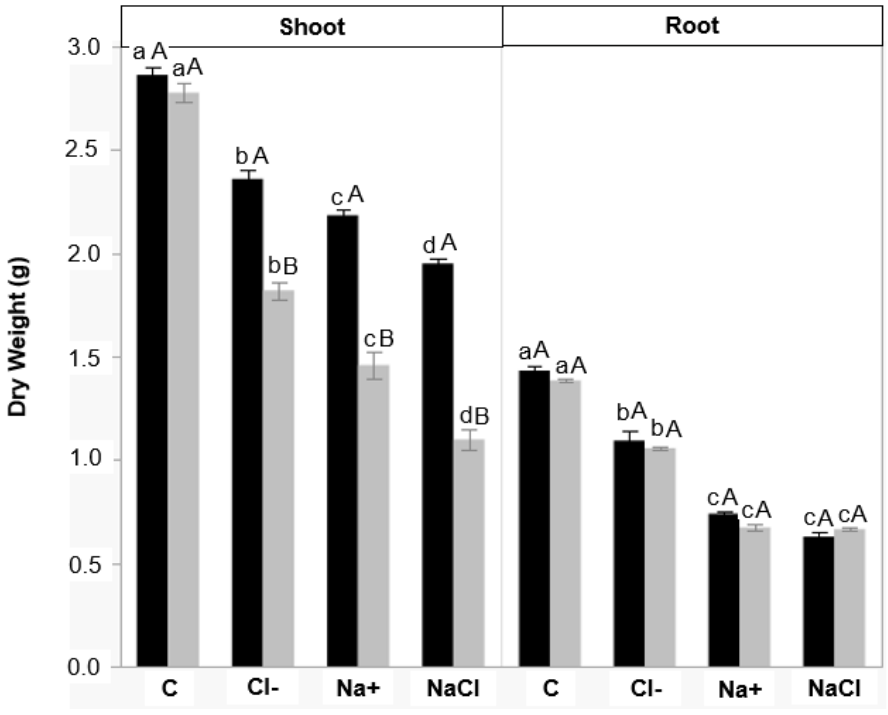


**Figure 1.** Dry weight (DW) of shoot and roots of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 (gray bars) cultivated during 2 weeks in control conditions (C) or in the presence of 50 mM of

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

*Mineral nutrition*

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

**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. 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.

| Cultivars | Treatments | Shoot length (cm) | Root length (cm) | (DW) root:shoot ratio |
|---|---|---|---|---|
| TOG5307 | C | $62.67 \pm 1.33^{aA}$ | $32.00 \pm 1.15^{aB}$ | $0.50 \pm 0.01^{aA}$ |
| | $Cl^-$ | $52.33 \pm 0.88^{bA}$ | $30.33 \pm 0.88^{aA}$ | $0.46 \pm 0.01^{aB}$ |
| | $Na^+$ | $36.67 \pm 1.86^{cA}$ | $32.33 \pm 1.20^{aA}$ | $0.34 \pm 0.003^{bB}$ |
| | NaCl | $33.33 \pm 1.45^{cA}$ | $18.33 \pm 0.88^{bA}$ | $0.32 \pm 0.01^{bB}$ |
| TOG5949 | C | $57.33 \pm 1.33^{aB}$ | $42.00 \pm 1.15^{aA}$ | $0.50 \pm 0.005^{bcA}$ |
| | $Cl^-$ | $41.67 \pm 1.86^{bB}$ | $27.00 \pm 0.58^{bB}$ | $0.58 \pm 0.02^{abA}$ |
| | $Na^+$ | $31.67 \pm 1.45^{cA}$ | $22.33 \pm 0.88^{cB}$ | $0.46 \pm 0.02^{cA}$ |
| | NaCl | $27.00 \pm 2.08^{cA}$ | $21.67 \pm 0.88^{cA}$ | $0.61 \pm 0.03^{aA}$ |

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.

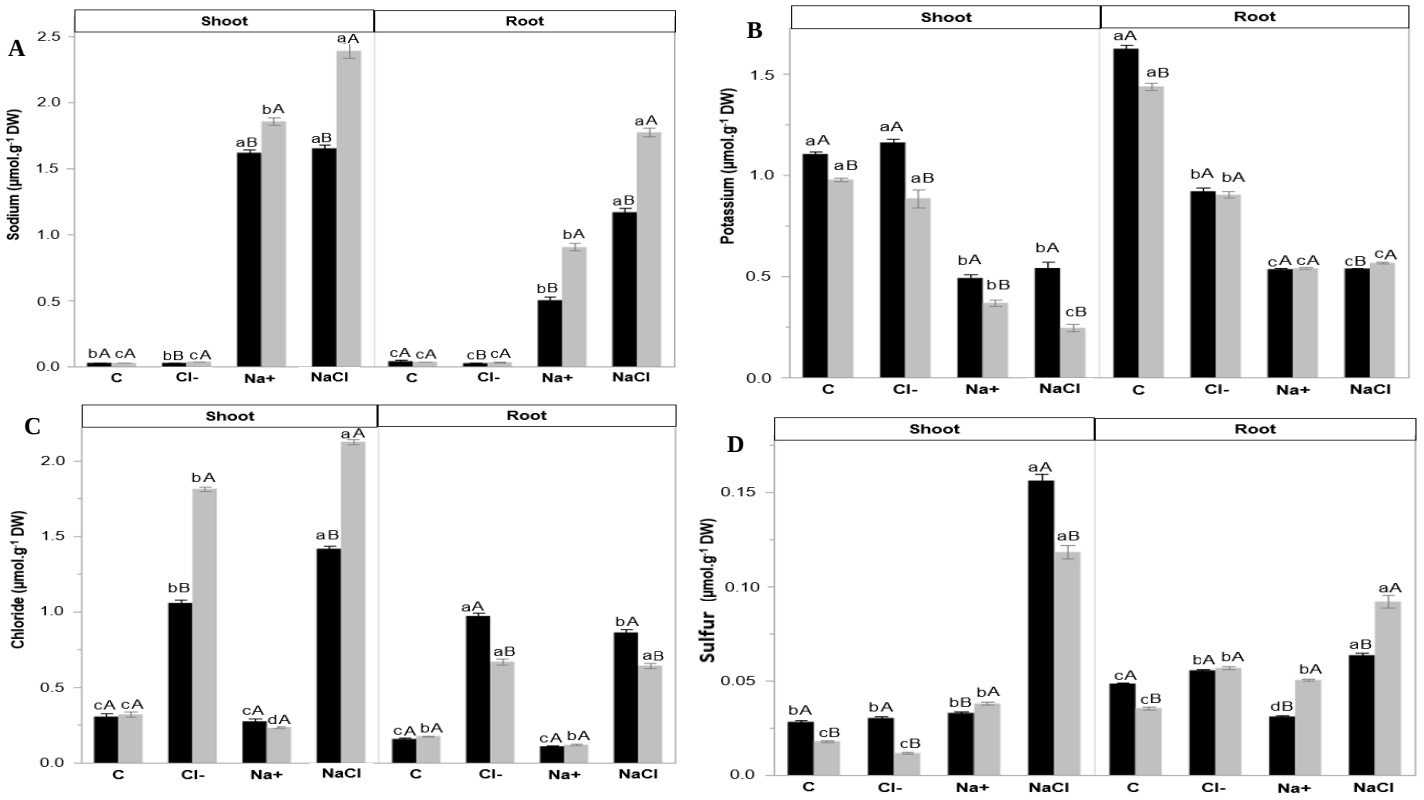


**Figure 2.** (A) sodium, (B) potassium, (C) chloride and (D) sulfur concentration in shoots and roots of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 (gray bars) cultivated during 2 weeks in control conditions (C) or in the presence of 50 mM of either $Cl^-$ (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 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 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.

| | Water content (%) | | | | Ψs (MPa) | | | |
|---|---|---|---|---|---|---|---|---|
| | Shoot | | Root | | Shoot | | Root | |
| Treatments | 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$^{aB}$ |

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 $Cl^-$ 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 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.

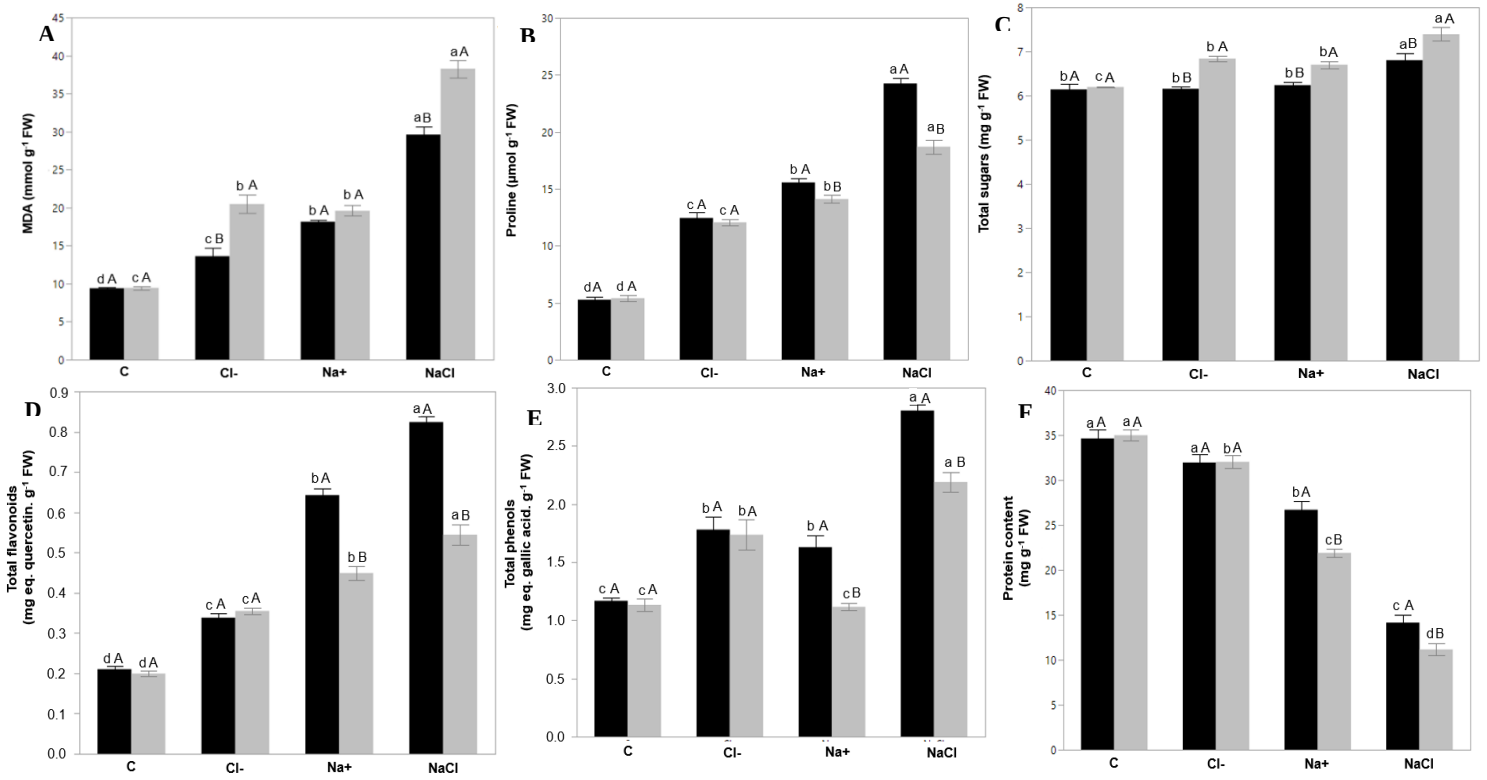


**Figure 3.** (A) Malondialdehyde, (B) proline, (C) total sugars, (D) total flavonoids, (E) total phenols and (F) protein concentration of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 (gray bars) cultivated during 2 weeks in control conditions (C) or in 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 latter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase latter in a particular condition do not differ statistically.

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

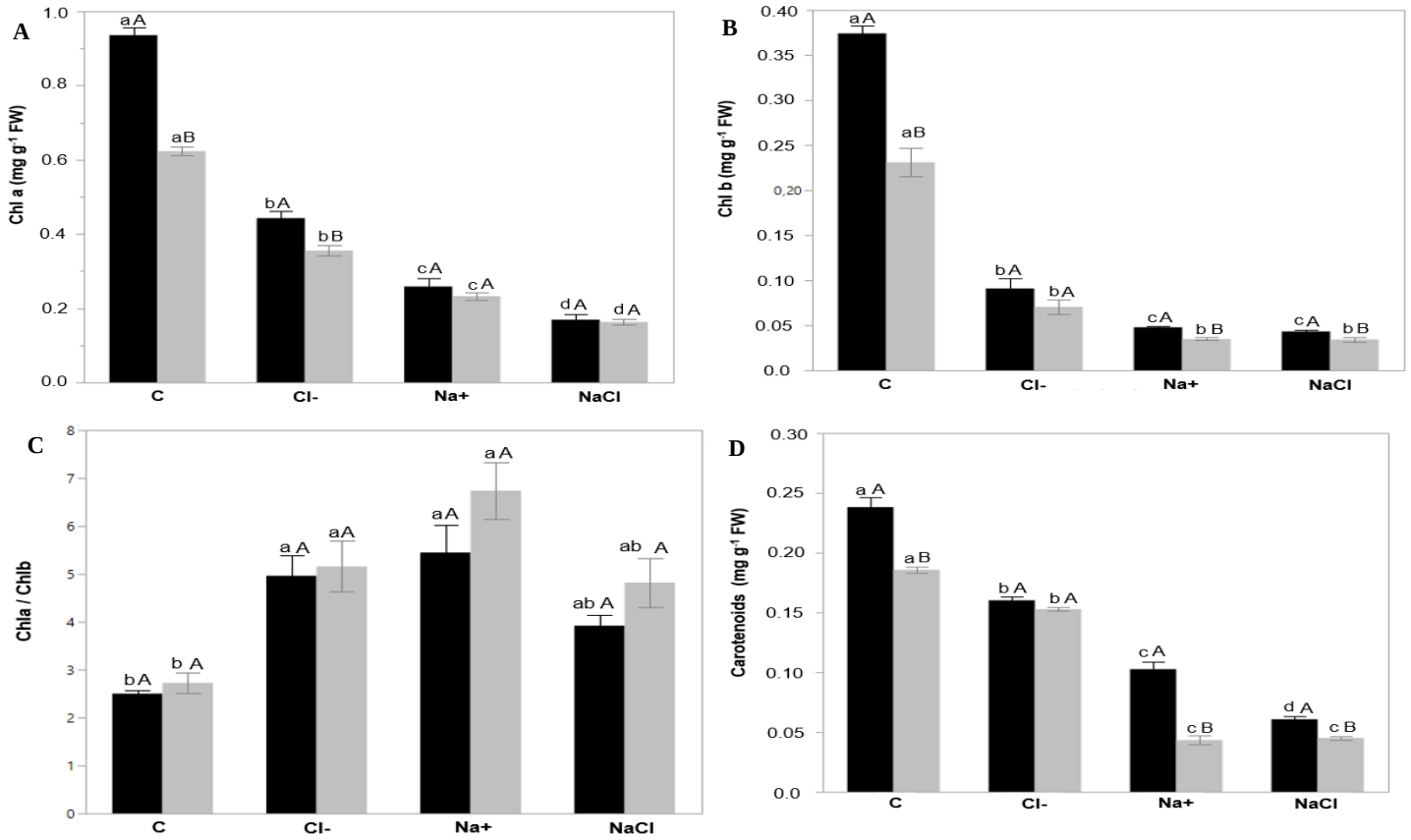


**Figure 4.** (A) chlorophyll a, (B) chlorophyll b, (C) chla/chlb and (D) carotenoids concentration of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 (gray bars) cultivated during 2 weeks in control conditions (C) or in the presence of 50 mM of either Cl- (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 latter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase latter in a particular condition do not differ statistically.

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 $\Phi_{PSII}$ (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 the following order $Cl^- > Na^+ >$ 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 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.

**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. 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.

| 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 | $6.48 \pm 0.12^{aA}$ | $3.49 \pm 0.05^{aA}$ | $339 \pm 4^{aA}$ | $461 \pm 6^{aA}$ |
| | $Cl^-$ | $5.58 \pm 0.14^{bA}$ | $3.20 \pm 0.10^{bA}$ | $342 \pm 3^{aA}$ | $334 \pm 7^{bA}$ |
| | $Na^+$ | $4.07 \pm 0.06^{cA}$ | $1.56 \pm 0.04^{cA}$ | $320 \pm 2^{bA}$ | $249 \pm 7^{cA}$ |
| | NaCl | $2.51 \pm 0.07^{dA}$ | $0.86 \pm 0.03^{dA}$ | $315 \pm 2^{bA}$ | $162 \pm 13^{dA}$ |
| TOG5949 | C | $6.42 \pm 0.08^{aA}$ | $3.45 \pm 0.04^{aA}$ | $348 \pm 2^{aA}$ | $454 \pm 10^{aA}$ |
| | $Cl^-$ | $4.73 \pm 0.13^{bB}$ | $2.88 \pm 0.07^{bA}$ | $341 \pm 3^{aA}$ | $324 \pm 6^{bA}$ |
| | $Na^+$ | $3.46 \pm 0.08^{cB}$ | $1.52 \pm 0.05^{cA}$ | $303 \pm 2^{bB}$ | $217 \pm 9^{cB}$ |
| | NaCl | $1.51 \pm 0.08^{dB}$ | $0.65 \pm 0.02^{dB}$ | $310 \pm 2^{bA}$ | $163 \pm 7^{dA}$ |

**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. 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.

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

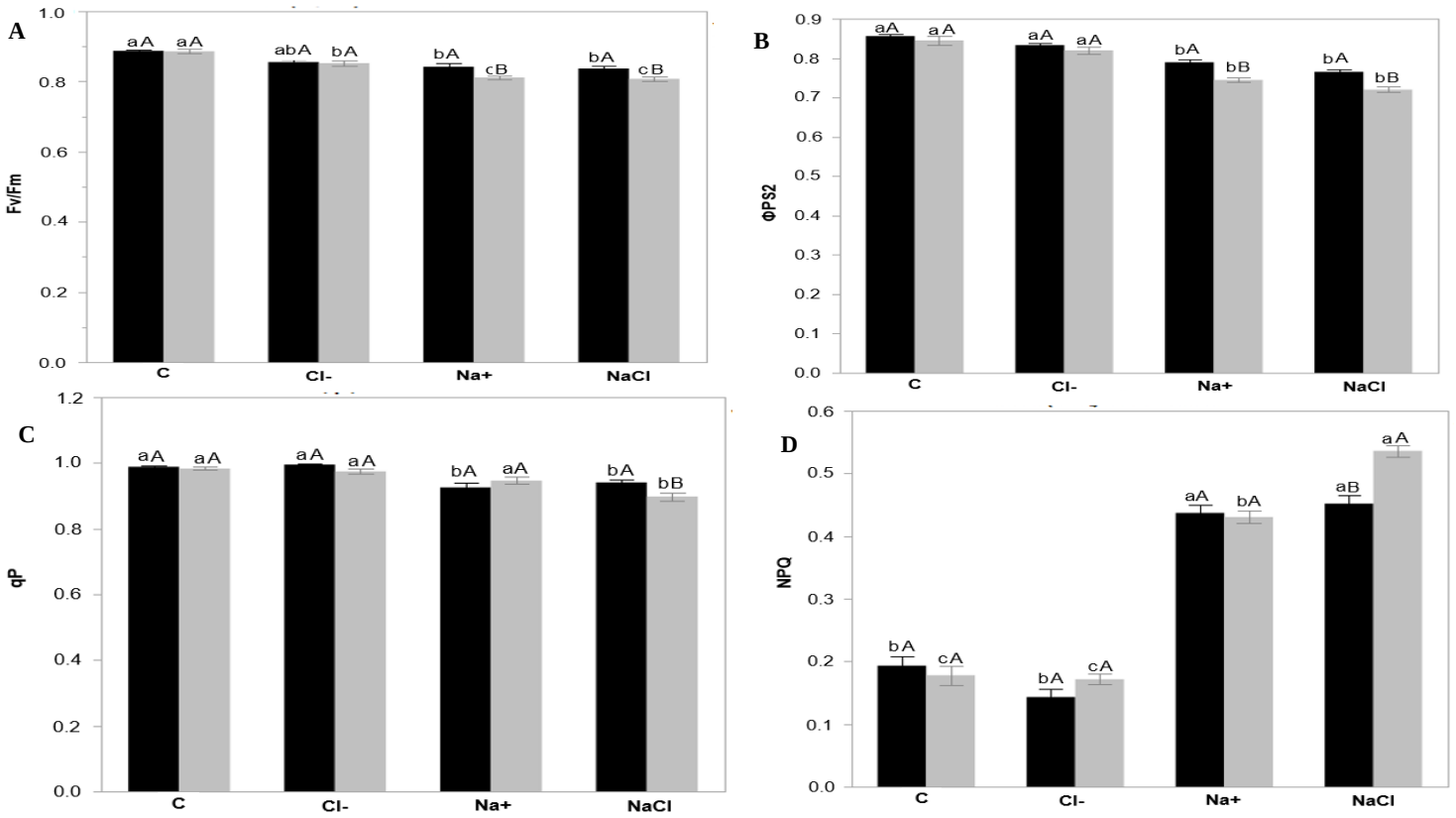


**Figure 5.** (A) Fv/Fm, (B) ΦPS2, (C) qP and (D) NPQ of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 (gray bars) cultivated during 2 weeks in control conditions (C) or in the presence of 50 mM of either Cl- (chloride dominant), Na+ (sodium dominant) or 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 latter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase latter in a particular condition do not differ statistically.

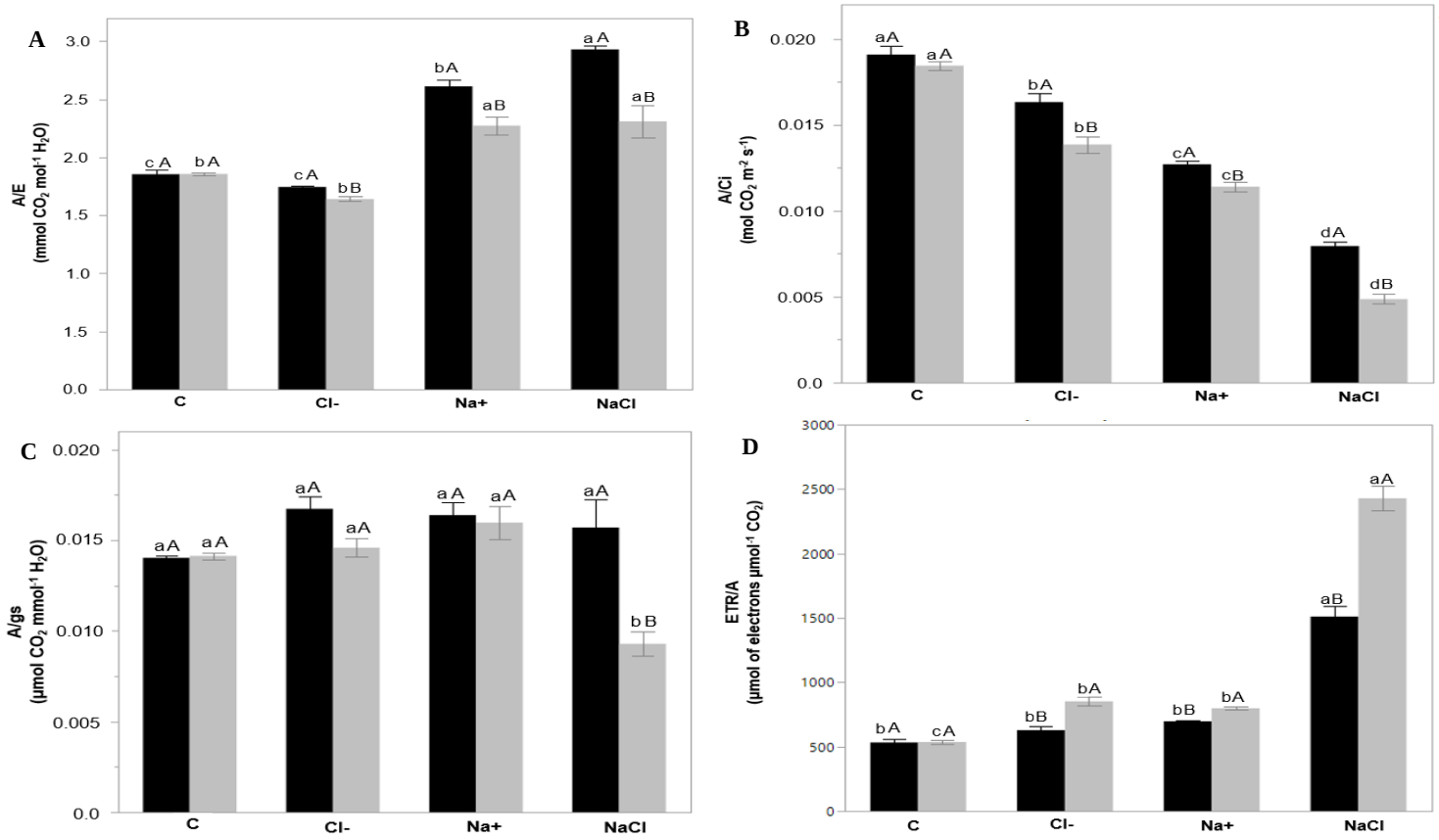


**Figure 6.** (A) A/E, (B) A/Ci, (C) A/gs and (D) ETR/A of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 (gray bars) cultivated during 2 weeks in control conditions (C) or in the presence of 50 mM of either Cl- (chloride dominant), Na+ (sodium dominant) 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 latter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase latter in a particular condition do not differ statistically.

*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 TOG5949. 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 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 $\Psi s$ values). Table 5 presents the ITI values for morphological 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 $\Psi_s$ 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 TOG5307 for *A*/*E*, ETR values, flavonoids, and total phenolics concentration as well as $\delta^{15}N$ and root $\Psi_s$. 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 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 $ITI_{Na} + ITI_{Cl}$ (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 Pearson 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.

**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 | 2786 |
| 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 |

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 | $ITI_{Na}/ITI_{Cl}$ |
| **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 |
| $\Psi$s root | 0.15 | 0.12 | 0.05 | 0.27 | 1.25 | 0.23 | 0.08 | 0.1 | 0.31 | 2.88 |
| $\Psi$s 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 | 0.12 | 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 |

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 | $ITI_{Na}/ITI_{Cl}$ |
| **Photosynthesis** | | | | | | | | | | |
| 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 |

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 | $ITI_{Na}/ITI_{Cl}$ |
| **Nutrition** | | | | | | | | | | |
| 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 |
| 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 |

# 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 $\Psi$s, 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 behaviour 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. glaberrima* 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) or as a strategy of resistance (as it was the case for the synthesis of the osmoprotectant osmolyte proline 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.

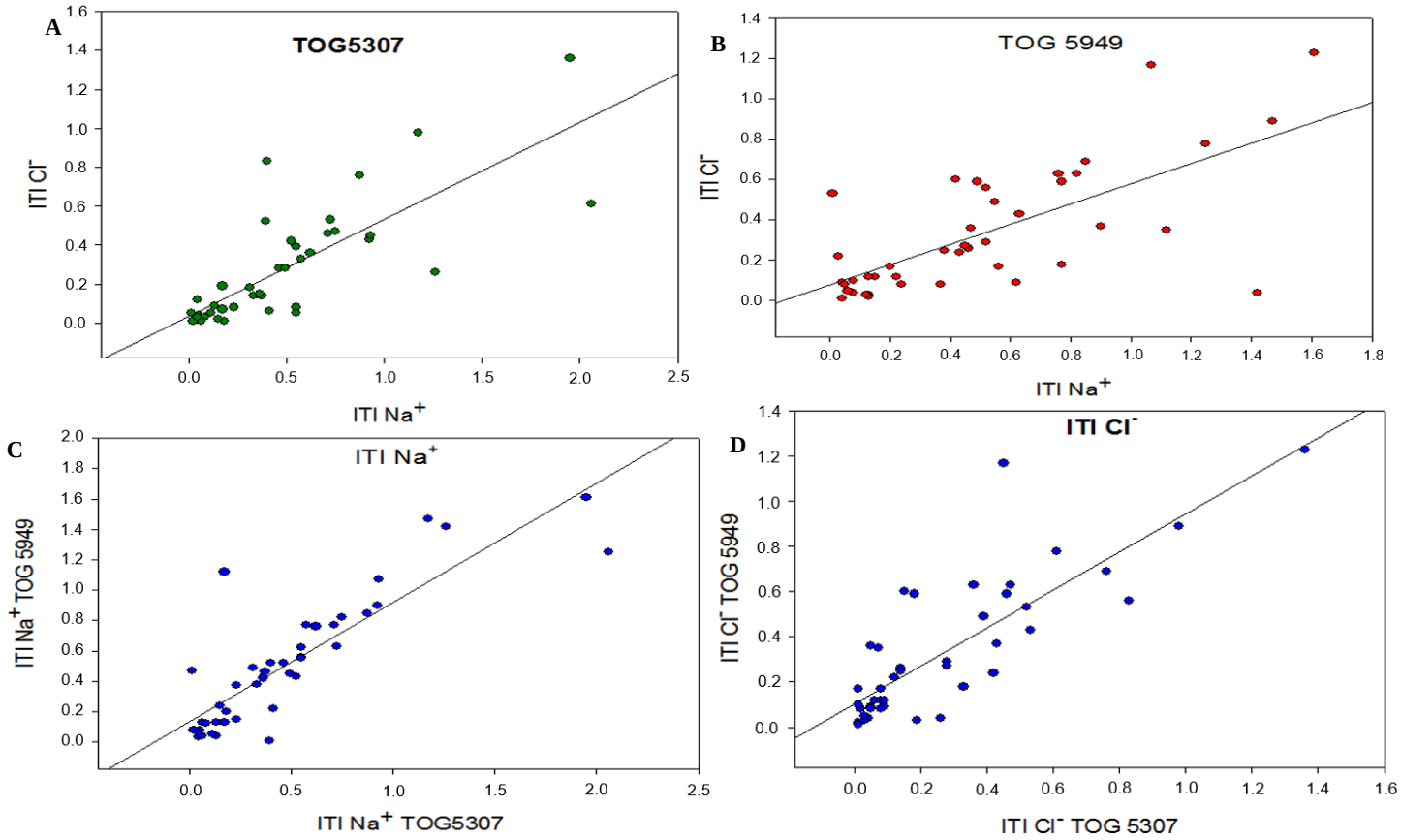


**Figure 7.** Correlations of ion toxicity index (ITI) on (A) sodium and (B) chloride of African rice seedlings (*Oryza glaberrima* Steud.) from cv. (C) TOG5307 and (D) TOG5949 cultivated during 2 weeks in control conditions (C) or in the presence of 50 mM of either Cl- (chloride dominant), Na+ (sodium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

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; $\Phi_{PSII}$, qP, and NPQ remained unaffected by $Cl^-$-treatment, although $Na^+$ and NaCl reduced $F_v/F_m$, qP and $\Phi_{PSII}$ 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 et al. 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 a synergetic 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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*Conflict of interest*

The authors declare no conflict of interest.

*Author's contribution*

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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# Interchapter : Chapter 3 – Chapter 4

## What do we already know ?

In the previous chapters, we considered two cultivars of *O. glaberrima* exhibiting contrasting levels of salinity resistance at both seedling and reproduction phases of development : TOG5307 is salt-resistant while TOG5949 is salt-sensitive. We demonstrated that TOG5307 accumulated lower amounts of $Na^+$ in both shoots and roots comparatively to TOG5949 and was able to sequester higher amounts of $Cl^-$ in their roots to protect photosynthetic tissues at the shoot level. For most analyzed physiological parameters, $Na^+$ was more toxic than $Cl^-$ in both cultivars. The toxic ions acted in an additive way, except for $Na^+$ and S accumulation where a synergistic interaction was recorded in response to NaCl treatment (see Figure A below). The two considered cultivars mainly differed for their response to the ionic component of salt stress but hardly differ for their osmotic behaviour.

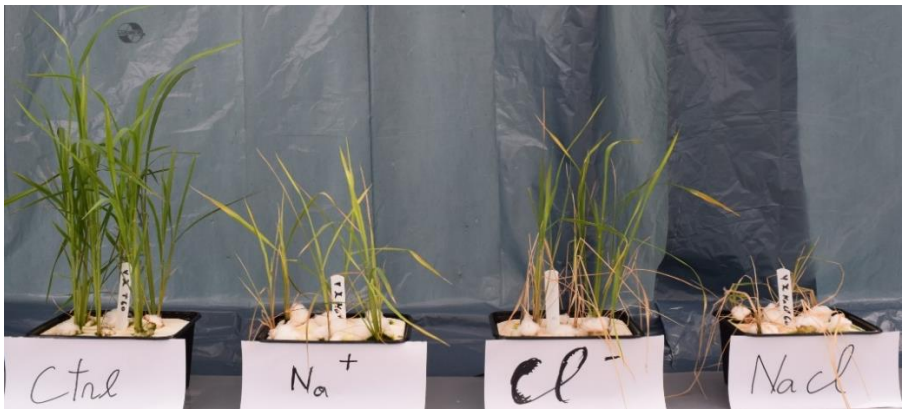



**Figure A** : African rice seedlings (*Oryza glaberrima* Steud.) cultivated in control conditions (Ctrl) or in the presence of 50 mM of either Cl- (chloride dominant), Na+ (sodium dominant) or NaCl.

## What do we want to know ?

Sodium is not considered as an essential element for C3 species, and is consequently absorbed by the roots through $K^+$ non-selective transporters and non-selective cation channels. Glycophyte plant species are nevertheless able to display some strategies allowing them to cope with an excess of $Na^+$ in the surrounding medium. This is especially the case for the Salt-Overly-Sensitive pathway, involving the plasma membrane protein SOS1 acting as a $Na^+/H^+$ antiporter to exclude $Na^+$ back to the surrounding medium at the root epidermis level, or to the apoplast in other tissues. Other $Na^+/H^+$ antiporters (NHX) are also present at the tonoplast level and contribute to sequester $Na^+$ within the vacuoles to protect cytosol from this toxic element. In contrast to $Na^+$, chloride is an essential elements involved in several steps of plant metabolism. Although this micro-element is required only in few amounts, some transporters are present at the cell level to ensure $Cl^-$ absorption from the external medium to the cytosol. This is the case for the Cation-Chloride-Transporter (CCC) which is a membrane carrier mediating electroneutral symport of $Cl^-$ coupled to $K^+$ and/or $Na^+$ transport (see Figure B below). We want to know to which extent these various transporters are involved in the plant response of the two considered cultivars to NaCl.

## What is our strategy ?

To the best of our knowledge, no mutant for gene coding for the studied transporters are available in *O. glaberrima*. An alternative will be to use a pharmacological approach using compounds able to inhibit the considered transporters. Amiloride is known as a powerful inhibitor of $Na^+/H^+$ antiporter while bumetanide is inhibiting the cation-chloride-transporter. We will thus use these products in the next chapter on both control and NaCl-treated plants and establish comparison with plants exposed to NaCl in the absence of inhibitors. Although these inhibitors are considered as rather specific, a side effect on other targets could not be excluded during a long term exposure ; for this reason, we will restrict the treatment to a 3 day exposure. To obtain comparable levels of $Na^+$ and $Cl^-$ accumulation as in Chapter 1 and 3, we will increase the NaCl dose to 75 mM. A 3 day exposure is too short to establish a reliable estimation of plant growth. We will thus consider mineral nutrition, and senescence-related parameters in the leaves which are reported to be quickly modified by toxic ion accumulation : cell viability index (estimated on the basis of TTC conversion to Formazan) cell membrane stability

estimated by both electrolyte leakage and relative leakage of UV-absorbing substances, and total soluble protein content. This set of parameters should allow us to gain information about the consequence of inhibitor-induced modification of $Na^+$ and $Cl^-$ content in the plant tissues.

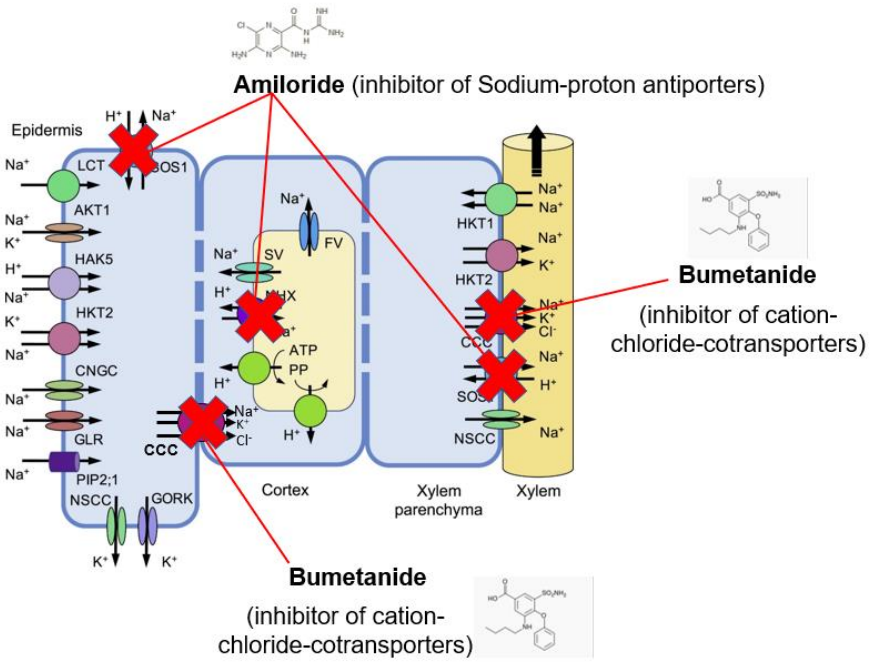
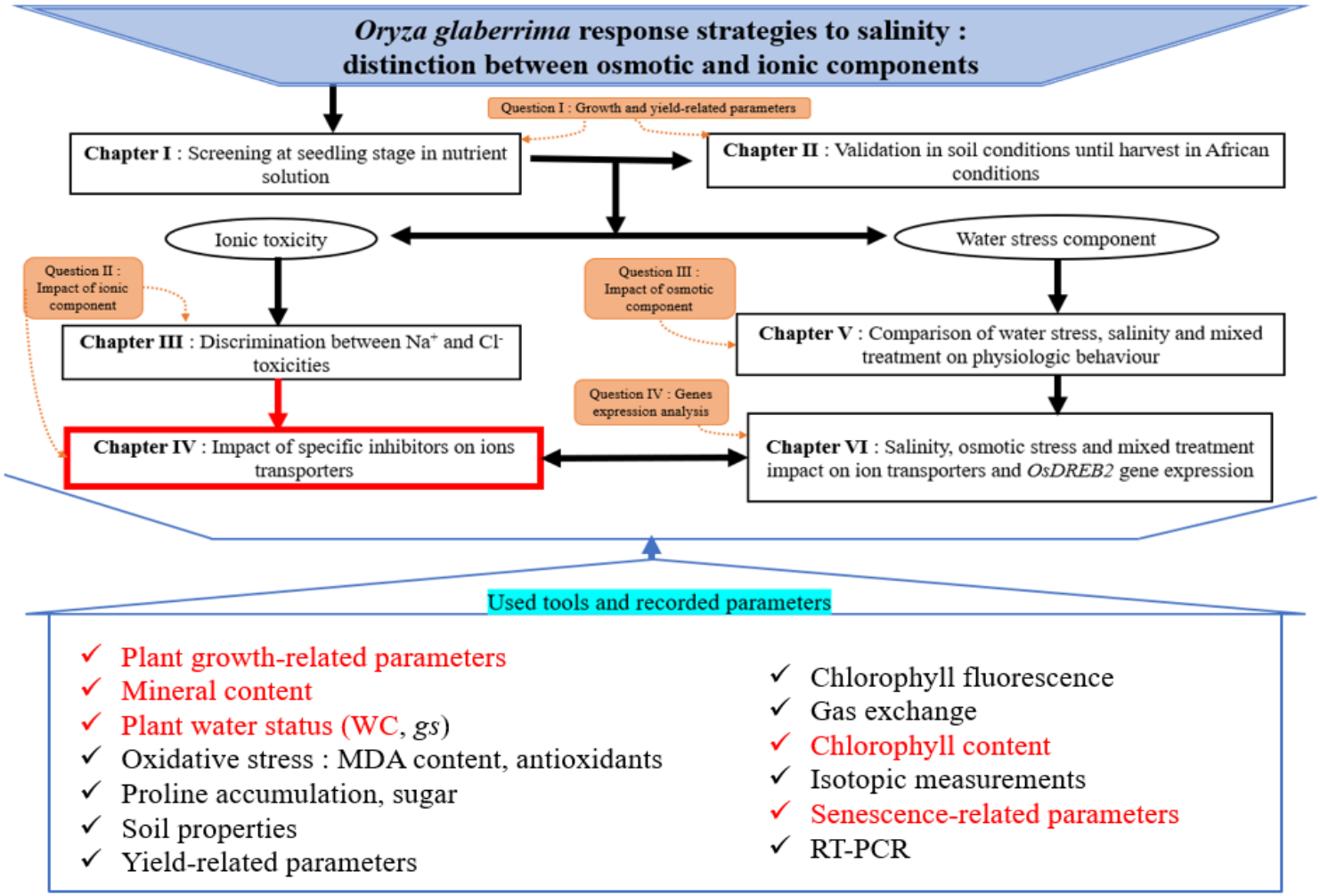



**Figure B** : Illustration of the action of amiloride on sodium-proton antiporter (SOS1) and bumetanide on cation-chloride cotransporters (CCC). (Adapted from Zhao et al., 2020)




**Overview of questions addressed during this thesis and their relationships with chapters.**

# Chapter IV. Inhibitors of $Na^+/H^+$ antiporter and cation-chloride-cotransporter have contrasting effects on two cultivars of *Oryza glaberrima* Steud. differing in salinity resistance.

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## Abstract

A pharmacological study was conducted to analyze the impact of inhibitors of $Na^+$-$H^+$ antiporters (amiloride 100 µM) and cation-chloride cotransporters (bumetanide 200 µM) on two cultivars of the African rice species (*Oryza glaberrima* Steud) differing in salt-resistant (TOG5307: salt-resistant and TOG5949: salt-sensitive) exposed in nutrient solution to 75 mM NaCl during 3 days. Amiloride increased $Na^+$ accumulation in roots and leaves to a higher extent in salt-resistant TOG5307 than in salt-sensitive TOG5949. Bumetanide reduced $Cl^-$ accumulation in both cultivars as well as $K^+$ accumulation in TOG5307 and $Na^+$ accumulation in TOG5949. Inhibitors mainly acted on the absorption step but had low impact on root-to-shoot translocation process. The salt-resistant cultivar was able to efficiently regulate $Na^+$ uptake and to cope with high concentration of accumulated toxic ions.

**Keywords**: African rice, ion accumulation, mineral nutrition, NaCl, salinity, senescence.

# Introduction

Limiting $Na^+$ and $Cl^-$ accumulation within tissues is an important challenge for plants exposed to salinity. Sodium and chloride uptakes by the roots of higher plants are controlled by a wide range of transporters (Teakle and Tyerman 2010; Zhang et al. 2010; Li et al. 2021). $Na^+/H^+$ antiporters assume key functions and were shown to extrude toxic $Na^+$ from the cytosol to the apoplasm (as it is the case for the Salt-Overly-Sensitive pathway, with SOS1 acting as a plasma membrane $Na^+/H^+$ antiporter (Guo et al. 2009; Deng et al. 2016)), or from the cytosol to the vacuoles, thus allowing $Na^+$ compartmentation (such as some members of the NHX family among which several are located in the tonoplast (Wang et al. 2016)).

As an essential element, $Cl^-$ may be absorbed by numerous transporters including the Cation-Chloride Cotransporters (CCC) which are membrane-integral solute carriers that mediate electroneutral translocation of $Cl^-$, coupled to $K^+$ and/or $Na^+$ (Henderson et al. 2018). A CCC-encoding gene has been identified in rice (*Oryza sativa* L.) (Kong et al. 2011) and is required for cell elongation and osmoregulation in the absence of salt (Chen et al. 2016). In the presence of high concentration of NaCl, this transporter may contribute to $Cl^-$ long-distance transport and impact $K^+/Na^+$ ratio (Zhu et al. 2017).

The classical Asian rice species *Oryza sativa* is very sensitive to salinity (Solis et al. 2020) but the marginal African crop *Oryza glaberrima* Steud. recently received considerable attention in relation to its high level of resistance to a wide range of biotic and abiotic constraints (Veltlman et al. 2019). As far as salt is concerned, a recent screening performed among numerous cultivars allowed to identify a salt resistant cultivar TOG5307 and a salt-sensitive genotype TOG5949 (Prodjinoto et al. 2018). The comparison between those cultivars suggests that an efficient regulation of $Na^+$ absorption and $Cl^-$ translocation from the root to the shoot may be involved in salinity resistance in *O. glaberrima* (Prodjinoto et al. 2018; 2021).

Amiloride was shown to act as a powerful inhibitor of $Na^+/H^+$ antiporter (Taleisnik et al. 1991 ; Fukuda et al. 1998) while bumetanide is inhibiting the cation-chloride-cotransporter (Colmenero-Flores et al. 2007). In order to gain additional information regarding the involvement of these transporters in salinity resistance in *Oryza glaberrima*, a pharmacological approach was performed by exposing young seedlings from the two cultivars of African rice

to 75 mM NaCl in the absence or in the presence of the inhibitors. Impacts of inhibitors were determined in relation to ion accumulation and to senescence-related parameters resulting from ion toxicity.

## Material and Methods

### Plant material and growth conditions

Seeds the two rice cultivars (*Oryza glabberima* Steud., cvs salt-resistant TOG5307 (Accession number WAB0021855) and salt-sensitive TOG5949 (Accession number WAB0020144)) were dehusked and surface-sterilized with formol/hypochlorite. For each cultivar, 250 seeds germinated on two layers of filter paper Whatman N°2 moistened with 7 mL of half-strength Yoshida solution (Yoshida et al. 1976) in a growth chamber under 12 h day light photoperiod at 95 µmoles.$m^{-2}$ $s^{-1}$ and a constant temperature of 25 °C. Ten-days old seedlings were then acclimated to nutrient solution: 180 seedlings for each cultivar were distributed among 30 tanks containing 1.2 L of full-strength Yoshida nutrient solution. In each tank, 6 seedlings were placed on a plugged polystyrene plate floating at the top of the solution. The tanks were randomly distributed in the phytotron (temperature at 29 °C during the day and 26 °C during the night; 16 $h.day^{-1}$ photoperiod with a photon flux density of 300 µmoles $m^{-2}$ $s^{-1}$ provided by LED LumiGrow lights, 650 W, red-blue).

After 10 days of growth (plants at the 3 leaves stage), inhibitors were added to 20 tanks per cultivars: 10 tanks received 150 µM amiloride and 10 others received 200 µM bumetanide (both provided by Sigma-Aldrich chemical). The remaining 10 tanks did not receive inhibitors. One day after inhibitor application, NaCl was added to half of the tanks (including half of those which did not receive inhibitor) to reach a concentration of 75 mM which was maintained for 3 days until plant harvest. Electrical conductivity was 0.82 ± 0.07 mS $cm^{-1}$ and 7.13 ± 0.24 mS $cm^{-1}$ for control and salt-solution, respectively; the presence of inhibitor had no impact on electrical conductivity.

**Mineral analysis**

Roots and shoots of 5 plants for each treatment were separated; roots were thoroughly rinsed during 60 s in cold distilled water to remove ions from the free spaces. Organs were weighed, dried in an oven at 70 °C until reaching constant weight. Dry matter was digested by $HNO_3$ (68%) + $HCl_{cc}$ (1:3, *v/v*); $Na^+$ and $K^+$ were determined by flame emission using Atomic Absorption Spectrometer (Thermo scientific S series model AAS4). Chloride was determined by liquid chromatography (HPLC-Dionex ICS2000, Dionex Corporation, Sunnyvale, California, U.S.A.) using a AS15/AG15 column/precolumn system and 20-38 mM KOH as eluant during 40 min.
Selective absorption capacity (SA) of the roots for $K^+$ over $Na^+$ was estimated according to Deng et al. (2016) as SA = ($Na^+/K^+$ in environment)/$Na^+/K^+$ in plant) while the selective transport (ST) of $K^+$ was defined as ST=($K^+/Na^+$ in shoots)/($K^+/Na^+$in roots)

**Senescence-related parameters**

Recorded senescence-related parameters mainly concern pigments and total soluble protein concentrations, cell membrane stability, and cell viability. Each parameter was quantified on five plants per treatment on leaf segments obtained from leaf n°2 and 3 (acropetal numbering). Chlorophyll and carotenoids were extracted with cold acetone 80%; absorbance of the extract was read at 445 and 663 nm and used to calculate pigment concentrations according to Lichtenthaler (1987).
Membrane permeability was assessed using two distinct methods. The electrolyte leakage technique was performed as detailed by Bajji et al. (2002). The leakage of UV-absorbing substances was quantified as a relative leakage ratio (RLR) according to Lutts et al. (1996).
Cell viability was determined on leaf segments (*c.a.* 50 mg FW) quickly rinsed in deionized water containing 0.05% Tween 20 and incubated in darkness at 30 °C in glass tubes containing 5 mL of 0.5% triphenyltetrazolium chloride (TTC; Sigma-Chemical) dissolved in 50 mM $K_2HPO_4$ at pH 7.0 during 15 h. Samples recovered after filtration were then incubated during 5 min in 3 mL glass vial containing 94% ethanol at 80 °C. The extracted formazan recovered after centrifugation at 5000 *g* was quantified spectrophotometrically at 487

nm and viability index was defined as absorbance measured per g FW according to Lutts et al. (2004). Total soluble proteins were quantified on both roots and leaves according to Bradford (1986).

**Statistic treatment of the data**

The experiment used a completely randomized design. ANOVA 2 was performed considering cultivar and treatment as main factors. Analysis were performed on 5 biological replicates. Normality of the data was preliminary checked using Shapiro-Wilk tests and data were transformed when required. Statistical analysis was performed using SAS Entreprise Guide 6.1 (SAS 9.4 system for Windows).

## Results

**Mineral nutrition**

In control conditions (absence of NaCl and inhibitors) the two cultivars contained similar low concentrations of $Na^+$ in roots (Fig. 1A) and shoots (Fig. 1B). The presence of NaCl increased $Na^+$ concentration to a higher extent in TOG5949 than in TOG5307 for both organs. Amiloride had no impact on $Na^+$ concentration in plants that were not exposed to NaCl but it increased $Na^+$ concentrations in roots and shoots of both cultivars in NaCl-treated plants: from a relative point of view, the recorded increase was higher in TOG5307 (47.3% in roots and 97.4% in leaves) than in TOG5949 (20.2% in roots and 27.7% in leaves). Bumetanide reduced $Na^+$ concentration in the roots and leaves of TOG5949 while it had no significant effect on the $Na^+$ content of TOG5307.

Control plants exposed to bumetanide presented deleterious symptoms of slight wilting: while the leaf water content was 90.3 ± 0.5 in the absence of the inhibitor, it dropped to 86.4 ± 0.8% in its presence. In the absence of bumetanide, TOG5949 accumulated higher amounts of $Cl^-$ in the leaves than TOG5307 (Fig. 1D) while an inverse trend was recorded for the roots (Fig. 1C). Bumetanide strongly decreased $Cl^-$ content in roots and leaves of the two cultivars but the recorded decrease in the leaves was higher for TOG5949 than

for TOG5307. Salinity decreased $K^+$ concentration in the roots and shoots of both *cvs* but $K^+$ remained slightly higher in roots of TOG5307 than in TOG5949. Amiloride had no significant impact on $K^+$ concentration. Conversely, bumetanide reduced the $K^+$ concentration in TOG5307 exposed to NaCl comparatively to plants exposed to NaCl in the absence of inhibitor while this was not observed in TOG5949 (Fig. 1E and 1F).

SA and ST values are provided in Table 1 for plants exposed to NaCl only, since only extremely low levels of $Na^+$ were present in control plants. In the absence of inhibitors, selective $K^+$ absorption in plants exposed to NaCl was higher in TOG5307 than in TOG5949, suggesting that salt-resistance in TOG5307 was related to a better capacity of this cultivar to efficiently discriminate among monovalent cations. Amiloride reduced SA to a higher extent in TOG5307 than in TOG5949. Bumetanide increased SA in TOG5949. In contrast, differences between cultivars were quite less obvious for ST values and this suggests that cultivars differed more for absorption selectivity of monovalent cations than for translocation selectivity. In NaCl-treated plants exposed to amiloride, ST was however higher in TOG5949 than in TOG5307.

**Table 1.** Selective absorption (SA) and selective transfer from root to shoot (ST) for $K^+$ over $Na^+$ in two cultivars of the African rice species *Oryza glaberrima* (TOG5307: salt-resistant and TOG5949: salt-sensitive). Plants were exposed during 72 h to 75 mM NaCl in the presence or absence of amiloride (150 µM) or bumetanide (200 µM). Each value is the mean of 5 biological replicates ± standard errors. For a given parameter, values with different letters are significantly different at $P = 0.05$ according to the Scheffé test.

| | SA | | ST | |
|---|---|---|---|---|
| | TOG5307 | TOG5949 | TOG5307 | TOG5949 |
| NaCl | 27.52 ± 1.07 a | 17.34 ± 1.21b | 1.27 ± 0.03 a | 1.19 ± 0.11 ab |
| NaCl + Amiloride | 14.53 ± 1.22 c | 14.90 ± 1.37 c | 0.88 ± 0.04 c | 1.01 ± 0.18 b |
| Bumetanide | 18.07 ± 0.65 b | 26.54 ± 2.30 a | 1.13 ± 0.10 ab | 1.15 ± 0.08 ab |

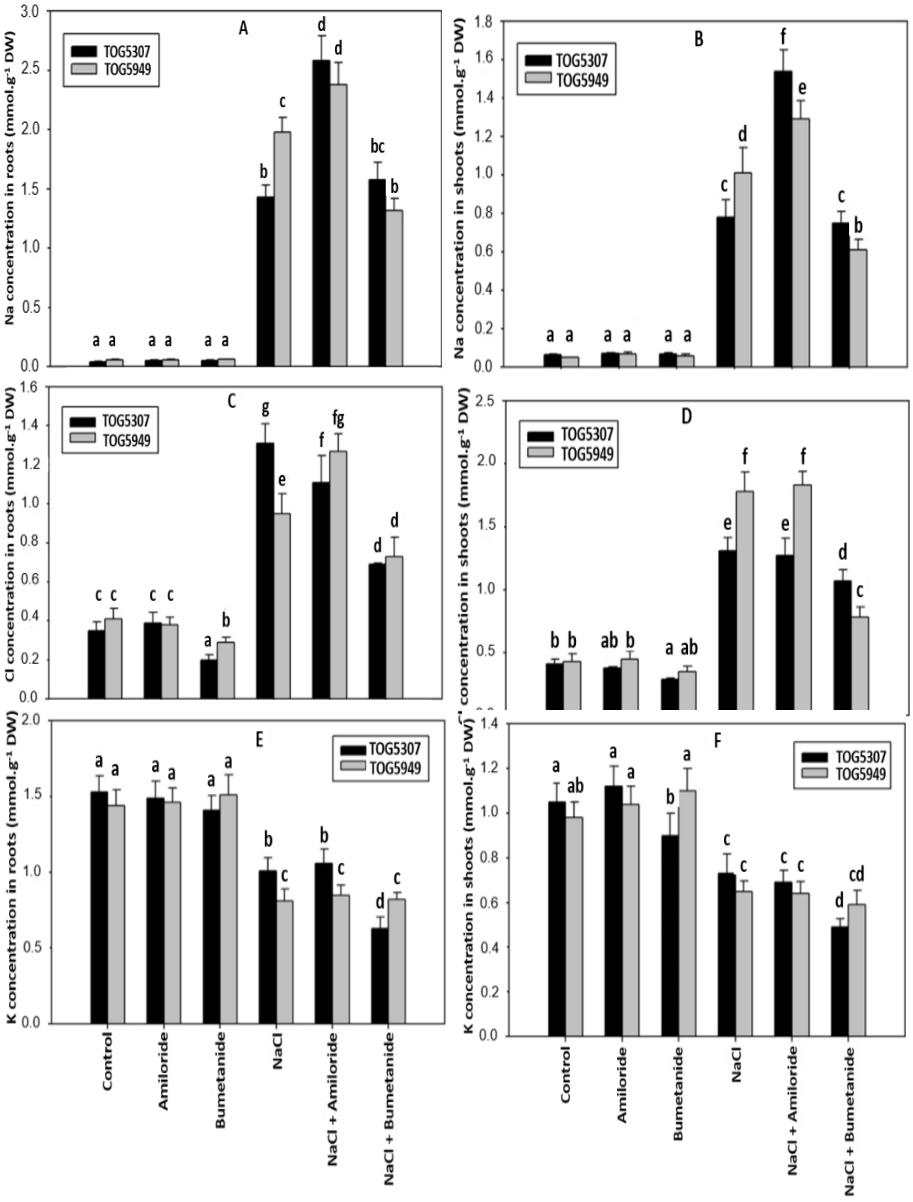


**Figure.1.** Concentrations of $Na^+$ (A;B), $Cl^-$ (C;D) and $K^+$ (E;F) in roots (A, C and E) and in shoots (B, D and F) in two cultivars of the African rice species *Oryza glaberrima* (TOG5307 : salt-resistant and TOG5949 : salt-sensitive). Plants were exposed during 72h to 0 (control) or 75 mM NaCl in the presence or absence of amiloride (150 µM) or bumetanide (200 µM). Each value is the mean of 5 biological replicates and bars represent standard errors. Values with different letters are significantly different at $P = 0.05$ according to the Scheffé test.

**Senescence-related parameters**

Cell viability index and total soluble protein concentrations are provided in Fig. 2, for roots and for leaves of the two cultivars exposed to the various treatments. Amiloride in the absence of NaCl had only minor impact on these parameters while bumetanide decreased the recorded values for both of them. Salinity significantly decreased cell viability index (Fig. 2A and 2B) but it remained higher in TOG5307 than in TOG5949. In the presence of NaCl, both amiloride and bumetanide increased cell viability in roots of TOG5307 but not in TOG5949. A contrasting behavior was observed at the shoot level, where bumetanide obviously increased cell viability in TOG5949 but not in TOG5307. Amiloride decreased protein content in NaCl-treated plants (Fig. 2C and 2D) while bumetanide mitigated the deleterious impact of NaCl on protein content in the shoots of TOG5949.

In the absence of NaCl, bumetanide significantly decreased Chla and Chlb in the two cultivars (Table 2). In NaCl-treated plants, Chla, Chlb and carotenoids concentrations were always higher in TOG5307 than in TOG5949. Amiloride decreased Chla and carotenoids in TOG5949 but not in TOG5307 while bumetanide increased Chla in the latter but not in the former. The two cultivars exhibited similar electrolyte leakage in the absence of NaCl (Table 2). Salt stress induced an obvious increase in EL values, and TOG5307 presented a higher EL value than TOG5949, the highest value being recorded in 75 mM NaCl + amiloride-treated plants. An inverse trend was observed for relative leakage ratio of UV-absorbing substance: it also increased in response to NaCl, but to a higher extent in TOG5949 than in TOG5307.

## Discussion

Salinity resistance in plants is related to the restriction of $Na^+$ and $Cl^-$ absorption, efficient compartmentation of toxic ions in vacuoles, restriction of $Na^+$ and $Cl^-$ xylem loading and osmoregulation allowing the plant to cope with the osmotic component of salt stress (van Zelm et al. 2020). Salt-overly-sensitive SOS1 is a $Na^+/H^+$ antiporter primarily expressed at the root tip epidermis, in parenchyma root cells and in xylem parenchyma at the xylem/ symplasm boundary (Shi et al. 2002; Guo et al. 2009). Other $Na^+/H^+$

antiporters NHX are located to tonoplast and involved in vacuolar sequestration (Fukuda et al. 1998).

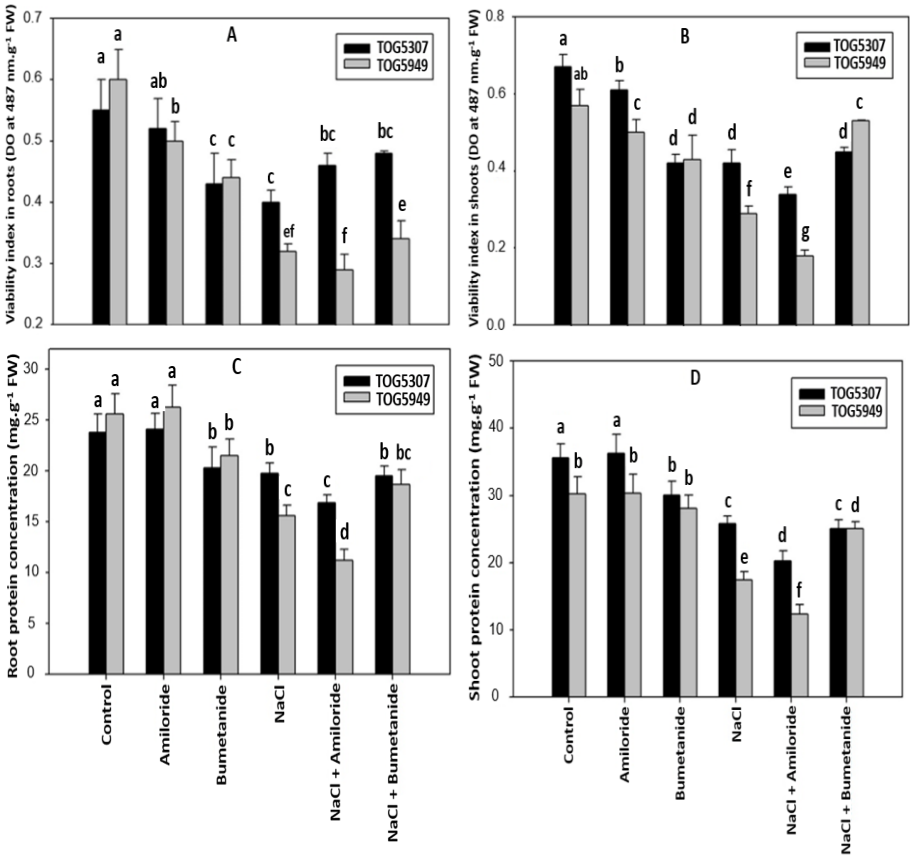


**Figure.2.** Total viability index recorded by TTC transformation to formazan (ΔDO at 487 nm.g-1 FW ; A and B) and total soluble protein concentration (mg.g$^{-1}$ FW ; C and D) in roots (A, C) and in shoots (B, D) of two cultivars of the African rice species *Oryza glaberrima* (TOG5307 : salt-resistant and TOG5949 : salt-sensitive). Plants were exposed during 72h to 0 (control) or 75 mM NaCl in the presence or absence of amiloride (150 µM) or bumetanide (200 µM). Each value is the mean of 5 biological replicates and bars represents standard errors. Values with different letters are significantly different at $P = 0.05$ according to the Scheffé test.

**Table 2.** Chlorophyll a (Chl a), chlorophyll b (Chl b), carotenoid (Car) concentrations (in $mg.g^{-1}$ FW), electrolyte leakage (EL, in %) and relative leakage of UV-absorbing substances (RLR; in %) in the leaves of two cultivars of the African rice species *Oryza glaberrima* (TOG5307: salt-resistant and TOG5949: salt-sensitive). Plants were exposed during 72 h to 75 mM NaCl in the presence or absence of amiloride (150 µM) or bumetanide (200 µM). Each value is the mean of 5 biological replicates. For a given parameter, values with different letters are significantly different at $P = 0.05$ according to the Scheffé test.

| Treatment | Cultivar | Chl a | Chl b | Car | EL (%) | RLR (%) |
|---|---|---|---|---|---|---|
| 0 mM NaCl | TOG5307 | 0.84 a | 0.37 a | 0.23 a | 9.8 a | 10.1 b |
| | TOG5949 | 0.78 b | 0.35 a | 0.21 a | 10.4 a | 9.5 ab |
| 0 mM NaCl | TOG5307 | 0.82 a | 0.35 a | 0.21 a | 9.5 a | 8.9 a |
| + Amiloride | TOG5949 | 0.80 ab | 0.32 ab | 0.19 ab | 10.3 a | 9.2 a |
| 0 mM NaCl | TOG5307 | 0.66 c | 0.30 b | 0.21 a | 12.6 ab | 12.4 b |
| + Bumetanide | TOG5949 | 0.69 c | 0.27 c | 0.17 b | 14.7 b | 15.8 c |
| 75 mM NaCl | TOG5307 | 0.54 d | 0.28 bc | 0.16 b | 24.3 d | 18.4 d |
| | TOG5949 | 0.45 e | 0.21 d | 0.11 c | 19.2 c | 25.3 e |
| 75 mM NaCl | TOG5307 | 0.51 d | 0.26 c | 0.14 bc | 30.4 e | 19.6 d |
| + Amiloride | TOG5949 | 0.33 f | 0.20 d | 0.07 d | 25.6 d | 30.8 f |
| 75 mM NaCl | TOG5307 | 0.63 c | 0.31 ab | 0.15 b | 25.2 d | 17.6 cd |
| + Bumetanide | TOG5949 | 0.46 e | 0.24 cd | 0.10 cd | 23.8 cd | 24.7 e |

SOS1 inhibition by amiloride may at least partly explain the recorded increase in root $Na^+$ content, considering that its presence in root meristem zone may contribute to $Na^+$ extrusion to the surrounding medium (Shi et al. 2002). Amiloride-induced $Na^+$ increase in the roots was higher in salt-tolerant TOG5307 than in salt-sensitive TOG5949 and this suggests that in NaCl-treated plants, SOS1 is more active in the former than in the latter. $K^+$ and $Na^+$

are similar in ionic radius and hydration energy (Zhang et al. 2010) and discrimination between these ions is an important component of salt tolerance in plants. The fact that amiloride reduced SA to a higher extent in TOG5307 than in TOG5949 reinforce the hypothesis that SOS1 is an efficient contributor to ion discrimination through its specific involvement in $Na^+$ efflux, mainly in the salt-resistant cultivar.

At the xylem/symplast boundary SOS1 mediates $Na^+$ loading and thus contribute to $Na^+$ delivery to the shoot (Shi et al. 2002; Zhu et al. 2017): blocking xylem loading by amiloride should contribute to reduce $Na^+$ translocation to the shoots but this was not supported by our data since amiloride increased rather than decreased $Na^+$ accumulation in the leaves. Other transporters such as the high affinity $Na^+/K^+$ permeable transporters from the HKT family are involved in $Na^+$ retrieval from the xylem (Horie et al. 2009) and it could therefore not be excluded that salinity had a detrimental impact on these transporters which are not sensitive to amiloride.

Amiloride and its analogues are permeable weak base and may also interfere with vacuolar $Na^+/H^+$ antiport in root cells. Sodium compartmentation inside vacuoles is driven by the electrochemical gradient of protons generated by the V-type $H^+$-ATPase and the $H^+$-PPiase (Zhang et al., 2010) and is a wise strategy to avoid $Na^+$-induced cytosolic damages. Reducing both SOS1 activity and vacuolar $Na^+$ compartmentation may increase $Na^+$ in the apoplast and its transport to the xylem by transpirational stream. This could partly explain the recorded increase in leaf $Na^+$ occurring despite partial amiloride inhibition of SOS1. In amiloride-treated plants exposed to NaCl, sodium may also remain in the cytosol of cells and induce a wide range of metabolic disorders.

Until recently, chloride received less attention than $Na^+$ in the plant response to NaCl. CCC belongs to cation-$Cl^-$ transporters and is essential for ion homeostasis. Bumetanide, which is a powerful inhibitor of CCC had a detrimental impact on control plants, reducing water content, cell viability index and protein content. This is in accordance with previous data who reported that CCC is required in the absence of NaCl stress for efficient cell elongation and osmoregulation processes in *O. sativa* (Kong et al. 2011; Chen et al. 2016).

Although the studied cultivars did not differ for their response to bumetanide in the absence of NaCl, they displayed contrasting behavior when the inhibitor was added to NaCl-containing solution. CCC is present in root hairs and root

epidermis and it also retrieve $Cl^-$ from the root xylem (Colmenero-Flores et al. 2007). Bumetanide strongly decreased $Cl^-$ content in roots and leaves but the recorded decrease was higher for TOG5949 than for TOG5307, which suggests that in salt stress conditions, CCC is a major contributor of $Cl^-$ uptake and transport in the salt-sensitive genotypes of *O. glaberrima*. The gene *OsCCC1* from *O. sativa* was cloned and its silencing lead to lower $K^+$ accumulation (Kong et al., 2011). CCC belongs to the NKCC cotransporter family and ensures $Na^+$:$K^+$:2$Cl^-$ transport (Han et al. 2020). In our study, however, we found that bumetanide reduced $Na^+$ in TOG5949 but not in TOG5307, while it reduced $K^+$ in TOG5307 but not in TOG5949. This is the first time, to the best of our knowledge, that cations involved in the symport mechanisms of CCC appeared different in salt-sensitive and salt-tolerant cultivars within a given species. According to Chen et al. (2016), affinity of OsCCC1 for $Na^+$ is weak in *O. sativa* but we suggest that it may differ depending on the cultivar in the close-related species *O. glaberrima* exposed to salinity.

Cell viability index, protein and pigment concentrations remained less affected in the salt-tolerant TOG5307 than in the salt-sensitive TOG5949. Although it might be suggested that this is related to a lower level of $Na^+$ accumulation (Fig. 1), this is not valid anymore for the NaCl+amiloride treatment: in this case, accumulation of $Na^+$ was indeed higher in TOG5307 comparatively to TOG5949, but cell viability and protein concentration also remained higher in the former than in the latter. This suggests that TOG5307 displayed a better strategy of tolerance allowing it to cope more efficiently with accumulated $Na^+$ and this could be partly due to a better maintenance of $K^+$ nutrition and/or to higher accumulation of $Na^+$ in the apoplasm comparatively to TOG5949. This hypothesis is supported by the data obtained for electrolyte leakage (Table 2): indeed, EL was the only senescence-related parameter which was higher in TOG5307 than in TOG5949, which is at first sight in contradiction with all other data. The possible explanation is that some of the ions leaking during the process and contributing to measured electrical conductivity in TOG5949 were in fact issued from apoplast and thus provided a false information regarding cell membrane stability as previously demonstrated in *Oryza sativa* (Lutts et al., 1996). It is not demonstrated that significant amounts of inhibitors added to the nutrient solution may reach the aerial part of the plant (Taleisnik et al. 1991) but it cannot be excluded that the two considered cultivars differed for ion distribution independently of inhibitors. The data obtained with leakage of UV-absorbing substances

necessarily issued from the symplast corroborated this view since RLR recorded in salt-treated plants was always lower in TOG5307 than in TOG5949.

Additional experiments are still required to precise the impact of the studied transporters in plant response to salinity, especially considering that inhibitor like amiloride could also marginally impact other transport proteins such as CHX (Qiu et al. 2021) and that some targets of the used inhibitors may also be localized in the endomembrane system (Henderson et al. 2015).

## Conclusions

Inhibition of $Na^+$-$H^+$ antiporters in roots induced an increase in $Na^+$ concentration in all organs and a higher impact was recorded in the salt-resistant genotype suggesting that antiporter such as SOS1 play a major role in salinity resistance of African rice *Oryza glaberrima*. The CCC transporter is directly involved in $Cl^-$ accumulation but the counterion involved in the symport was preferentially $K^+$ in the salt-resistant cultivar and $Na^+$ in the salt-sensitive one. The salt-resistant cultivar was able to regulate $Na^+$ uptake and to cope with internal $Na^+$ accumulation which might be related to a higher accumulation of toxic ions in the apoplast.

**Authors contributions**

HP performed the whole experiment, SL and CG managed the project, SL conceived he experimental design, WI conducted the statistical treatment of the data, SL and HP wrote the first draft, all authors prepared the final version and approved submission.

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# Interchapter : Chapter 4 – Chapter 5

## Wo do we already know ?

Two contrasting cultivars of *O. glaberrima* were selected : TOG5307 is a salt-resistant genotype while TOG5949 is a salt-sensitive genotype. TOG5307 accumulated lower amounts of $Na^+$ than TOG5949 in all organs. We showed that $Na^+$ and $Cl^-$ acted on the same physiological and biochemical targets and that $Na^+$ was more toxic than $Cl^-$. Moreover, when present together as it is the case when plants are exposed to NaCl, the two ions acted in an additive way, except for a limited number of parameters. The use of specific inhibitors (amiloride : inhibitors of $Na^+/H^+$ antiporters ; bumetanide : inhibitor of CCC transporter) provided evidence that the SOS1 pathway may at least partly explain the higher salt-resistance of TOG5307 and that the two considered cultivars are using different ion in the CCC system during co-transport of $Cl^-$ ($K^+$ in TOG5307 and $Na^+$ in TOG5949, hence contributing to differences in $K^+/Na^+$ ratios between the two cultivars). Measurements of senescence-related parameters also suggest that, beside a limitation of $Na^+$ absorption and root-to-shoot translocation, TOG5307 also displays a tolerance strategy allowing it to more efficiently cope with accumulated $Na^+$ than TOG5949.

## What do we want to know ?

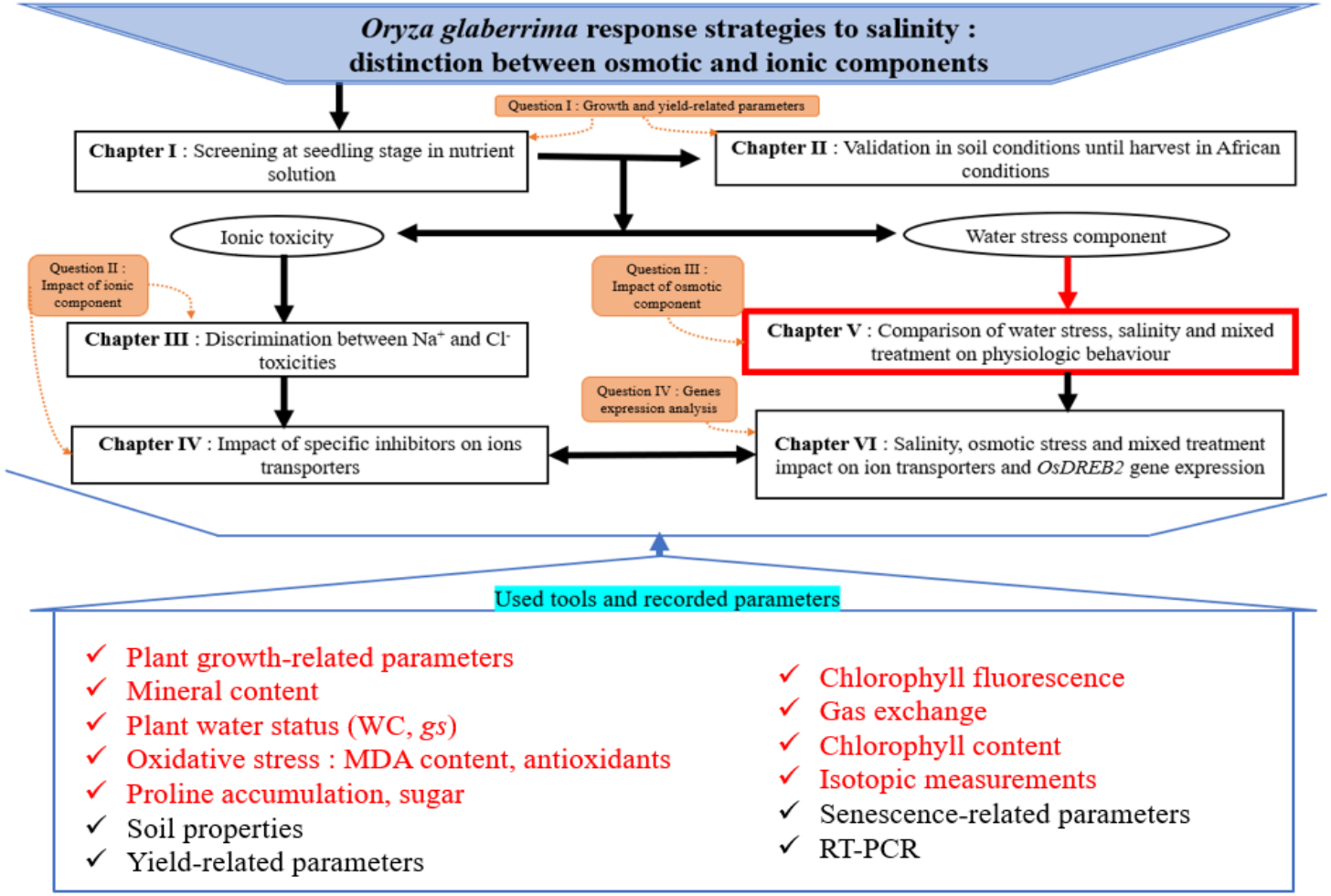
Until now, we mainly focused on the ionic component of salt stress. However, it is obvious that salinity induces a « double constraint » at the plant level : an osmotic component which is related to the fact that external salt drastically reduces external osmotic potential hampering the plant to absorb water, and an ionic constraint related to progressive accumulation of toxic ions. According to the classical « biphasic model » established by Munns, the first component to act on the plant is the osmotic one since it requires some time for $Na^+$ and $Cl^-$ ions to reach toxic levels. It might be argued that these two types of constraint are not clearly independent : osmotic stress may act on ion transporters, or induce stomatal closure which could reduce the rate of transpiration and thus delay toxic ion accumulation. On another hand, it has been postulated that a brutal increase of $Na^+$ and $Cl^-$ may occur in stressed plants for a brief period to ensure osmotic adjustment, and thus reduce the osmotic constraint until organic osmocompatible solute may be synthesized.

In natural field conditions, drought and salinity may occur together, but this co-occurrence is expected to imply a strong interaction since a decrease in the soil water content will lead to an increase in concentration of soluble salts, thus to an increase in electrical conductivity which means an increase in salt stress intensity. The aim of the next Chapter will be to more clearly discriminate the impact of ionic and osmotic stress in the two cultivars of *O. glaberrima*.

## What is our strategy ?

In order to discriminate between ionic and osmotic constraints, we will use in the next Chapter polyethyleneglycol 6,000 (PEG) to induce osmotic stress. In contrast to NaCl, PEG is indeed a non-penetrating agent which could not be absorbed by the roots and thus could not contribute to internal osmotic adjustment. Plants will be cultivated in hydroponic culture under fully-controlled environmental conditions and exposed at the seedling stage to a solution containing PEG 8.6%, or NaCl 50 mM or to a mix-treatment (PEG 4.3%+NaCl 25 mM). In the case of mix treatment, NaCl and PEG doses are reduced by 50%, so that all three solutions will have similar osmotic potential ($\Psi s$ = -0.31 MP). The purpose of the mix treatment is to pinpoint putative interactions between osmotic and ionic components. Considering the above-mentioned aspects related to kinetics aspects of osmotic adjustment and ion accumulation, one harvest will be performed after 2 days and another one after 2 weeks. All recorded parameters are the same than in Chapter 3.

*Chapter V. Comparison of the effects of water stress, salinity and mixed treatment (water stress + salinity) on the physiological behavior of two cultivars of the African rice (Oryza glaberrima Steud.) differing in salinity resistance*




**Overview of questions addressed during this thesis and their relationships with chapters.**

*Chapter V. Comparison of the effects of water stress, salinity and mixed treatment (water stress + salinity) on the physiological behavior of two cultivars of the African rice (Oryza glaberrima Steud.) differing in salinity resistance*

# Chapter V. Comparison of the effects of water stress, salinity and mixed treatment (water stress + salinity) on the physiological behavior of two cultivars of the African rice (*Oryza glaberrima* Steud.) differing in salinity resistance.

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## Abstract

*Aims*

This study was conducted to compare the impacts of salinity, water stress and a combination of the two constraints on 40 morphophysiological properties of two cultivars of African rice (*Oryza glaberrima* Steud.) differing in salinity resistance.

*Methods* Seedlings from cultivars TOG5307 (salt-resistant) and TOG5949 (salt-sensitive) were exposed during 2 and 15 days to iso-osmotic nutrient solution ($\Psi s$ = -0.32±0.02 MPa) containing PEG6000 8.6% (water stress), NaCl 50 mM (salt stress) and PEG 4.3% + NaCl 25 mM (MIX-treatment). Plants were analyzed for photosynthetic parameters, water status, mineral nutrition and oxidative stress-related parameters.

*Results*

The duration of treatment did not affect root Na:K ratio only, but was significant for all other parameters. Cultivars displayed a significant disparity for all these parameters, except for root magnesium concentration, root:shoot ratio, instantaneous water use efficiency (A/E) and root- shoot- water content. The NaCl and MIX treatments induced an increase of Na+ content and a decrease in K+ concentration in shoot and root. TOG5307 accumulated less sodium than TOG5949. TOG5307 also exhibited a higher net carbon assimilation rate and a higher water use efficiency. The shoot MDA, proline, total soluble sugars concentrations and antioxidant activity changed little after 2 days of treatment but were drastically increased in stressed plants after 15 days. The isotope elements obviously decreased in response to all stressing agents.

*Conclusion*

The differential impact of osmotic stress on two African rice (*Oryza glaberrima*) cultivars is mainly observed at short-term stress. At long-term stress, the accumulation of toxic ions in the organs of the plant accentuates the effect of salt stress. This underlines the importance of ionic toxicity in *O. glaberrima* response to salt stress.


**Keywords** African rice, combined stress, NaCl, PEG *Oryza glaberrima*, salinity, water stress.

# Introduction

Salinity is an important challenge posing serious threat to agriculture (Van Zehm et al. 2020). Beside salinity, drought seriously hampers plant water uptake and this environmental constraint is expected to increase in the future as a result of climate change (Tardieu et al. 2018). In the past decades, most physiological studies mainly focused on the impact of one single abiotic stress but in field conditions, plants are rarely exposed to one single constraint. In their natural environments, plants are exposed to a myriad of constraints and this prompted physiologists to consider combination of individual stress, underlying the urgency to breed plants for their behavior in relation to several interacting targets (Mittler 2006).

Even if distinct abiotic stresses may act on the same morpho-physiological parameters or molecular cues, some recent studies suggested that the simultaneous occurrence of the two stress often confer to the plant a specific physiological status which does not simply correspond to the additive action of individual stress (Loudet and Hasegawa 2017; Zandallina et al. 2020; Blanchard-Gros et al. 2021). The complexity may also result from the kinetics of stress application which drastically influence not only the kinetic of the plant response but also the nature of the plant response (Almansouri et al. 1999).

The situation appears rather complex for salt stress and drought. Salinity by itself is a complex stress since it implies two types of constraints. The first one in an "osmotic stress" due to the decrease in the external water potential which compromises the plant water uptake. The second one, the so-called "ionic constraint", is related to the progressive accumulation of toxic ions (mainly $Na^+$ and high concentration of $Cl^-$) which alter cellular metabolism. Prodjinoto et al. (2021) recently demonstrated that in the African rice species *Oryza glaberrima*, $Na^+$ is more toxic than $Cl^-$. It has to be mentioned that salinity may involve other types of salt than NaCl and that it was recently shown that $Na_2SO_4$ induces a specific behavior in rice (Irakoze et al. 2020, 2021).

According to the well-known biphasic model, it is commonly considered that the osmotic component is the first to have an impact on plants since the toxic constraint requires the progressive accumulation of toxic ions (Munns 2005; Munns and Tester 2008; Munns et al., 2020). Munns and Gilliham (2015) and Munns et al. (2020) however hypothesized that, even in glycophyte species a

burst in $Na^+$ may occur very quickly to perform osmotic adjustment for a short time with a cheap osmoticum in order to ensure plant water uptake and cell turgor before the accumulation of organic osmoprotectant whose synthesis require a delay. Such $Na^+$ accumulation should occur during a brief period before the synthesis of soluble organic compounds may occur (Alzahrani 2021). Lefevre et al. (2001) also reported that rice may accumulate high amounts of $Na^+$ and $Cl^-$ before proline accumulation.

After an initial burst, $Na^+$ absorption is more tightly regulated to avoid overaccumulation of toxic ions. This justifies to discriminate between short- and long-term exposure to exogenous stressor. Molecular studies aiming to identify stress-induced regulated genes frequently uses short term exposure to high (and sometimes unrealistic) stress intensities. In contrast, agronomic approaches whose purpose is to determine the impact of stress on yield components uses a more realistic approach but analyze the ultimate consequence of stress and not necessarily the strategy of plant response. The experimental approaches are so different that there is no clear evidence that these two types of studies complement each other.

Quinet et al. (2012) demonstrated using a transcriptomic approach in *Oryza sativa* exposed to iron toxicity that only a few percent of differentially-regulated genes were modified in the same way in response to 3 days and to 3 weeks of stress exposure and that the vast majority of identified EST (Expressed Sequence Tag) are specific to only one duration of exposure. This reinforces the interest to perform short- and long-term exposure to stress in the framework of one single experiment.

Soil solution always contains some soluble salts. In areas which are prone to salinization, drought implies a progressive decline in the soil water content and thus a progressive increase in the soil electrical conductivity (EC). Accordingly, this implies that water stress and salt stress develop simultaneously in a dynamic scheme. Under these circumstances, it is difficult to accurately distinguish and compare the effects of drought from the effect of salinity. The use of hydroponic culture may somewhat help to obtain a more static system when the non-penetrating osmotic solute polyethylene glycol (PEG) is used to mimic the impact of drought on plants. In order to simultaneously impose salt and water stress Goharrizi et al. (2020) used a MIX treatment by adding a given concentration of NaCl to a PEG-containing nutrient solution.

The African rice species is by far less cultivated than its Asian counterpart *Oryza sativa*. Although it is often considered as less productive than *O. sativa*, *O. glaberrima* is now considered as a promising species for breeding purposes because it kept during domestication interesting rustic properties, including resistance to numerous biotic and abiotic stresses in its native environment (Linares 2002; Sarla and Swamy 2005; Majerus et al. 2007; Wambugu et al. 2021). The present work was therefore undertaken in order to compare the impact of salt and water stress on two cultivars of *Oryza glaberrima* differing in their mean level of salt resistance and to analyze the plant behavior in response to the simultaneous presence of both constraints. For this purpose, iso-osmotic nutrient solutions containing PEG and/or NaCl were applied during two days and two weeks on *O. glaberrima* seedlings and morphological parameters, ion content, plant water status, photosynthesis, and oxidative stress-related parameters were quantified.

## Material and Methods

### Plant material, growth conditions and stress application

Two contrasted cultivars of *Oryza glaberrima* [salt-tolerant (TOG5307; Accession number WAB0021855) and salt-sensitive (TOG5949; Accession number WAB0020144)], as described by Prodjinoto et al. (2018) were used. Seeds were obtained from AfricaRice (Bouaké, Ivoiry coast). For each cultivar, non-dehusked seeds were surface-sterilized and germinated on two layers of filter papers (Whatman 85 mm, Grade 1) moistened with sterile deionized water in glass vessels at 25 °C and under 12 h daylight period. Illumination was provided by Sylvania fluorescent tubes (F36W/840-T8, cool white, 150 µmole $m^{-2}$ $s^{-1}$). After 10 days, equally grown seedlings were transferred into tanks and fixed on polystyrene plates floating on 16 l of Yoshida nutritive solution [(YNS); 1.43 mM $NH_4NO_3$, 323 µM $NaH_2PO_4$, 512 µM $K_2SO_4$, 750 µM $CaCl_2$, 1.64 mM $MgSO_4$, 11.4 µM $MnSO_4$, 0.14 µM $Na_2Mo_4$, 57.8 µM $H_3BO_3$, 0.96 µM $ZnSO_4$, 0.4 µM $CuSO_4$, 42.7 µM Fe-EDTA]. For each cultivar, 216 young seedlings were distributed among 12 tanks with a plant-to-plant distance of 10 cm. Seedlings were cultivated inside a phytotron at 29 °C during the day and 26 °C during the night. The daytime humidity was maintained at 70% and 16 h of illumination was provided by

PHILIPS metal iodide lamp (HPIT/400W) with a minimal light intensity (PAR) of 250 µmole $m^{-2}$ $s^{-1}$. The renewal of solutions and the random re-arrangement of tanks in the phytotron were performed every week.

After two weeks' acclimatization, four different stresses were applied on plants using either ionic and penetrating (NaCl), or non-ionic and non-penetrating [polyethylene glycol (PEG) 6,000 (Aldrich; Sigma Chemical)] stressing agents or a combination of two stressing agents (hereafter called MIX). The following treatments were considered: 1) Control (no NaCl and no PEG; $\Psi$s = -0.08±0.002 MPa; EC = 0.74±0.008 mS/cm); 2) PEG 6,000 8.6% ($\Psi$s = -0.31±0.02 MPa; EC = 0.73±0.001 mS/cm); 3) NaCl 50 mM ($\Psi$s = -0.32±0.02 MPa; EC = 5.83±0.11 mS/cm) and 4) MIX: PEG 6,000 4.3% + NaCl 25 mM ($\Psi$s = -0.31±0.02 MPa; EC = 3.04±0.04 mS/cm). Hence, all stressing treatments had the same osmotic potential but EC varied depending on the NaCl dose. In order to discriminate between ionic and osmotic constraints, we used PEG 6,000 to induce osmotic stress. In contrast to NaCl, PEG is indeed a non-penetrating agent which could not be absorbed by the roots and thus could not contribute to internal osmotic adjustment. In the case of MIX treatment, NaCl and PEG doses are reduced by 50%, so that all three solutions had similar osmotic potential ($\Psi s$ = -0.31 MP). The purpose of the MIX treatment is to pinpoint putative interactions between osmotic and ionic components. To avoid plants from suffering hypoxia due to PEG application (Verslues et al. 1998), all tanks were oxygenated by aerating Eheim aquarium pumps.

After short term (2 days) and long-term exposure (15 days), rice seedlings were harvested: shoots and roots were separated, the latter being quickly rinsed with sterile deionized water to remove ions from the free space or PEG adhering to the root surface, and then gently blotted dry with a paper towel. Six seedlings were used for ion quantification and morphological evaluations while the others were quickly frozen in liquid nitrogen and then stored at -80 °C until analyses.

Determination of growth rate, water content and ion concentration

The root length was measured on six plants per treatment from the tip of the longest root to the root-shoot junction and the length of the shoot was

estimated from that junction to the tip of longest leaf. The fresh weight (FW) of roots and shoots were estimated and samples were incubated for 72 h in an oven at 70 °C for of the dry weight (DW) estimation. The water content (WC) was calculated by:

$$WC = (FW- DW)/ FW * 100$$

For the estimation of ion content, c.a. 100 mg dry weight of shoot and root of six plants per treatment were digested in the presence of hot nitric acid (68%) and minerals WERE dissolved in 1:3 (v/v) of nitric acid (68%) and concentrated hydrochloric acid. The concentrations of $Na^+$, $K^+$, $Mg^{2+}$ and $Ca^{2+}$ were estimated by flame emission using atomic spectrometry (Thermo scientific S series model AAS4).

Osmotic potential ($\Psi$s) and malondialdehyde quantification

For $\Psi$s estimation, samples of roots and leaves collected from 9 individual plants per cultivar per stress were cut in small pieces (c.a. 5 mm length), quickly placed in an Eppendorf tubes perforated with four small holes and immediately frozen in liquid nitrogen during 30 s. Then, samples warmed at ambient temperature (c.a. 25 °C) for 5 mins to rupture membranes (Lutts et al. 1999). Three cycles of frozen/warmed were applied on samples. Each Eppendorf tube was then encased in a second unperforated Eppendorf tube and centrifugated at 15,000 *g* at 4 °C for 15 min. The osmolarity (mosmoles $Kg^{-1}$, C) of the collected tissular sap was assessed with a Wescor 5600 vapor pressure osmometer and converted to $\Psi$s according to Van't Hoff equation.

The method of Heath and Packer (1968) was used for malondialdehyde (MDA, routinely used as an indicator of membrane lipid peroxidation) quantification. Malondialdehyde was estimated as the 2-thiobarbituric acid-reactive substances (TBARS). For each sample, *c.a.* 250 mg of FW were ground in liquid nitrogen in the presence of 1.25% glycerol and 5% trichloroacetic acid. Samples were centrifuged at 4 °C during 10 min at 6,700 *g*. Solutions were filtered with one layer of Whatman paper (8 mm, Grade 1) and 2 ml of the filtrate were mixed with 2 ml of 0.67% thiobarbituric acid. Samples were incubated at 100 °C during 30 min and then 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 $mM^{-1}$ $cm^{-1}$ used to calculate MDA concentration.

Gas exchange and photosynthesis-related parameters

The middle part of the second fully expanded leaf (counting by the top) of 9 plants per cultivar per treatment were used for gas exchange and photosynthesis-related parameters estimation between 10:00 am and 2:00 pm. The porometer (type AP4-UM-3, Delta T-devices, UK) was used to measure the stomatal conductance ($g_s$). PLC Parkinson leaf cuvette (20 records/min) and an air flow of 300 ml/min of an infrared gas analyzer (LCA4; ADC Bioscientific, Hoddesdon, Hertfordshire, UK) were used to record gas exchange. Ambient air was sent to a chamber containing of leaf portion of 6.25 $cm^2$. The net carbon assimilation rate (µmoles $CO_2$ $m^{-2}$ $s^{-1}$, $A$) was estimated under constant photosynthetic photon flux. The intercellular $CO_2$ content (µmoles $moles^{-1}$, $C_i$) and the instantaneous transpiration (µmoles $H_2O$ $m^{-2}$ $s^{-1}$, E) were measured using water vapor analyzer (LCA 28.7, ADC, Great Amwell, England) and an air supply unit (ASU 10.87, ADC, Hertfordshire, UK).

A portable pulse-modulated chlorophyll fluorimeter (FMS2, Hansatech, King's Lynn, UK) was used to determine the chlorophyll fluorescence. During 30 mins leaf portions were acclimated to darkness and the minimal fluorescence level ($F_0$) was estimated by assessing the modulated light (0.1 µmol $m^{-2}$ $s^{-1}$). The maximal fluorescence level ($F_m$) with all photosystem II (PSII) reaction centers closed was determined by 0.8 s saturation pulse at 18,000 µmol $m^{-2}$ $s^{-1}$ in the dark-adapted portions of leaves. The latter were then continuously illuminated with white actinic light (600 µmol $m^{-2}$ $s^{-1}$) for 3 min. The steady-state value of fluorescence ($F_s$) was measured 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's portion with a 3 s pulse of fared. Using both light and dark fluorescence parameters, the actual PSII efficiency ($\Phi_{PSII}$), the photochemical quenching coefficient ($q_P$), the non-photochemical quenching (NPQ) and the maximal efficiency of PSII photochemistry in the dark-adapted state ($F_v/F_m$) were assessed according to Maxwell and Johnson

(2000). The intrinsic ($A/g_s$) and instantaneous ($A/E$) water use efficiency, the efficiency of the instantaneous carboxylation ($A/C_i$) and the efficiency of electron transport (ETR/$A$) were calculated.

Chlorophyll content, proline and total soluble sugar quantification

Pigments (chlorophyll a, chlorophyll b and total carotenoids) were estimated in the dark from leaf segments harvested on second fully expanded leaf (basipetal numbering) of 9 plants per treatments. Samples were ground in the presence of 8 ml of cold acetone 80%. The extracts were centrifuged at 1,000 *g* for 10 min at 4 °C and the absorbance was read at 663.2, 646.8 and 470 mm with a Beckman DU640 spectrophotometer. The leaf pigment concentrations were then calculated according to Lichtenthaler (1987).

For proline quantification, c. a. 200 mg FW of shoots were ground in liquid nitrogen in a mortar containing 10 ml of 3% sulfosalicylic acid. Samples were then centrifuged at 1,000 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 (sigmaAldrich) as standards (Bates et al. 1973).

Soluble sugar estimation was performed on *c.a*. 300 mg FW of leaves ground in liquid nitrogen in the presence of 7 ml of ethanol 70% (w/v) for 5 min on ice and centrifuged at 4 °C at 8,000 *g* during 20 min; 200 µl of the supernatant were then added to 1 ml of an anthrone solution (0.5 anthrone, 250 ml 95% $H_2SO_4$ and 12.5 ml distilled water). A calibration curve was established using glucose as standard and the absorbance was read at 625 nm according to Yemm and Willis (1954).

Total antioxidant capacity, flavonoids and total phenolics quantification

Total antioxidant capacities (ferric reducing antioxidant power, FRAP) were determined in shoots of 3 plants per cultivar and treatment according to Benzie and Strain (1996). One gram of shoots FW was ground in a pre-chilled mortar

with liquid nitrogen. The homogenous powder was dissolved in 10 ml of methanol and then centrifuged at 10,000 *g* for 12 h at 4 °C. The supernatant corresponded to the antioxidant activity measured in methanol extract (AOAM) fraction. The pellet was re-dissolved in 10 ml of dichloromethane and centrifuged again at 10,000 *g* for 12 h at 4 °C. This second supernatant corresponded to antioxidant activity measured in dichloromethane extract (AOAD) fraction. AOAM and AOAD fractions were respectively used for determining hydrophilic and lipophilic antioxidant capacity (Arnoa et al. 2001): 150 µl sample were added to 2.85 ml of FRAP solution. The mixture was incubated in the dark for 30 min at room temperature and the absorbance was measured at 593 nm.

For flavonoids and total phenolics estimation, a solution of 80% methanol was added to c.a. 100 mg of leaves. Concentration of flavonoids in this methanolic extract was measured at 510 nm according to the method of Dewanto et al. (2002) using phosphomolybdic-phosphotungstic reagent and catechin as standard. The total phenolics were quantified in the same methanolic extract using Folin-Ciocalteu method and absorbance was read at 750 nm according to Singleton and Rossi (1965).

Elemental and stable isotope analyses of carbon and nitrogen

Prior to elemental and stable isotope analysis, dry matter of shoots was ground to homogenous powder and *c.a.* 2 mg were weighed and placed in tin capsules (3 x 5 mm). Carbon and nitrogen elemental composition were reported in % of the sample dry weigh. Carbon and nitrogen stable isotope measurements were performed on each sample with an isotope ratio mass spectrometer (Optima Isoprime, VG Instrument, Cambridge, U. K.) coupled to C-N-S elemental analyzer (Carlo Erba). 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:

$$\delta X = [(Rsample/Rstandard) - 1] \times 1000$$

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 in relationship to the V-PDB (Vienna Pee Dee Belemnite) standard and to 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 biological discrimination process that causes variation in this ratio (Shaheen and Hood-Nowotny, 2005). The $^{13}C$ discrimination ($\Delta^{13}C$) was then calculated from $\delta^{13}C$ sample according to Farquhar et al. (1982) formula:

$$\Delta^{13}C = (\delta^{13}C \text{ air} - \delta^{13}C\text{shoot}) / (1+ (\delta^{13}C \text{ sample}/1000)).$$

Where $\delta^{13}C$air and $\delta^{13}C$shoot are respectively the carbon isotope composition of air ($\delta^{13}C$air= – 8.15 ‰) and shoot (Keeling 2010 ; Munjonji et al. 2016).

Toxicity index

In the case of non-mixed treatment (NaCl and PEG), the toxicity index (TI) is defined for each recorded parameter as:

$$TI = | (V_y - V_c)/V_c) |$$

Where $V_y$ is the value recorded for the stressed plants and $V_c$ is the value recorded for the control.

If NaCl and PEG act in an independent and additive way, then the expected toxicity index for the MIX treatment should correspond to the following value, considering that doses of NaCl and PEG were reduced by 50% in this treatment:

$$TI_{MIX\ EXP} = | [(V_{NaCl}/2)/V_c] + (V_{PEG}/2)/V_c] – 1|$$

This expected theoretical value is compared with the observed value ($TI_{MIX\ OBS}$) defined as:

$$TI_{MIX\ OBS} = | (V_{MIX}/V_c) - 1 |$$

Where $V_{MIX}$ is the value recorded for the MIX treatment during the experiment

Statistical analysis

All the data in this research were calculated with JMP Pro 14 software and subjected to three-way analysis of variance (three-way ANOVA). At $P < 0.05$ a post-hoc test Tukey's HSD all-pairwise comparisons were used to compare means. Plant growth data (a composite of shoot length, root length, DW of shoot, DW of root, WC of shoot, WC of root, $F$v/$F$m, $\Phi_{PS2}$, $q_P$, NPQ, $A$, $E$, $g_s$, $C_i$, $A/E$, $A/C_i$, $A/g_s$, chla, chlb, carotenoids, root:shoot ratio, shoot MDA content, and osmotic potential of shoot and root) were converted into a dissimilarity matrix using the Euclidean distances without standardization. For spatial ordinations, principal coordinate analysis (PCoA) was carried out using the cmdscale function in "vegan" package of R (version 4.0.3) (Oksanen et al. 2020; R core Team 2020).

## Results

Table 1 provides a summary of the statistical impact of the main factors cultivar (CV), treatment (T) and duration of the treatment (D) for all recorded parameters. Table 1 showed a statistical significance of effects of cultivars, treatments and durations by three-way analysis of variance. There was a significant effect of stress for all recorded parameters, except for the shoot water content. The duration of treatment did not affect root Na:K ratio only, but was significant for all other parameters. Cultivars displayed a significant disparity for all these parameters, except for root magnesium concentration, root:shoot ratio, instantaneous water use efficiency ($A/E$) and root- shoot-water content. As far as interactions are concerned, T x D was not significant for root Na:K ratio. CV x T did not have effect on morphological parameters. Photosynthesis machinery was not affected by CV x D while only ions

contents, some photosynthetic parameters and antioxidants contents were significantly affected by CV x Tx D.

**Table 1**. Statistical effects of cultivar (CV), treatment (T) and duration of exposure and their interactions as determined by 3-way analysis of variance of all parameters in of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (salt-resistant) and TOG5949 (salt-sensitive). Symbols: *, **, *** and **** represent statistical significance at $P < 0.05$, 0.01, 0.001 and 0.0001, respectively; and NS = not significant at $P = 0.05$.

| Parameters | | Analyse of Variance (ANOVA 3) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | CV | T | D | CVxT | CVxD | TxD | CVxT xD |
| Ψs (MPa) | Root | ** | **** | * | NS | NS | * | NS |
| | Shoot | **** | **** | **** | **** | * | **** | NS |
| Sodium | Root | **** | **** | **** | **** | * | **** | ** |
| | Shoot | **** | **** | **** | **** | **** | **** | **** |
| Potassium | Root | **** | **** | **** | **** | **** | **** | **** |
| | Shoot | **** | **** | **** | **** | **** | **** | **** |
| Na : K ratio | Root | **** | **** | NS | **** | **** | NS | **** |
| | Shoot | **** | **** | **** | **** | **** | **** | **** |
| Calcium | Root | **** | **** | **** | NS | **** | **** | **** |
| | Shoot | **** | **** | **** | ** | *** | **** | NS |
| Magnesium | Root | NS | **** | * | NS | NS | **** | NS |
| | Shoot | **** | **** | **** | * | * | **** | NS |
| Length | Root | ** | **** | **** | NS | NS | *** | NS |
| | Shoot | ** | **** | **** | NS | * | **** | NS |
| Dry Weight | Root | ** | **** | **** | NS | NS | **** | NS |
| | Shoot | **** | **** | **** | *** | **** | **** | ** |
| Water content | Root | NS | **** | **** | NS | * | **** | * |
| | Shoot | NS | NS | **** | NS | NS | * | NS |
| Fv/Fm | | **** | **** | **** | NS | NS | **** | *** |
| PS | | **** | **** | **** | * | NS | **** | NS |
| qP | | *** | **** | **** | * | NS | **** | NS |
| NPQ | | **** | **** | **** | **** | ** | **** | * |
| A/E | | NS | **** | **** | * | NS | **** | ** |
| A/Ci | | **** | **** | **** | **** | *** | **** | **** |
| A/gs | | **** | **** | **** | **** | **** | **** | **** |
| ETR/A | | **** | **** | **** | **** | **** | **** | **** |
| Chla | | **** | **** | **** | **** | **** | **** | *** |
| Chlb | | **** | **** | **** | **** | NS | **** | NS |
| Carotenoids | | **** | **** | **** | **** | **** | **** | **** |
| Chla /Chlb | | *** | **** | **** | **** | NS | **** | **** |
| Root : Shoot ratio | | NS | **** | **** | NS | **** | **** | * |
| A | | **** | **** | **** | **** | **** | **** | **** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| E | **** | **** | **** | **** | NS | **** | NS |
| Ci | **** | **** | **** | * | NS | **** | NS |
| gs | **** | **** | **** | **** | **** | **** | **** |
| MDA | **** | **** | **** | **** | **** | **** | **** |
| Proline | **** | **** | **** | **** | **** | **** | **** |
| Total Sugars | **** | **** | **** | **** | ** | **** | Ns |
| Total Flavonoids | **** | **** | **** | **** | NS | **** | **** |
| Total phenols | **** | **** | **** | NS | **** | **** | **** |
| AOAM | **** | **** | **** | *** | * | **** | NS |
| AOAD | **** | **** | **** | **** | NS | **** | **** |
| $\Delta^{13}C$ (‰) | **** | **** | **** | - | - | - | - |
| $\delta^{15}N$ (‰) | * | **** | **** | - | - | - | - |
| C(%) | **** | **** | *** | - | - | - | - |
| N(%) | **** | **** | **** | - | - | - | - |

Plant growth, water content and osmotic potential

The root length, the root dry weight and the root water content decreased in stressed plant throughout the experiment (Table 2). Only the interaction between treatment and duration was significantly different ($p < 0.0001$; Table 1). After 2 days of exposure, NaCl and MIX treatments decreased the root length in TOG5307 while the stress had no impact on the root length of TOG5949, whatever the nature of stressing agent. In contrast, after 15 days of treatment, all stressing agents significantly decreased root length in TOG5307 while PEG had no impact on TOG5307.

The shoot length and the shoot water content did not change after 2 days of treatments, whereas the shoot dry weight decreased in response to MIX treatment only. After 15 days, the root- and shoot-length, the root- and shoot-dry weight and the root water content were significantly reduced in stressed plants (Table 2). It is noteworthy that the shoot WC remained unaffected after 15 days of treatment, whatever the cultivar or the stressing agent.

As far as root:shoot ratio is concerned, NaCl-stressed plants had the highest values after 15 days ($p <0.0001$; Figure 1.A). While root:shoot ratio decreased after 2 days of stresses, it increased after 15 days. However, root:shoot ratio did not show a significant difference between cultivars (Table 1).

The $\Psi_s$ values were higher in roots than in shoots whatever the duration of the treatment (Figure 1.B). As far as the shoots are concerned, $\Psi_s$ was lower after 15 days than after 2 days whatever the treatment and cultivar ($p < 0.0001$, Table 1). After 2 days, the shoot $\Psi_s$ decreased only in NaCl-stressed plants for TOG5307, whereas it decreased in response to all stressing agents in TOG5949. After 15 days, the $\Psi$s of stressed shoots was lower in TOG5949 than in TOG5307. The $\Psi_s$ values were lower for NaCl-treated plants than for PEG-exposed ones and mix treatment ($p < 0.0001$, Table 1).

**Table 2.** Impact of iso-osmotic solutions of PEG 8.6%, NaCl 50 mM or mix treatment (PEG 4.3% + NaCl 25 mM) applied for 2 or 15 days on root and shoot length, dry weight and water content of two contrasting cultivars of African rice (*Oryza glaberrima* Steud. cv TOG5307 (salt-resistant) and TOG5949 (salt-sensitive)). For a particular cultivar, values followed by similar lower case (2 days) or uppercase (15 days) letters are not statistically different according to Tukey post hoc test.

| | | | Length | | Dry weight | | Water content | |
|---|---|---|---|---|---|---|---|---|
| Cultivar | Treatment | Duration | Root | Shoot | Root | Shoot | Root | Shoot |
| TOG5307 | Control | 2 days | 32.67 ± 0.67 a | 39.67 ± 1.45 a | 0.32 ± 0.002 a | 0.57 ± 0.005 a | 74.36 ± 0.34 a | 69.74 ± 0.69 a |
| TOG5949 | | | 29.67 ± 0.88 a | 39.67 ± 0.88 a | 0.32 ± 0.004 a | 0.57 ± 0.01 a | 74.04 ± 0.22 a | 69.13 ± 1.11 a |
| TOG5307 | PEG | | 31.67 ± 0.88 a | 37.67 ± 1.20 a | 0.29 ± 0.003 b | 0.56 ± 0.01 ab | 75.53 ± 0.93 a | 67.42 ± 1.18 a |
| TOG5949 | | | 29.33 ± 0.33 a | 39.33 ± 1.67 a | 0.28 ± 0.003 b | 0.56 ± 0.01 ab | 77.76 ± 0.26 b | 65.73 ± 1.18 a |
| TOG5307 | NaCl | | 27.33 ± 0.67 b | 37 ± 1.53 a | 0.28 ± 0.003 bc | 0.54 ± 0.01 ab | 71.17 ± 0.39 b | 68.10 ± 0.57 a |
| TOG5949 | | | 27.33 ± 0.67 a | 35 ± 1.15 a | 0.26 ± 0.003 bc | 0.53 ± 0.01 ab | 73.70 ± 0.99 b | 65.69 ± 1.98 a |
| TOG5307 | Mix | | 27.67 ± 0.88 b | 36.67 ± 0.67 a | 0.27 ± 0.003 c | 0.52 ± 0.01 b | 74.97 ± 0.73 a | 68.07 ± 0.60 a |
| TOG5949 | | | 28 ± 0.58 a | 35 ± 1 a | 0.26 ± 0.003 c | 0.52 ± 0.01 b | 73.95 ± 1.04 b | 68.88 ± 0.76 a |
| TOG5307 | Control | 15 days | 36 ± 1.15 A | 61.33 ± 0.67 A | 1.15 ± 0.02 A | 2.78 ± 0.03 A | 87.09 ± 0.53 A | 78.31 ± 0.46 A |
| TOG5949 | | | 36.67 ± 0.67 A | 58.67 ± 1.33 A | 1.152 ± 0.01 A | 2.74 ± 0.02 A | 86.49 ± 0.38 A | 78.32 ± 0.41 A |
| TOG5307 | PEG | | 33 ± 0.58 AB | 55.67 ± 1.20 B | 1.11 ± 0.01 A | 2.22 ± 0.04 B | 83.04 ± 0.87 B | 78.79 ± 1.18 A |
| TOG5949 | | | 29 ± 0.58 B | 55 ± 1.53 A | 1.10 ± 0.01 B | 2.10 ± 0.02 B | 83.49 ± 0.18 B | 79.21 ± 0.75 A |
| TOG5307 | NaCl | | 29.67 ± 0.67 B | 42 ± 1.15 D | 0.96 ± 0.01 B | 1.87 ± 0.02 D | 82.39 ± 0.90 B | 78.64 ± 0.58 A |
| TOG5949 | | | 29.33 ± 0.67 B | 38.33 ± 0.88 C | 0.93 ± 0.01 C | 1.62 ± 0.01 D | 80.07 ± 0.40 C | 79.74 ± 0.69 A |
| TOG5307 | Mix | | 28.97 ± 0.88 B | 50.67 ± 0.67 C | 0.95 ± 0.003 B | 2.05 ± 0.02 C | 80.01 ± 0.66 B | 78.92 ± 0.33 A |
| TOG5949 | | | 28 ± 1.15 B | 46 ± 0.58 B | 0.94 ± 0.004 C | 1.94 ± 0.01 C | 79.95 ± 1.17 C | 78.15 ± 0.37 A |

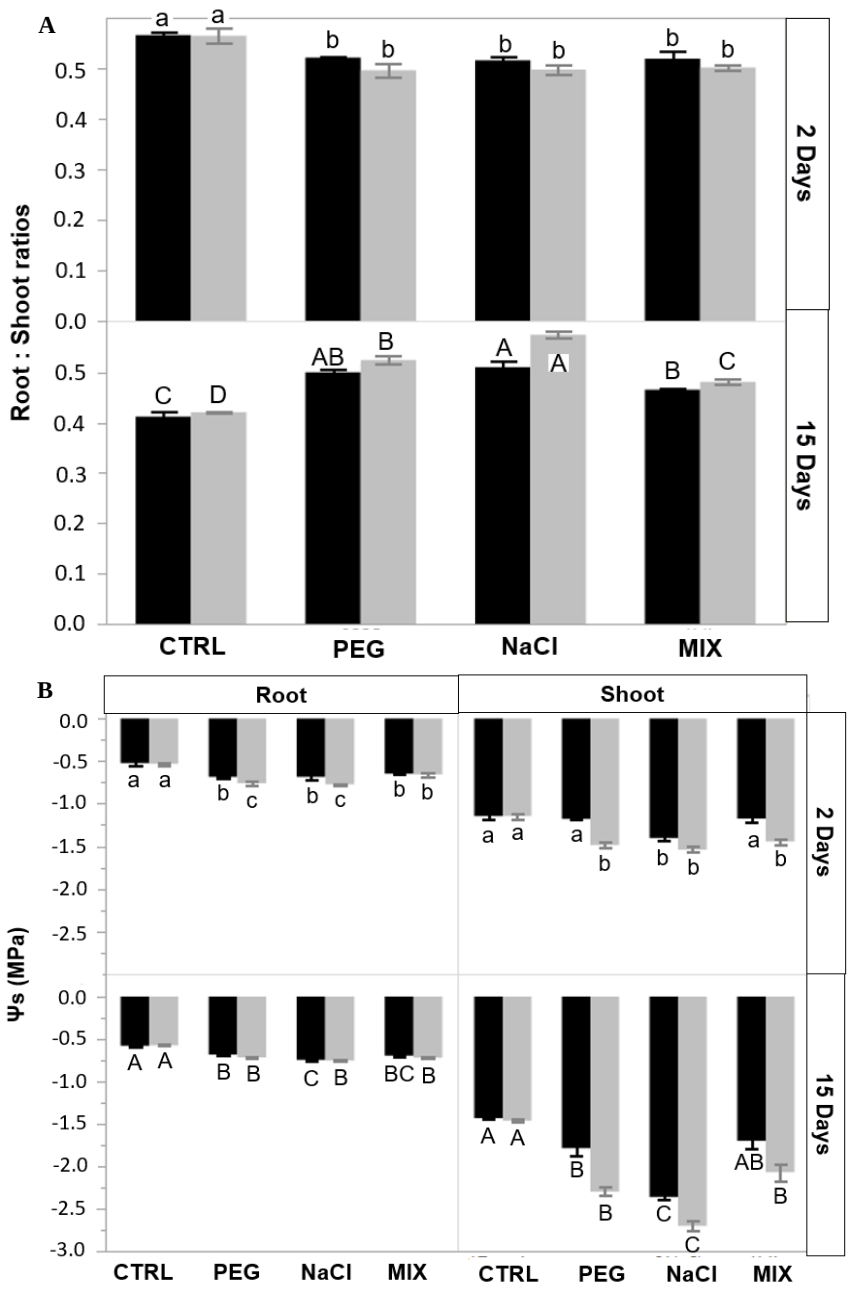


**Figure 1.** Impact of iso-osmotic concentration of PEG 8.6%, NaCl 50 mM or mix treatment (PEG 4.3% + 25 mM) on (A) root:shoot ratio, (B) osmotic potential of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (salt-resistant; black bars) and TOG5949 (salt-sensitive; grey bars). Seedlings were exposed during 2 and 15 days. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments with by the same lowercase latter (2 days) or the same uppercase (15 days) for a particular cultivar do not differ statistically according to Tukey HSD test.

Effects on mineral nutrients

The NaCl and MIX treatments induced an increase in shoot and root $Na^+$ content ($p < 0.0001$, Figure 2.A, Table 1) as well as Na:K ratio ($p < 0.0001$, Figure 2.C, Table 1). Sodium accumulated to a higher extent in roots than in shoots after 2 days, while an opposite trend was observed for 15 days. Throughout the experiment, TOG5307 accumulated less sodium than TOG5949. Sodium concentration was lower for MIX treatment than for NaCl-treated plants. After 15 days, $Na^+$ accumulation was still higher in TOG5949 than in TOG5307.

The NaCl and MIX treatment also induced a decrease in $K^+$ concentration in both roots and shoots ($p < 0.0001$, Figure 2.B, Table 1). In NaCl-treated plants and plants exposed to MIX treatment, $K^+$ concentration remained higher in TOG5307 than in TOG5949 after 15 days of stress($p < 0.0001$, Figure 2.B, Table 1).

The magnesium (Figure 2.D) and the calcium (Figure 2.E) concentrations were higher in shoots than in roots. Magnesium concentration decreased in NaCl- and MIX-stressed plant. It also decreased in response to PEG in the shoots after 15 days of treatment. Calcium concentration decreased in the shoot of NaCl and MIX-stressed plants, especially after 15 days of treatment. Treatments and duration of exposure influenced considerably magnesium and calcium content ($p < 0.0001$) but no significant difference between cultivars was recorded for root magnesium concentration (Table 1).

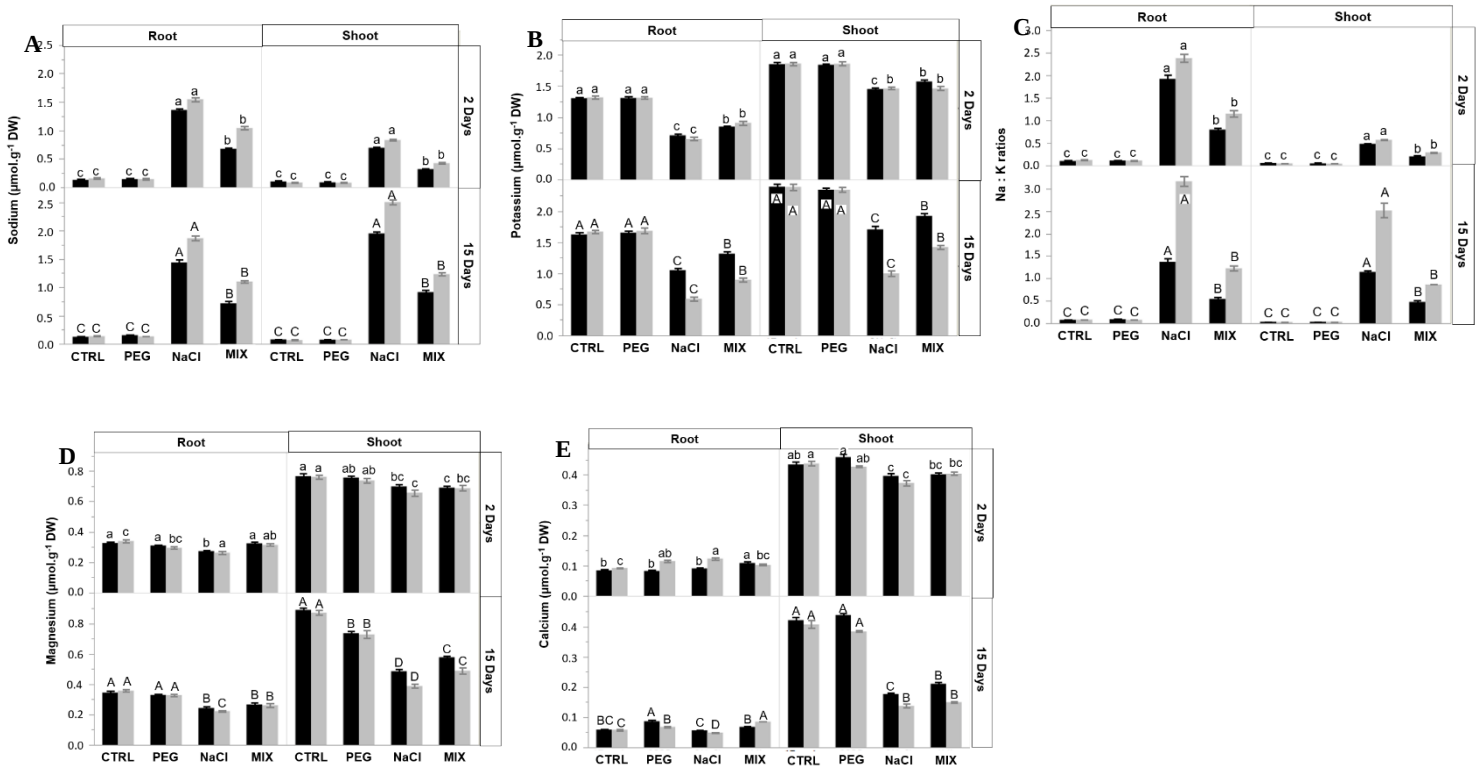


**Figure 2.** Impact of iso-osmotic concentration of PEG 8.6%, NaCl 50 mM or MIX treatment (PEG 4.3% + 25 mM) on (A) sodium concentration (B) potassium concentration (C) Na:K ratio (D) magnesium concentration and (E) calcium concentration in roots and shoots of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (salt-resistant; black bars) and TOG5949 (salt-sensitive; grey bars). Seedlings were exposed to stress during 2 and 15 days. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments with by the same lowercase letter (2 days) or the same uppercase letter (15 days) for a particular cultivar do not differ statistically according to Tukey HSD.

Effects on photosynthetic, chlorophyll fluorescence and total chlorophyll

The net photosynthesis (*A*) increased in TOG5307 plants submitted to PEG treatment (Fig. 3A) after 2 days, It significantly decreased in plants exposed to NaCl. The minimal value is recorded for the MIX treatment. It was not affected in TOG5949 exposed to PEG stress but decreased in response to NaCl and MIX treatment. After 15 days of stress, *A* decreased in response to all treatments and, for both cultivars, the minimal value was observed for NaCl-exposed plants. The *A* values after 15 days were similar for TOG5307 in response to PEG and MIX treatment while *A* values for MIX treatment was intermediate between NaCl and PEG in TOG5949. For both NaCl and MIX treatment, *A* values were higher in TOG5307 than in TOG5949.

Instantaneous transpiration (*E*; Fig. 3B) was not affected in response to PEG after 2 days of exposure but it significantly decreased in response to NaCl stress in the two cultivars and in response to MIX treatment in TOG5949. In contrast, *E* was drastically reduced after 15 days of treatment, minimal values being recorded for NaCl-treated plants. Instantaneous transpiration after 15 days was significantly higher in TOG5307 than in TOG5949.

After 2 days of treatment, $C_i$ (Fig. 3C) was not modified for PEG- and NaCl-treated plants but significantly increased in response to MIX treatment. After 15 days, this parameter was slightly but significantly decreased in response to PEG and MIX treatment in TOG5307, and in response to NaCl and MIX treatment for TOG5949. The stomatal conductance ($g_s$; Fig. 3D) was strongly affected in TOG5949 already after 2 days of exposure. The recorded decrease was limited in TOG5307 for plants exposed to PEG and MIX treatment, but more marked for plants exposed to NaCl. A similar pattern was observed after 15 days, although the difference between cultivars was less marked than after 2 days. While all parameters and their interactions were significantly affected for *A* ($p < 0.0001$) and $g_s$ ($p < 0.0001$), there was not significant interaction between cultivars and duration for parameters such as *E* and $C_i$ (Table 1).

As shown in Table 3, the instantaneous water use efficiency (*A*/*E*) increased in response to PEG after 2 days in TOG5307 and remained unaffected in TOG5949 but it decreased in both cultivars in response to the two other treatments. After 15 days of treatment, *A*/*E* increased in response to NaCl in TOG5307 but not in TOG5949. In all cases, including controls, water use efficiency was higher after 15 days than after 2 days. After 2 days, $A/C_i$

decreased in the two cultivars exposed to NaCl and MIX treatment, while it increased in PEG-treated plants in TOG5307 only. It decreased in response to all treatments in both cultivars after 15 days of treatment, reaching minimal value in NaCl-treated plants. After 2 days, the intrinsic water use efficiency ($A/g_s$) increased in response to all treatments, except for TOG5307 exposed to the MIX treatment. After 15 days, a significant decrease was observed in TOG5307 for plants exposed to PEG and NaCl, and in TOG5949 for plants exposed to NaCl and to the MIX treatment. The $A/g_s$ ratio was always higher after 15 days comparatively to 2 days, whatever the cultivar or the stressing agent. After 2 and 15 days, the efficiency of electron transport (ETR/*A*) increased in both cultivars exposed to stress, except for TOG5307 exposed to PEG during 2 days (detailed data not shown).

The maximal efficiency of PSII photochemistry in the dark-adapted state ($F_v/F_m$), the photochemical quenching coefficient ($q_P$) and the actual PSII efficiency ($\Phi_{PS2}$) were not affected by stresses (Table 4) while the non-photochemical quenching (NPQ) was higher in PEG treatment after 2 days. After 15 days of treatment, NPQ strongly increased in response to NaCl and MIX treatment in TOG5307 and in response to all stressing agents in TOG5949. (Table 4).

For TOG5307, chlorophyll a, chlorophyll b and carotenoids contents increased in response to all stressing agents after 2 days while these pigments decreased in response to the MIX treatment in TOG5949 (Table 5). In contrast, the pigments decreased in both cultivars and in response to all stressing agent after 15 days of treatment. As far as Chla: Chlb ratio is concerned, there was not significant difference between treatments after 2 days. After 15 days, Chla:Chlb ratio was significantly reduced showing the minimal value in response to PEG in TOG5307 and in response to NaCl in TOG5949 (Table 5).

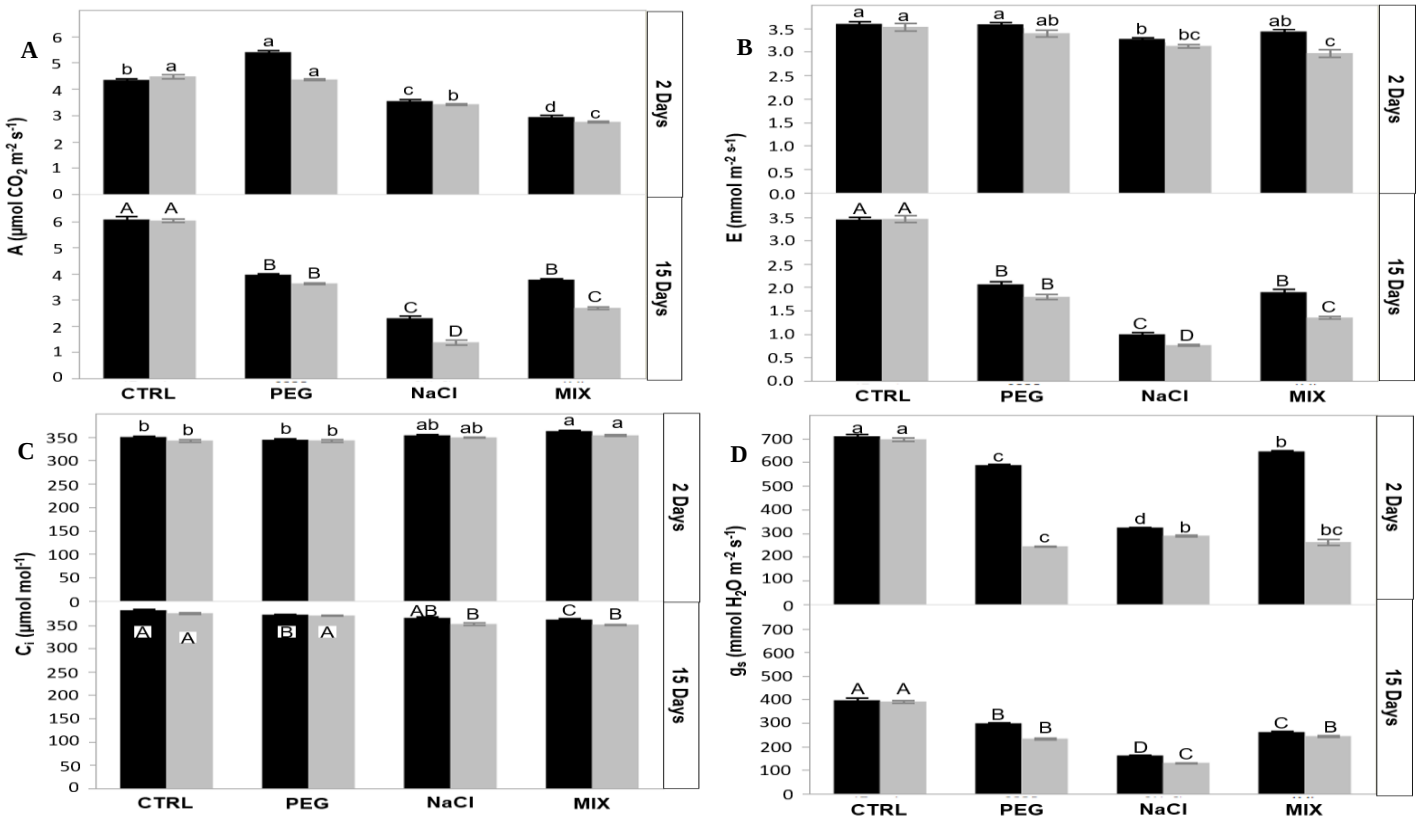


**Figure 3.** Impact of iso-osmotic concentration of PEG 8.6%, NaCl 50 mM or MIX treatment (PEG 4.3% + 25 mM) on (A) net photosynthesis (*A*; in µmol $CO_2$ $m^{-2}$ $s^{-1}$), (B) instantaneous transpiration (*E*; in mmol $H_2O$ $m^{-2}$ $s^{-1}$), (C) intercellular $CO_2$ concentration ($C_i$, µmol $mol^{-1}$) and (D) stomatal conductance ($g_s$; mmol $H_2O$ $m^{-2}$ $s^{-1}$) of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (salt-resistant; black bars) and TOG5949 (salt-sensitive; grey bars). Seedlings were exposed to stress during 2 and 15 days. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments with by the same lowercase letter (2 days) or the same uppercase letter (15 days) for a particular cultivar do not differ statistically according to Tukey HSD.

**Table 3.** Impact of iso-osmotic solutions of PEG 8.6%, NaCl 50 mM or mix treatment (PEG 4.3% + NaCl 25 mM) applied for 2 or 15 days on instantaneous water use efficiency (*A*/*E*), A/Ci and intrinsic water use efficiency ($A/g_s$) of two contrasting cultivars of African rice (*Oryza glaberrima* Steud. cv TOG5307 (salt-resistant) and TOG5949 (salt-sensitive)). For a particular cultivar, values followed by similar lower case (2 days) or uppercase (15 days) letters are not statistically different according to Tukey post hoc test.

| Cultivar | Treatment | Duration | *A*/*E* | *A*/Ci | $A/g_s$ |
|---|---|---|---|---|---|
| TOG5307 | Control | 2 days | 1.21 ± 0.03 b | 0.012 ± 0.0001 b | 0.006 ± 0.0001 c |
| TOG5949 | | | 1.27 ± 0.04 a | 0.013 ± 0.0003 a | 0.006 ± 0.0001 c |
| TOG5307 | PEG | | 1.51 ± 0.04 a | 0.016 ± 0.0003 a | 0.009 ± 0.0001 b |
| TOG5949 | | | 1.29 ± 0.02 a | 0.013 ± 0.0001 a | 0.018 ± 0.0003 a |
| TOG5307 | NaCl | | 1.08 ± 0.03 c | 0.010 ± 0.0003 c | 0.011 ± 0.0002 a |
| TOG5949 | | | 1.09 ± 0.01 b | 0.010 ± 0.0002 b | 0.012 ± 0.0003 b |
| TOG5307 | Mix | | 0.85 ± 0.01 d | 0.008 ± 0.0003 d | 0.005 ± 0.0001 d |
| TOG5949 | | | 0.93 ± 0.02 c | 0.008 ± 0.0001 c | 0.010 ± 0.001 b |
| TOG5307 | Control | 15 days | 1.76 ± 0.06 B | 0.016 ± 0.0003 A | 0.015 ± 0.0001 A |
| TOG5949 | | | 1.74 ± 0.05 A | 0.016 ± 0.0001 A | 0.015 ± 0.0003 A |
| TOG5307 | PEG | | 1.92 ± 0.05 AB | 0.011 ± 0.0001 B | 0.013 ± 0.0001 C |
| TOG5949 | | | 2.02 ± 0.08 A | 0.010 ± 0.0001 B | 0.016 ± 0.0004 A |
| TOG5307 | NaCl | | 2.32 ± 0.15 A | 0.006 ± 0.0002 C | 0.014 ± 0.0004 BC |
| TOG5949 | | | 1.81 ± 0.15 A | 0.004 ± 0.0002 D | 0.011 ± 0.001 B |
| TOG5307 | Mix | | 1.99 ± 0.04 AB | 0.010 ± 0.0002 B | 0.014 ± 0.0003 AB |
| TOG5949 | | | 1.99 ± 0.09 A | 0.008 ± 0.0001 C | 0.011 ± 0.0001 B |

**Table 4.** Impact of iso-osmotic solutions of PEG 8.6%, NaCl 50 mM or mix treatment (PEG 4.3% + NaCl 25 mM) applied for 2 or 15 days on maximal efficiency of PSII photochemistry in the dark-adapted state ($F_v/F_m$), actual PSII efficiency ($\Phi_{PSII}$), photochemical quenching ($q_P$) and non-photochemical quenching (NPQ) of two contrasting cultivars of African rice (*Oryza glaberrima* Steud. cv TOG5307 (salt-resistant) and TOG5949 (salt-sensitive)). For a particular cultivar, values followed by similar lower case (2 days) or uppercase (15 days) letters are not statistically different according to Tukey post hoc test.

| Cultivar | Treatment | Duration | $F_v/F_m$ | $\Phi_{PSII}$ | $q_P$ | NPQ |
|---|---|---|---|---|---|---|
| TOG5307 | Control | 2 days | 0.90 ± 0.004 a | 0.82 ± 0.01 a | 0.94 ± 0.01 a | 0.25 ± 0.01 b |
| TOG5949 | | | 0.89 ± 0.01 a | 0.81 ± 0.01 a | 0.95 ± 0.01 a | 0.26 ± 0.01 b |
| TOG5307 | PEG | | 0.90 ± 0.002 a | 0.85 ± 0.01 a | 0.98 ± 0.01 a | 0.29 ± 0.01 a |
| TOG5949 | | | 0.88 ± 0.004 a | 0.81 ± 0.01 a | 0.95 ± 0.01 a | 0.34 ± 0.01 a |
| TOG5307 | NaCl | | 0.91 ± 0.005 a | 0.85 ± 0.005 a | 0.95 ± 0.01 a | 0.22 ± 0.01 bc |
| TOG5949 | | | 0.90 ± 0.01 a | 0.82 ± 0.004 a | 0.94 ± 0.01 a | 0.25 ± 0.01 b |
| TOG5307 | Mix | | 0.90 ± 0.01 a | 0.84 ± 0.003 a | 0.96 ± 0.005 a | 0.21 ± 0.004 c |
| TOG5949 | | | 0.90 ± 0.01 a | 0.83 ± 0.004 a | 0.94 ± 0.01 a | 0.28 ± 0.01 b |
| TOG5307 | Control | 15 days | 0.89 ± 0.003 A | 0.81 ± 0.01 A | 0.95 ± 0.01 A | 0.22 ± 0.01 C |
| TOG5949 | | | 0.90 ± 0.005 A | 0.81 ± 0.01 A | 0.95 ± 0.003 A | 0.23 ± 0.01 D |
| TOG5307 | PEG | | 0.88 ± 0.01 A | 0.78 ± 0.01 AB | 0.92 ± 0.01 A | 0.26 ± 0.01 C |
| TOG5949 | | | 0.87 ± 0.004 B | 0.75 ± 0.02 B | 0.91 ± 0.01 B | 0.33 ± 0.01 C |
| TOG5307 | NaCl | | 0.82 ± 0.002 C | 0.74 ± 0.004 B | 0.88 ± 0.02 B | 0.46 ± 0.01 A |
| TOG5949 | | | 0.81 ± 0.004 C | 0.71 ± 0.002 B | 0.84 ± 0.003 C | 0.56 ± 0.01 A |
| TOG5307 | Mix | | 0.85 ± 0.003 B | 0.78 ± 0.01 AB | 0.91 ± 0.004 AB | 0.37 ± 0.01 B |
| TOG5949 | | | 0.82 ± 0.002 C | 0.75 ± 0.01 B | 0.85 ± 0.01 C | 0.45 ± 0.01 B |

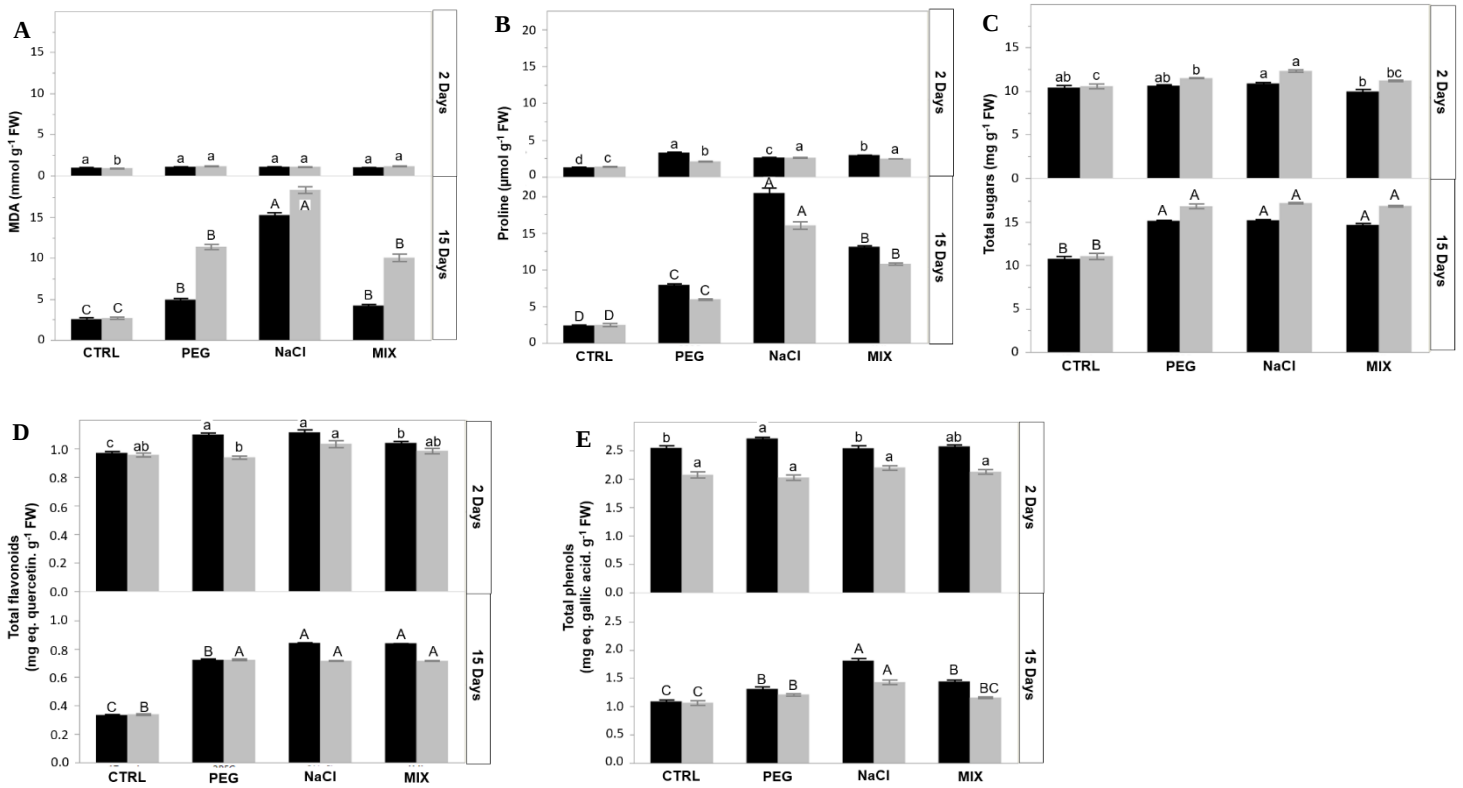


**Figure 4.** Impact of iso-osmotic concentration of PEG 8.6%, NaCl 50 mM or MIX treatment (PEG 4.3% + 25 mM) on shoot (A) malondialdehyde (MDA) (B) proline; (C) total soluble sugars, (D) total flavonoids (E) total phenols of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (salt-resistant; black bars) and TOG5949 (salt-sensitive; grey bars). Seedlings were exposed to stress during 2 and 15 days. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments with by the same lowercase letter (2 days) or the same uppercase letter (15 days) for a particular cultivar do not differ statistically according to Tukey HSD.

**Table 5.** Impact of iso-osmotic solutions of PEG 8.6%, NaCl 50 mM or mix treatment (PEG 4.3% + NaCl 25 mM) applied for 2 or 15 days on chlorophyll a (*Chl a*; in $mg.Kg^{-1}$ FW), chlorophyll b (*Chl b*; in $mg.g^{-1}$ FW), total carotenoids (Caro; in $mg.g^{-1}$ FW) and *Chl* a/*Chl* b ratio in two contrasting cultivars of African rice (*Oryza glaberrima* Steud. cv TOG5307 (salt-resistant) and TOG5949 (salt-sensitive)). For a particular cultivar, values followed by similar lower case (2 days) or uppercase (15 days) letters are not statistically different according to Tukey post hoc test.

| Cultivar | Treatment | Duration | *Chl a* | *Chl b* | Caro | *Chl a/Chl b* |
|---|---|---|---|---|---|---|
| TOG5307 | Control | 2 days | 0.82 ± 0.01 c | 0.25 ± 0.01 b | 0.23 ± 0.002 d | 3.35 ± 0.07 a |
| TOG5949 | | | 0.81 ± 0.003 b | 0.26 ± 0.004 a | 0.23 ± 0.01 a | 3.07 ± 0.04 a |
| TOG5307 | PEG | | 1.04 ± 0.01 a | 0.30 ± 0.01 a | 0.27 ± 0.001 a | 3.44 ± 0.07 a |
| TOG5949 | | | 0.82 ± 0.01 b | 0.26 ± 0.01 a | 0.22 ± 0.01 a | 3.13 ± 0.12 a |
| TOG5307 | NaCl | | 0.96 ± 0.02 b | 0.32 ± 0.01 a | 0.25 ± 0.0001 c | 3.31 ± 0.07 a |
| TOG5949 | | | 0.92 ± 0.005 a | 0.28 ± 0.01 a | 0.24 ± 0.01 a | 2.96 ± 0.06 a |
| TOG5307 | Mix | | 0.92 ± 0.01 b | 0.29 ± 0.01 a | 0.26 ± 0.0002 b | 3.17 ± 0.09 a |
| TOG5949 | | | 0.73 ± 0.02 c | 0.21 ± 0.01 b | 0.19 ± 0.01 b | 3.46 ± 0.23 a |
| TOG5307 | Control | 15 days | 0.91 ± 0.03 A | 0.24 ± 0.005 A | 0.21 ± 0.004 A | 3.82 ± 0.18 A |
| TOG5949 | | | 0.94 ± 0.03 A | 0.25 ± 0.002 A | 0.20 ± 0.003 A | 3.80 ± 0.13 A |
| TOG5307 | PEG | | 0.29 ± 0.01 D | 0.13 ± 0.003 C | 0.08 ± 0.001 D | 2.19 ± 0.05 C |
| TOG5949 | | | 0.27 ± 0.003 D | 0.10 ± 0.001 C | 0.08 ± 0.004 D | 2.68 ± 0.03 B |
| TOG5307 | NaCl | | 0.38 ± 0.004 C | 0.17 ± 0.01 B | 0.11 ± 0.0005 C | 3.46 ± 0.03 AB |
| TOG5949 | | | 0.39 ± 0.01 C | 0.11 ± 0.001 C | 0.10 ± 0.001 C | 2.31 ± 0.07 C |
| TOG5307 | Mix | | 0.69 ± 0.003 B | 0.24 ± 0.003 A | 0.17 ± 0.001 B | 3.04 ± 0.07 B |
| TOG5949 | | | 0.60 ± 0.01 B | 0.20 ± 0.01 B | 0.16 ± 0.001 B | 2.88 ± 0.03 B |

Malondialdehyde, proline, total sugars, flavonoids, phenol and antioxidant activity (FRAP)

The shoot MDA concentration did not change after 2 days of treatment but was drastically increased in stressed plants after 15 days. NaCl-stressed plants exhibited the highest concentration, while PEG- and MIX-treated plants presented similar concentration. The MDA content was commonly higher in TOG5949 than in TOG5307, especially in response to PEG and to MIX-treatment (Figure 4.A).

The shoot proline (Fig. 4B) concentration was slightly increased in response to all stresses after 2 days of exposure. After 15 days, an obvious increase in proline concentration was observed for both cultivars. PEG treatment had a lower impact on proline accumulation, while MIX treatment was intermediate: in all stressed plants, cv. TOG5307 had a higher proline concentration than TOG5949 while the two cultivars had a similar proline concentration in the absence of stress.

After two days, stress had no significant impact on total soluble sugars in TOG5307 and slightly increased it in response to PEG and NaCl but not in response to the MIX treatment in TOG5949. After 15 days, the total soluble sugar concentrations increased significantly in response to all treatments, especially in TOG5949 (Fig. 4.C). Concentrations of total flavonoids (Figure 4D) and total phenols (Fig. 4E) were higher after 2 days than after 15 days of treatment, even in control plants. Total flavonoids increased after 2 days in TOG5307 in response to all stresses but it remained unaffected in TOG5949.

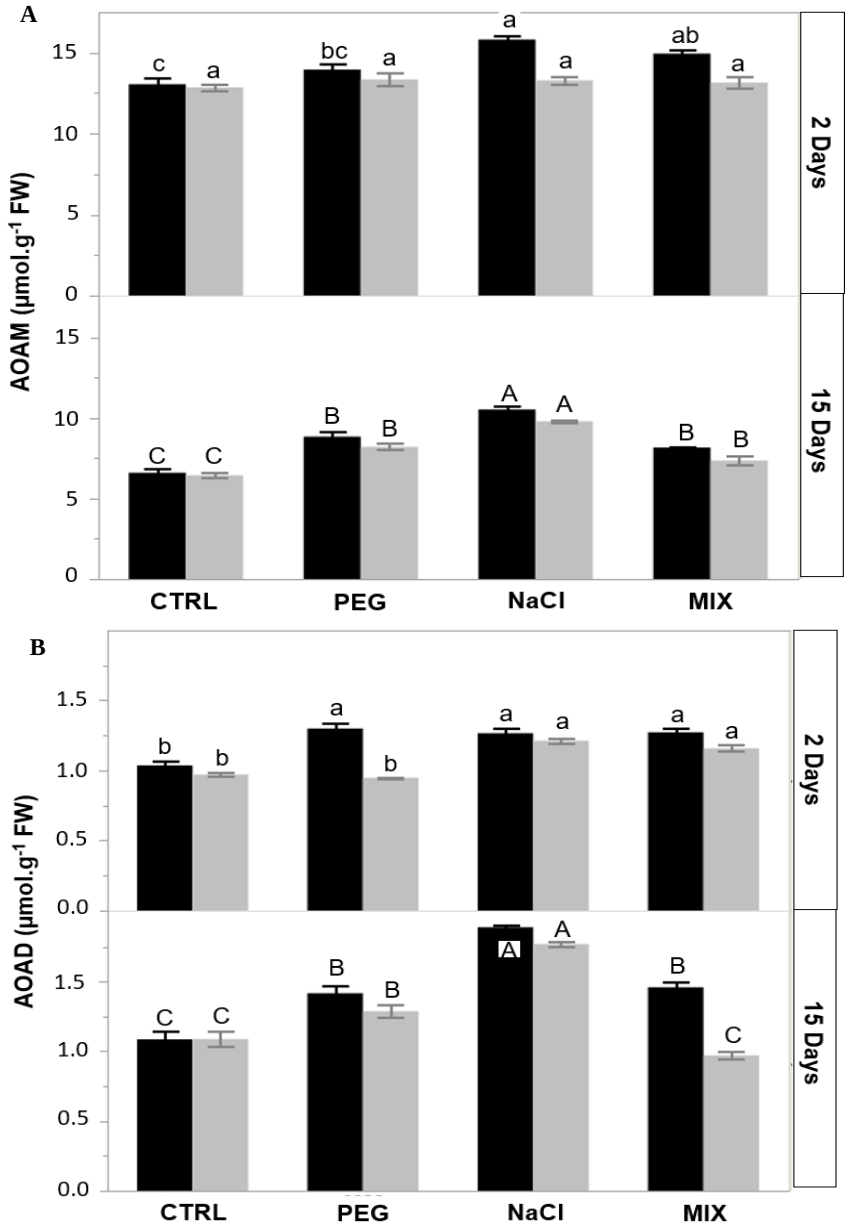


**Figure 5.** Impact of iso-osmotic concentration of PEG 8.6%, NaCl 50 mM or MIX treatment (PEG 4.3% + 25 mM) on antioxidant activity (A)AOAM, (B) AOAD fractions (expressed as µmol.g$^{-1}$ FW of Trolox equivalent) determined by the FRAP method of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (salt-resistant; black bars) and TOG5949 (salt-sensitive; grey bars). Seedlings were exposed to stress during 2 and 15 days. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments with by the same lowercase letter (2 days) or the same uppercase letter (15 days) for a particular cultivar do not differ statistically according to Tukey HSD.

After 15 days, the stress-induced increase in flavonoid concentration was similar for all types of constraints in TOG5949, and was higher in response to NaCl and MIX treatment in TOG5307. After 2 days, total phenol remained unaffected in TOG5949 and increased only in response to PEG in TOG5307 which also exhibited a higher content in total phenol than TOG5949, including in controls. After 15 days, the highest total phenol was recorded for NaCl-treated plants in both cultivars, while plants exposed to PEG and to MIX treatment exhibited similar values.

As far as total antioxidant activity (ferric reducing antioxidant power, FRAP) is concerned, AOAM activity (Figure 5.A) increased after 2 days in response to NaCl and MIX treatment in TOG5307 only. In control plants, the antioxidant activity of the AOAM fraction decreased after 15 days comparatively to 2 days: it was the highest in NaCl-treated plants, while PEG- and MIX-treated plants exhibited similar values. The activity of the AOAD fraction increased in a similar way in response to all stressing agents after 2 days of exposure in TOG5307 and in response to NaCl and MIX treatment in TOG5949. After 15 days of treatment, the highest AOAD activity was recorded for NaCl-treated plants. Plants exposed to PEG and MIX treatment had similar lower AOAD activity in TOG5307, while the value recorded in TOG5949 was similar to control for plants exposed to MIX treatment.

Effects on Isotopic and elemental compositions

The $\Delta^{13}C$ obviously decreased in response to all stressing agents, the minimal values was observed for TOG5949 exposed to NaCl stress (Fig. 6A). The $\delta^{15}N$ was significantly increased in PEG-stressed plants and obviously reduced in NaCl-treated ones: once again, the MIX treatment had an intermediate impact in both cultivars (Fig. 6B). The elemental C composition decrease in PEG-treated plants of cv. TOG5949, while it was reduced in both cultivars in NaCl-treated plants. Unexpectedly, the MIX treatment induced an increase in C elemental composition in both cvs. (Fig. 6C). The nitrogen elemental composition (Fig. 6D) decreased to similar values in TOG5307 exposed to the different stressing agents; in TOG5949, a strong decrease was observed in PEG-treated plants.

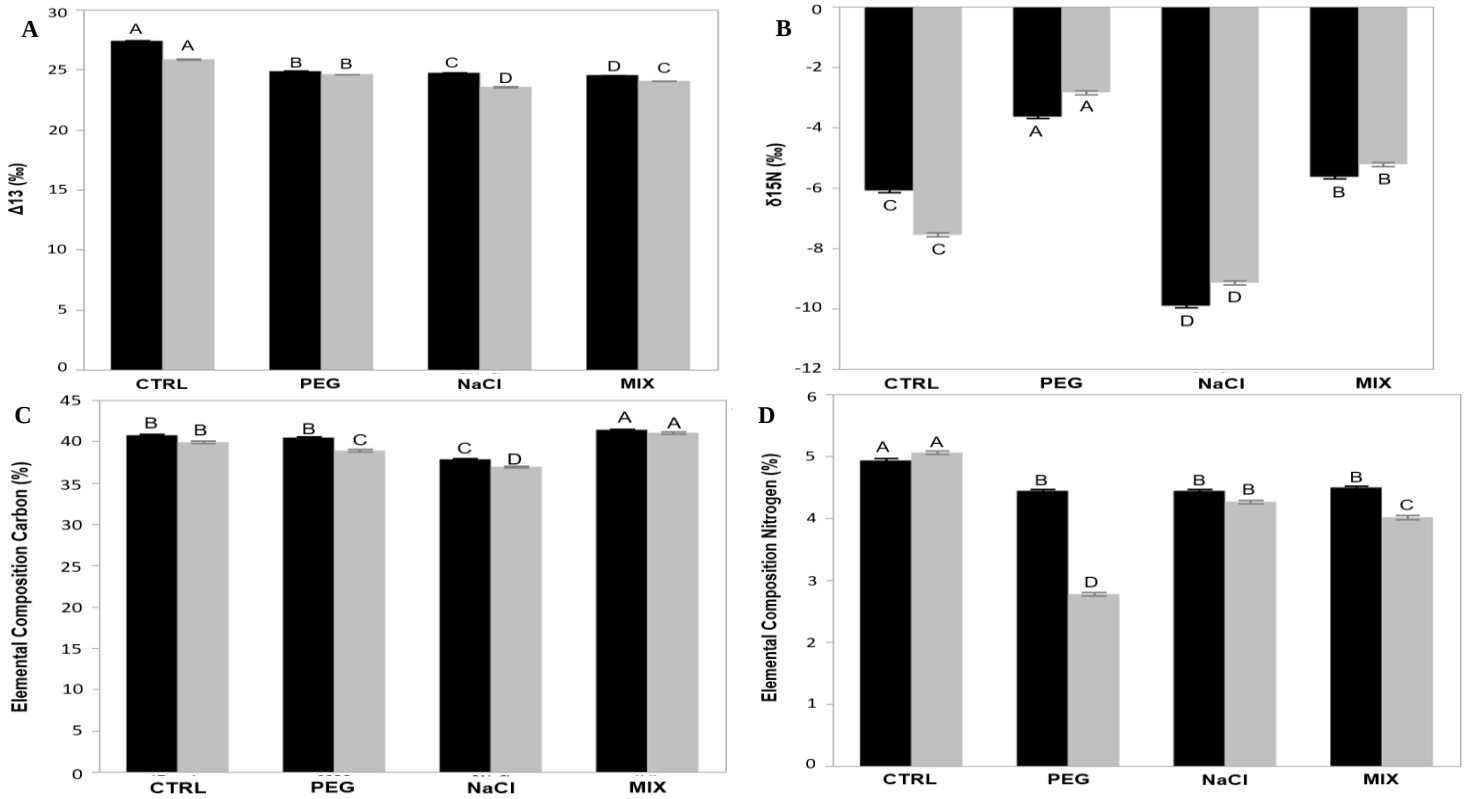


**Figure 6.** Impact of iso-osmotic concentration of PEG 8.6%, NaCl 50 mM or MIX treatment (PEG 4.3% + 25 mM) on (A) carbon isotope discrimination (B) Nitrogen isotope composition (C) carbon elemental composition (in%) and (D) nitrogen elemental composition (in %) of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (salt-resistant; black bars) and TOG5949 (salt-sensitive; grey bars). Seedlings were exposed to stress during 15 days. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments with by the same uppercase letter for a particular cultivar do not differ statistically according to Tukey HSD.s HSD

## Toxicity index

Toxicity index measurements was calculated for 40 morphophysiological parameters listed in supplemental data (Table S1). After 2 days, $TI_{PEG}$ was lower than $TI_{NaCl}$. TIPEG exhibited similar values in both cultivars while $TI_{NaCl}$ was higher for TOG5949 than for TOG5307. The TI MIX OBS value was similar to $TI_{NaCl}$ in TOG5307 but was significantly higher than $TI_{NaCl}$ in TOG5949. The observed toxicity index ($TI_{MIX\ OBS}$) was lower than the expected value ($TI_{MIX\ EXP}$) in TOG5307 while no difference was recorded in TOG5949.

Toxicity indexes increased after 15 days comparatively to 2 days for all treatments, and was higher in TOG5949 than in TOG5307, except for NaCl stress. For both cultivars $TI_{NaCl}$ was higher than $TI_{PEG}$. Once again, $TI_{MIX\ OBS}$ was lower than $TI_{MIX\ EXP}$ in TOG5307 while the two values were statistically similar for TOG5949.

## Principal coordinate, Boxplots analysis

In order to better understand the impact of stresses in both cultivars, PCoA (principal coordinate analysis) was performed to compare the growth parameters of African rice at 2 days and 15 days of treatment (Figure 7). The results displayed that the first principal axis accounts for 99.83% at 2 days (Figure 7.A) and for 99.05% at 15 days (Figure 7.B). Furthermore, boxplots were conducted based on the data mapped on the first axis of PCoA to visualize the impact of stresses (Figures 7.C & 7.D). At 2 days, PEG-stressed and MIX-stressed TOG5949 plants had the largest distances from controls whereas TOG5307 presented an opposite trend. The distance between NaCl and control was large for both varieties (Figures 7.A & 7.C). However, at 15 days, the distance between NaCl and control was larger than that between MIX and control, and that between PEG and control for both cultivars. Nevertheless, stressed TOG5307 plants were closer to control than stressed TOG5949 plants (Figure 7.B & 7.D).

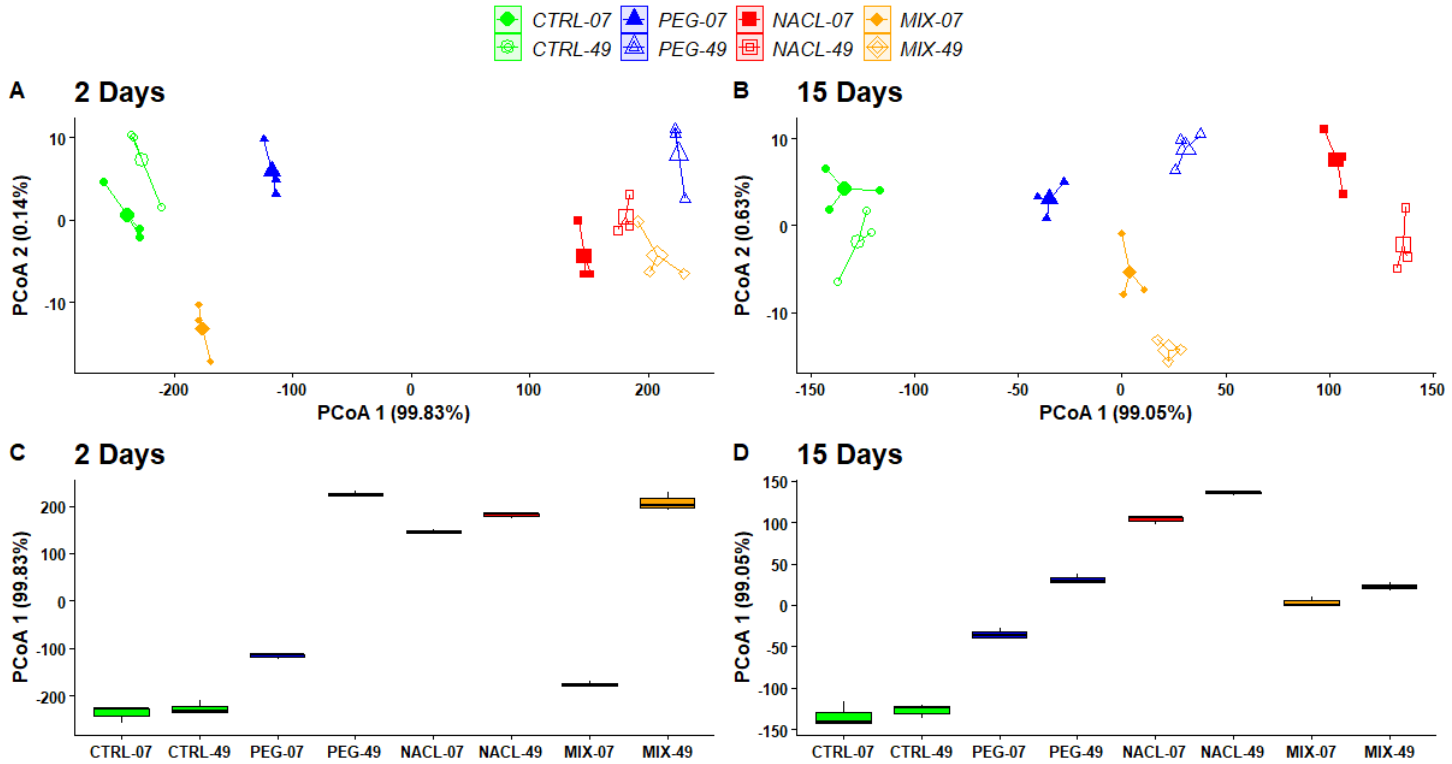


**Figure 7.** Principal coordinate analysis (PCoA) of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (.-07) and TOG5949 (.-49) cultivated during (A) 2 days and (B) 15 days in control conditions (CTRL) or in the presence of 50 mM of either NaCl, PEG-6000 (PEG) or MIX (PEG +NaCl). Boxplots for visualizing the Euclidean distance of the first axis of PCoA of (C) 2 days and (D) 15 days parameters.

## Discussion

The aim of the present work was to compare the impacts of salt and water stress, and a combination of these two constraints, on two cultivars of the African rice species (*Oryza glaberrima* Steud.) differing in their level of salt resistance. Our data confirms that the type of constraint had a significant impact on all recorded parameters, except the shoot water content (Table 1). Goharrizi et al. (2020) recently reported that a combination of drought and salinity was more detrimental that individual stresses on pistachio rootstocks but these authors compared an addition of two stress intensities and it was therefore not surprising that the combined treatment with a high stress intensity was more detrimental that individual stresses with low stress intensity. Our approach was somewhat different since we used iso-osmotic concentration for the three considered stress treatments ($\Psi s$ = -0.32 MPa). Indeed, PEG was used to mimic drought stress. This is a bit different from situation occurring in real field conditions since the major difference between low-water-potential environment caused by salinity versus drought is the total amount of available water (Ahmed et al. 2015): in our experimental conditions we only manipulated availability of water.

The duration of stress also had a significant effect on all recorded parameters (Table1). This is not surprising since rice is a salt-sensitive species and a long-term exposure to the constraint is expected to aggravate the stress symptoms comparatively to a short-term period. Accordingly, the toxicity index was higher after 15 days than after 2 days (Table 1) and this is valid for almost all recorded parameters (Table S1).

As far as a 2 days exposure is considered, it might be hypothesized that the impact of NaCl is mainly attributable to the osmotic component of salt stress. Indeed, according to the biphasic model of Munns (Munns et al. 1995), salinity first compromises plant water uptake before accumulation of toxic ions may occur. The time limit between the two components is dependent on the considered species and the "osmotic" component is probably extremely short in rice and that things could not be so simple. Indeed, we demonstrated that $Na^+$ accumulation already occurred after 48h in both roots and shoots of the two cultivars (Fig. 2) while a significant decrease in $K^+$ was also recorded after such a brief period. After 2 days of stress, $g_s$ was already reduced (Fig 3D); as far as the salt-resistant TOG5307 is concerned, the recorded decrease was even more important in response to NaCl than in response to PEG and

should have led to a marked decrease in transpiration stream, while $Na^+$ accumulation was similar for the two cultivars after this period. This suggests that $Na^+$ accumulation in both cultivars occurred very rapidly and supports earlier view (Lefèvre et al. 2001; Roshandel and Flowers 2009) that the "osmotic component" of salt stress in rice is extremely brief.

**Table 6**. Mean toxicity index calculated for plants of the African rice species (*Oryza glaberrima* Steud) exposed to stressing agent during 2 and 15 days. Toxicity index was provided for plants exposed to PEG 8.6% ($TI_{PEG}$), NaCl 50 mM ($TI_{NaCl}$), MIX treatment (PEG 4.3% + NaCl 25 mM) ($TI_{MIX\ OBS}$). Values were also calculated for theoretical expected toxicity index for MIX treatment ($TI_{MIX\ EXP}$). Mean values were calculated for 40 morphophysiological parameters as listed in Table S1 (± SE). Means followed by the same letters are not statistically different according to the Tukey HSD test.

| Duration | Cultivar | $TI_{PEG}$ | $TI_{NaCl}$ | $TI_{MIX\ OBS}$ | $TI_{MIX\ EXP}$ |
|---|---|---|---|---|---|
| 2 days | TOG5307 | 0.145±0.006 a | 0.574±0.042 d | 0.526±0.033 d | 0.624±0.042 e |
| | TOG5949 | 0.140±0.008 a | 0.642±0.046 e | 0.724±0.044 f | 0.719±0.046 f |
| 15 days | TOG5307 | 0.284±0.01 b | 1.507±0.103 h | 1.181±0.071 g | 1.477±0.08 h |
| | TOG5949 | 0.346±0.014 c | 1.845±0.144 hi | 2.023±0.137 i | 2.563±0.190 i |

The metabolic consequences of accumulated $Na^+$ after 2 days remained however limited. Indeed, it is known that salinity induces an oxidative stress in plants (van Zelm et al. 2020) but MDA accumulation (commonly considered as a marker of oxidative stress; Fig. 4A) was rather low in the shoot of both cultivars after 2 days. The total phenol acting as major antioxidants did not accumulated after this short-term exposure (Fig. 4E). However, both AOAM and AOAD antioxidant activities quantified by the FRAP assay increased after 2 days, which suggests that *O. glaberrima* is able to efficiently trigger the synthesis of other antioxidant such as glutathione and tocopherols

which were not quantified in this study but may act as preventive agent to avoid stress-induced oxidative damages.

Proline accumulation (Fig. 4B), which is a major solute involved in osmotic adjustment (Alzahrami 2021) was observed after 2 days: from a quantitative point of view, however, it remained rather low and could not fully explain the recorded decrease in the shoot osmotic potential (Fig. 1B). Hence, it could not be excluded that $Na^+$ itself (and probably $Cl^-$ as well), although being toxic at high concentration, could be used as cheap osmoticum to ensure the maintenance of cell turgor through a decrease in internal $\Psi s$. Such strategy should rely on tolerance mechanism to accumulated ions (Yeo et al. 1991) and probably has a high energy cost (Munns et al. 2020). After 15 days of stress, proline accumulation was quite higher than after 2 days (Fig. 2B). Proline accumulation was quite higher for NaCl-treated plants than for PEG-exposed ones and the difference in proline accumulation was by far higher than the difference in $\Psi s$ recorded between treatments. This suggests that proline may assume other functions than osmotic adjustment: it has been repeatedly reported to act as an antioxidant (Alhasnawi 2019). However, our data do not fully support this assumption since the highest proline accumulation recorded for shoots of NaCl-treated plants also corresponds to the highest MD accumulation (Fig. 4B).

The mean toxicity index calculated for 40 morphophysiological parameters (Table 6; Table S1) indicates that salt stress was more toxic than PEG on *O. glaberrima*. Undoubtedly, this is due to the very high values recorded for $Na^+$ content (see Table S1) which as a strong impact on the calculation of the mean $TI_{NaCl}$. If we remove root and shoot $Na^+$ content and $Na^+/K^+$ ratio from the calculation, however, mean $TI_{NaCl}$ after two days dropped to 0.182 ± 0.005 in TOG5307 and to 0.198 ± 0.004 in TOG5949. After 15 days, these values dropped respectively to 0.624 ± 0.034 and 0.675 ± 0.033. In all cases, the $TI_{NaCl}$ was still higher than the $TI_{PEG}$, whatever the cultivar or the stress duration, and this underlines the importance of ionic toxicity in *O. glaberrima* response to salt stress. Recent data confirm that the plant may react extremely rapidly to the ionic component of salt stress: according to Choi et al. (2014), local salt application at the root level may induce the propagation of long-range calcium wave only 10 minutes after salt application and such a wave was not observed in response to PEG. These authors also demonstrated that this calcium wave may reach the leaves within 30s. According to Boursiac et al. (2005), a concentration of 50 mM NaCl outside the roots decreases the hydraulic conductivity of the root by 35%. It is interesting to mention that Sun

et al. (2021) recently reported that the rice aquaporin OsPIP2;3 contributed to rice resistance to water deficit induced by PEG but not in response to NaCl. Unfortunately, these authors did not test a MIX treatment.

In the present study, the combination of stress (MIX treatment) was applied by reducing PEG and NaCl doses by 50%. It may be expected that the toxicity of the MIX treatment should correspond to the sum of half of the toxicity of NaCl + half of the toxicity of PEG (assuming for each recorded property that the response is a linear function of the applied dose). If the two types of stress act on an additive way, then $TI_{MIX\ OBS}$ should be equal to $TI_{MIX\ EXP}$ (see Material and Methods). This is valid for the salt-sensitive cultivar TOG5949, whatever the duration of stress (Table 6) but this is not true for the salt-resistant cultivar TOG5307: in this latter case, $TI_{MIX\ OBS}$ was significantly lower than $TI_{MIX\ EXP}$ (Table 6) and this suggests an antagonist action between the two considered constraints or, in other words, that the presence of one constraint helped the plant to more efficiently cope with the presence of the other constraint.

According to Mittler (2006), plant acclimation to a particular abiotic stress condition requires a specific response that is tailored to the precise environment the plant encounters. This is supported by transcriptomic data performed on plants exposed to heat, wounding, cold and high UV for which each stress condition prompter a somewhat unique response with little overlap in transcript expression. But, as stated above, osmotic stress and salt stress are not purely independent since salt stress has an osmotic component. $TI_{MIX\ OBS}$ (Table 6) was similar to $TI_{NaCl}$ in the resistant cultivar after 2 days, but it was higher than $TI_{NaCl}$ in TOG5949. After 15 days, this is not the case anymore and $TI_{MIX\ OBS}$ was significantly lower than $TI_{NaCl}$ for TOG5307. It is well established that environment act on plants through a complex network of stress perception and transduction signals leading to specific gene expression triggered by transcription factors. Even if genes induced by salt stress and osmotic stress are different, they act on similar physiological targets, at least if we do not consider mineral nutrition. PEG and NaCl rarely acted in an opposite way: $\delta^{15}N$ is probably the unique example of an opposite action of PEG and NaCl. Both osmotic stress and NaCl are known to alter the plant water status, to impair photosynthesis and to induce oxidative stress. It could therefore not be excluded that TOG5307 is able to recruit molecular tools induced by PEG to more efficiently cope with NaCl. This could be illustrated by the fact that A and E values for TOG5307 exposed to MIX treatment for 15 days were close to the values recorded for the less detrimental PEG

treatment while the values recorded for TOG5949 exposed to MIX-treatment were intermediated between values recorded for NaCl and PEG-treated plants. The same observation is valid for $g_s$ after 2 days of treatment. Additional transcriptomic and proteomic approaches (Kashyap et al. 2020) are required to decipher the differences recorded between the two cultivars exposed to the MIX treatment: this should help us to identify specific genes and transcription factors involved in the plant response to MIX-treatment comparatively to NaCl or PEG alone. Analyzing of hormonal profile involved in stress signaling may also be of paramount importance to perform a comprehensive approach.

Ion discrimination, especially $\Delta^{13}C$ is, beside growth, the only integrative parameter which provide an information regarding the plant behavior for the whole stressing period (Chen et al. 2011). For both NaCl and MIX-treatment $\Delta^{13}C$ was higher for TOG5307 than for TOG5949, which confirms that the resistant cultivar was able to maintain a higher WUE than the sensitive one. This is confirmed by the instantaneous water use efficiency $A/E$ after 15 days for NaCl-treated plants, but not for plants exposed to the MIX-treatment. Although $g_s$ was strongly reduced by salt stress, Ci remained hardly affected, suggesting that the carboxylation phase of photosynthesis was impaired. No obvious difference was regarded between the two tested cultivars. This is supported by the NaCl-induced decrease in $A$ value recorded after 15 days. The light phase of photosynthesis remained hardly affected by the stress after 2 days and only NPQ values increased after 15 days in response to NaCl and MIX-treatment.

$\delta^{15}N$ is also related to salinity resistance (Yousfi et al. 2009). We found that in control conditions, TOG5949 had a lower $\delta^{15}N$ than TOG5307. In both cultivars, $\delta^{15}N$ strongly increased (became less negative) while NaCl had an opposite effect. We also found that in PEG conditions, TOG5307 had a lower $\delta^{15}N$ than TOG5949. More interested, values recorded in PEG conditions were higher than values recorded in Mix and NaCl solutions for both varieties. So, we hypothesis that the PEG would not be a stress which negatively impacts the nitrogen isotopes. However, $\delta^{15}N$ is a complex parameter influenced by a several factors such as uptake, efflux, nitrogen assimilation, nitrogen release, and reallocation between organs (Evans 2001).The values of $\delta^{15}N$ observed in our study would justify the fact that all the growth parameters of the two varieties of African rice are not greatly affected despite the osmotic stress due to PEG. Our results converge with the work of Saud et al. (2020) who demonstrated that nitrogen isotopes contributed to drought tolerance in all

three clonal ramets of Kentucky bluegrass by maintaining a better osmoprotectant and antioxidant defense system, which helped the plants eliminate reactive oxygen species.

According to Kumar et al. (2021), salinity and drought have become a severe threat to ensure food security in the world. In our study, the effects of osmotic stress on the plant growth of African rice varieties during 2 days and 15 days under iso-osmotic nutrient solutions containing PEG and/or NaCl were globally assessed using principal coordination analysis (PCoA; Fig. 7). The sensitive variety TOG5949 was greatly affected by the effects of stresses from the start of application. This supposes that the resistant variety TOG5307 uses either $Na^+$ ions acting as osmoticum to counterbalance the low osmotic potential of the medium or its ability to secrete flavonoids and total phenols from the first hours of stress could explain its resistance to osmotic stress (Fig. 4D&E) . On the other hand, over the long term, the two varieties are much more affected by Mix and NaCl stresses, although TOG5307 is always more resistant. This mean that the initial growth reduction in African rice varieties caused by studied stresses was due to a limitation of water supply. And long-term salt effects on plant growth is related to direct ion toxicity due to the accumulation of high ion concentrations in plant tissue. (Sibole et al. 1998 ; Farsiani and Ghobadi. 2009).

## Conclusion

We can concluded that the duration of stress had a significant effect on all recorded parameters. Indeed, the differential impact of osmotic stress on two African rice (*Oryza glaberrima*) cultivars is mainly observed at short-term stress. At long-term stress, the accumulation of toxic ions in the organs of the plant accentuates the effect of salt stress. This underlines the importance of ionic toxicity in *O. glaberrima* response to salt stress. NaCl solution is more toxic than Mix solution followed by PEG solution on *O. glaberrima*. Isotope discrimination data suggest that nitrogen isotopes might contribute to drought tolerance.

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**Table S1** : [supplemental data] Toxicity index of iso-osmotic solutions of PEG 8.6%, NaCl 50 mM or mix treatment (PEG 4.3% + NaCl 25 mM) applied for 2 or 15 days on two contrasting cultivars of African rice (*Oryza glaberrima* Steud. cv TOG5307 (salt-resistant) and TOG5949 (salt-sensitive)).

| | | **TOG5307** | | | | **TOG5949** | | | |
|---|---|---|---|---|---|---|---|---|---|
| Moment | Parameters | Index-PEG | Index-NaCl | Index-Mix | Index-Mix predicted | Index-PEG | Index-NaCl | Index-Mix | Index-Mix predicted |
| **2 Days** | A | 0.244 | 0.185 | 0.324 | 0.030 | 0.025 | 0.236 | 0.385 | 0.131 |
| | A/Ci | 0.264 | 0.194 | 0.348 | 0.035 | 0.026 | 0.251 | 0.405 | 0.138 |
| | A/E | 0.246 | 0.106 | 0.294 | 0.070 | 0.012 | 0.138 | 0.270 | 0.063 |
| | $A/g_s$ | 0.500 | 0.780 | 0.259 | 0.640 | 1.774 | 0.838 | 0.634 | 1.306 |
| | Caro | 0.187 | 0.100 | 0.147 | 0.143 | 0.036 | 0.059 | 0.184 | 0.011 |
| | *Chl a* | 0.271 | 0.167 | 0.123 | 0.219 | 0.011 | 0.133 | 0.096 | 0.072 |
| | *Chl a* / *Chl b* | 0.026 | 0.011 | 0.053 | 0.008 | 0.018 | 0.037 | 0.128 | 0.009 |
| | *Chl b* | 0.238 | 0.322 | 0.187 | 0.280 | 0.004 | 0.051 | 0.194 | 0.024 |
| | Ci | 0.016 | 0.011 | 0.037 | 0.002 | 0.001 | 0.019 | 0.033 | 0.010 |
| | Calcium root | 0.024 | 0.073 | 0.284 | 0.024 | 0.247 | 0.338 | 0.108 | 0.292 |
| | Calcium shoot | 0.057 | 0.087 | 0.075 | 0.015 | 0.025 | 0.148 | 0.077 | 0.086 |
| | DW root | 0.100 | 0.133 | 0.159 | 0.117 | 0.135 | 0.179 | 0.190 | 0.157 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | DW shoot | 0.024 | 0.049 | 0.082 | 0.036 | 0.015 | 0.069 | 0.088 | 0.042 |
| | E | 0.002 | 0.089 | 0.044 | 0.045 | 0.038 | 0.114 | 0.158 | 0.076 |
| | Flavonoids | 0.133 | 0.149 | 0.072 | 0.141 | 0.018 | 0.081 | 0.030 | 0.031 |
| | Fv/Fm | 0.002 | 0.008 | 0.002 | 0.005 | 0.008 | 0.012 | 0.017 | 0.002 |
| | $g_s$ | 0.171 | 0.542 | 0.089 | 0.357 | 0.649 | 0.584 | 0.622 | 0.616 |
| | K root | 0.002 | 0.456 | 0.349 | 0.227 | 0.003 | 0.505 | 0.309 | 0.254 |
| | K shoot | 0.006 | 0.216 | 0.150 | 0.111 | 0.001 | 0.214 | 0.214 | 0.107 |
| | Length root | 0.031 | 0.163 | 0.153 | 0.097 | 0.011 | 0.079 | 0.056 | 0.045 |
| | Length shoot | 0.050 | 0.067 | 0.076 | 0.059 | 0.008 | 0.118 | 0.118 | 0.063 |
| | MDA | 0.104 | 0.099 | 0.046 | 0.101 | 0.260 | 0.186 | 0.246 | 0.223 |
| | Na/K root | 0.104 | 0.951 | 6.945 | 9.108 | 0.096 | 0.949 | 8.391 | 9.170 |
| | Na/K shoot | 0.155 | 0.886 | 2.606 | 3.675 | 0.055 | 0.921 | 5.309 | 5.629 |
| | Na root | 0.107 | 9.385 | 4.185 | 4.746 | 0.098 | 8.618 | 5.480 | 4.260 |
| | Na shoot | 0.156 | 5.684 | 2.069 | 2.764 | 0.055 | 8.712 | 3.977 | 4.328 |
| | NPQ | 0.159 | 0.122 | 0.169 | 0.018 | 0.326 | 0.021 | 0.080 | 0.153 |
| | AOAM | 0.069 | 0.211 | 0.145 | 0.140 | 0.039 | 0.034 | 0.022 | 0.036 |
| | AOAD | 0.253 | 0.222 | 0.227 | 0.237 | 0.023 | 0.250 | 0.194 | 0.113 |
| | $\Psi_S$ root | 0.307 | 0.303 | 0.234 | 0.305 | 0.423 | 0.449 | 0.234 | 0.436 |
| | $\Psi_S$ shoot | 0.027 | 0.224 | 0.026 | 0.125 | 0.299 | 0.344 | 0.261 | 0.321 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Polyphenols | 0.064 | 0.003 | 0.010 | 0.030 | 0.022 | 0.061 | 0.025 | 0.019 |
| | Proline | 1.555 | 1.043 | 1.301 | 1.299 | 0.487 | 0.843 | 0.756 | 0.665 |
| | PS2 | 0.035 | 0.030 | 0.026 | 0.032 | 0.008 | 0.010 | 0.016 | 0.001 |
| | qP | 0.036 | 0.010 | 0.017 | 0.023 | 0.002 | 0.020 | 0.016 | 0.011 |
| | Root/shoot | 0.078 | 0.088 | 0.083 | 0.083 | 0.121 | 0.119 | 0.111 | 0.120 |
| | Mg root | 0.052 | 0.164 | 0.005 | 0.108 | 0.129 | 0.223 | 0.073 | 0.176 |
| | Mg shoot | 0.011 | 0.088 | 0.099 | 0.050 | 0.032 | 0.137 | 0.099 | 0.085 |
| | Total sugars | 0.020 | 0.044 | 0.044 | 0.032 | 0.088 | 0.165 | 0.062 | 0.126 |
| | WC root | 0.016 | 0.043 | 0.008 | 0.014 | 0.050 | 0.005 | 0.001 | 0.023 |
| | WC shoot | 0.033 | 0.024 | 0.024 | 0.028 | 0.049 | 0.050 | 0.004 | 0.050 |
| | | | | | | | | | |
| | | **TOG5307** | | | | **TOG5949** | | | |
| | Parameters | Index-PEG | Index-NaCl | Index-Mix | Index-Mix predicted | Index-PEG | Index-NaCl | Index-Mix | Index-Mix predicted |
| **15 Days** | A | 0.347 | 0.622 | 0.379 | 0.484 | 0.398 | 0.771 | 0.553 | 0.585 |
| | A/Ci | 0.330 | 0.605 | 0.345 | 0.467 | 0.390 | 0.756 | 0.522 | 0.573 |
| | A/E | 0.091 | 0.314 | 0.128 | 0.203 | 0.159 | 0.036 | 0.142 | 0.098 |
| | $A/g_s$ | 0.134 | 0.077 | 0.058 | 0.106 | 0.008 | 0.319 | 0.285 | 0.156 |
| | Caro | 0.601 | 0.477 | 0.185 | 0.539 | 0.622 | 0.499 | 0.210 | 0.560 |
| | *Chl a* | 0.683 | 0.584 | 0.244 | 0.633 | 0.710 | 0.592 | 0.364 | 0.651 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Chl a* / *Chl b* | 0.426 | 0.095 | 0.205 | 0.261 | 0.295 | 0.391 | 0.242 | 0.343 |
| | *Chl a* | 0.449 | 0.303 | 0.002 | 0.376 | 0.589 | 0.558 | 0.204 | 0.573 |
| | Ci | 0.025 | 0.044 | 0.052 | 0.034 | 0.013 | 0.062 | 0.066 | 0.038 |
| | Calcium root | 0.466 | 0.037 | 0.160 | 0.215 | 0.162 | 0.165 | 0.455 | 0.001 |
| | Calcium shoot | 0.040 | 0.581 | 0.500 | 0.270 | 0.057 | 0.661 | 0.632 | 0.359 |
| | DW root | 0.033 | 0.168 | 0.169 | 0.101 | 0.045 | 0.192 | 0.187 | 0.118 |
| | DW shoot | 0.203 | 0.328 | 0.265 | 0.265 | 0.233 | 0.408 | 0.290 | 0.321 |
| | E | 0.401 | 0.711 | 0.449 | 0.556 | 0.480 | 0.779 | 0.608 | 0.629 |
| | Flavonoids | 1.162 | 1.517 | 1.510 | 1.339 | 1.132 | 1.108 | 1.111 | 1.120 |
| | Fv/Fm | 0.015 | 0.079 | 0.054 | 0.047 | 0.034 | 0.104 | 0.095 | 0.069 |
| | $g_s$ | 0.246 | 0.590 | 0.341 | 0.418 | 0.403 | 0.664 | 0.375 | 0.533 |
| | K root | 0.018 | 0.353 | 0.190 | 0.168 | 0.008 | 0.647 | 0.463 | 0.319 |
| | K shoot | 0.022 | 0.288 | 0.196 | 0.155 | 0.020 | 0.582 | 0.405 | 0.301 |
| | Length root | 0.083 | 0.176 | 0.176 | 0.130 | 0.209 | 0.200 | 0.236 | 0.205 |
| | Length shoot | 0.092 | 0.315 | 0.174 | 0.204 | 0.063 | 0.347 | 0.216 | 0.205 |
| | MDA | 0.924 | 4.958 | 0.646 | 2.941 | 3.236 | 5.808 | 2.738 | 4.522 |
| | Na/K root | 0.200 | 0.944 | 6.025 | 8.395 | 0.034 | 0.974 | 13.989 | 18.830 |
| | Na/K shoot | 0.008 | 0.971 | 13.586 | 17.027 | 0.110 | 0.988 | 28.756 | 42.800 |
| | Na root | 0.224 | 10.391 | 4.687 | 5.308 | 0.029 | 12.612 | 7.022 | 6.292 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Na shoot | 0.013 | 23.918 | 10.699 | 11.952 | 0.089 | 35.016 | 16.714 | 17.552 |
| | NPQ | 0.180 | 1.034 | 0.638 | 0.607 | 0.455 | 1.443 | 0.963 | 0.949 |
| | AOAM | 0.340 | 0.595 | 0.237 | 0.468 | 0.273 | 0.518 | 0.141 | 0.396 |
| | AOAD | 0.304 | 0.737 | 0.343 | 0.521 | 0.182 | 0.624 | 0.108 | 0.403 |
| | $\Psi s$ root | 0.177 | 0.286 | 0.204 | 0.231 | 0.247 | 0.314 | 0.253 | 0.281 |
| | $\Psi s$ shoot | 0.248 | 0.649 | 0.187 | 0.449 | 0.574 | 0.851 | 0.419 | 0.713 |
| | Polyphenols | 0.202 | 0.663 | 0.324 | 0.432 | 0.136 | 0.342 | 0.085 | 0.239 |
| | Proline | 2.299 | 7.498 | 4.451 | 4.898 | 1.406 | 5.475 | 3.365 | 3.441 |
| | PS2 | 0.044 | 0.088 | 0.038 | 0.066 | 0.083 | 0.133 | 0.080 | 0.108 |
| | qP | 0.026 | 0.072 | 0.041 | 0.049 | 0.040 | 0.108 | 0.096 | 0.074 |
| | Root/shoot | 0.214 | 0.237 | 0.130 | 0.226 | 0.246 | 0.365 | 0.144 | 0.306 |
| | Mg root | 0.042 | 0.292 | 0.223 | 0.167 | 0.085 | 0.376 | 0.269 | 0.230 |
| | Mg shoot | 0.172 | 0.454 | 0.350 | 0.313 | 0.163 | 0.554 | 0.438 | 0.359 |
| | Total sugars | 0.407 | 0.412 | 0.361 | 0.409 | 0.517 | 0.552 | 0.521 | 0.534 |
| | WC root | 0.047 | 0.054 | 0.081 | 0.050 | 0.035 | 0.074 | 0.076 | 0.054 |
| | WC shoot | 0.006 | 0.004 | 0.008 | 0.005 | 0.011 | 0.018 | 0.002 | 0.015 |
| | $\Delta^{13}C$ | 0.092 | 0.097 | 0.104 | 0.095 | 0.048 | 0.089 | 0.070 | 0.068 |
| | $\delta^{15}N$ | 0.402 | 0.629 | 0.074 | 0.113 | 0.624 | 0.210 | 0.309 | 0.207 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Elemental Composition Carbon (%C) | 0.006 | 0.071 | 0.016 | 0.039 | 0.026 | 0.074 | 0.028 | 0.050 |
| | Elemental Composition Nitrogen (%N) | 0.099 | 0.099 | 0.089 | 0.099 | 0.451 | 0.157 | 0.206 | 0.304 |

# Interchapter : Chapter 5 – Chapter 6

## What do we already know ?

The comparison between a salt resistant cultivar (TOG5307) and a salt-sensitive (TOG5949) cultivar of the African rice *Oryza glaberrima* confirmed that the ionic component of salt stress plays a major role in the plant response (see figure A below). For most recorded physiological parameters related to plant water status, mineral nutrition, oxidative stress and photosynthesis, $Na^+$ was more toxic than $Cl^-$ and the two considered ions acted on similar target according to an additive model. It was suggested that $Na^+/H^+$ antiporters (SOS1 and NHX) assume key functions in the salinity resistance of TOG5307 and that this cultivar is also able to display some salt-tolerance mechanisms allowing it to cope with high amounts of accumulated $Na^+$. It was suggested that cation-chloride-cotransporter (CCC) uses different cations in relation to $Cl^-$ co-transport in the two considered cultivar. Salinity was by far more detrimental than osmotic stress induced by PEG. The duration of salt stress also had a significant impact on all recorded parameters. Modification of mineral status may occur very quickly, suggesting that the osmotic component of salt stress in the biphasic model is rather short in this species. Beside a more efficient ionic regulation, carbon isotope discrimination also suggested that TOG5307 exhibited a higher water use efficiency than TOG5949 in the presence of NaCl.

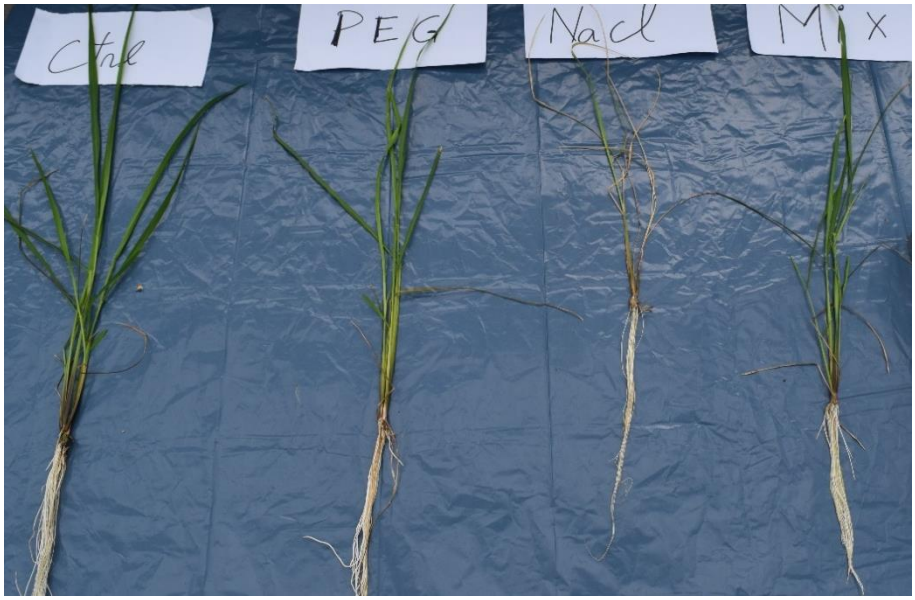



**Figure A** : African rice seedlings (*Oryza glaberrima* Steud.) cultivated in control conditions (Ctrl) or in the presence of PEG 8.6%, NaCl 50 mM or mix treatment (PEG 4.3% + 25 mM).

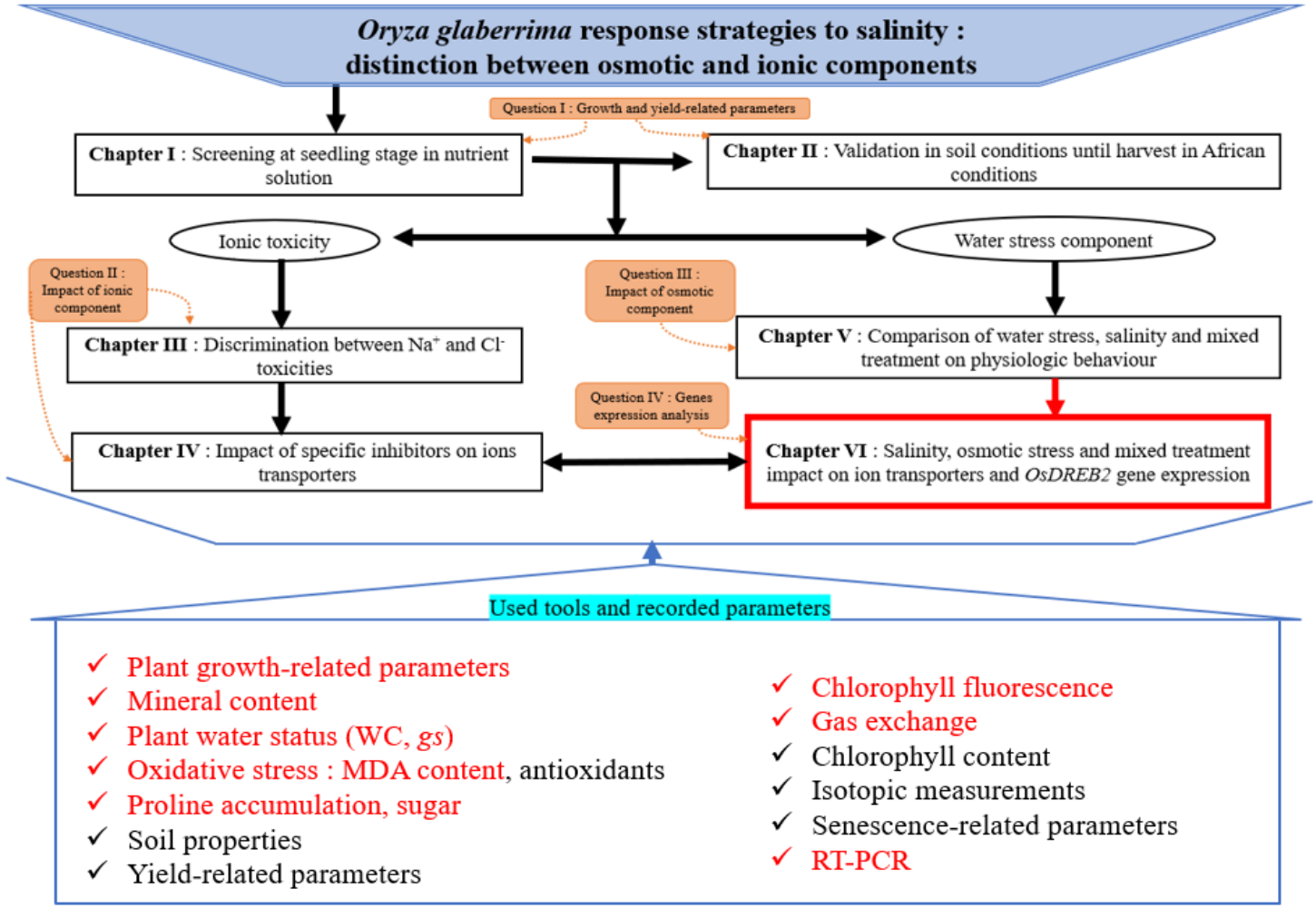
## What do we want to know ?

Previous data showed that the ionic component of salt stress is of paramount importance in the plant response of *O. glaberrima* to salinity. Indirect pharmacological approach also suggested that some specific transporters such as SOS1 or members of the NHX family assume key functions in salinity resistance of the African rice cultivar TOG5307. Beside $Na^+/H^+$ transporters, numerous members of the HKT family are thought to play major in salinity resistance since they are involved in $Na^+$ retrieval from the xylem sap to the phloem in order to avoid $Na^+$ accumulation in photosynthetic tissues through cycling. Other transporters may contribute to salinity resistance through chloride vacuolar sequestration (CLC ; NRT). It may be hypothesized that genes coding for these transporters could be regulated by salt stress. It could even not be excluded that osmotic stress had an impact on gene expression and could also impact the expression of the gene coding for desiccation responsive element (DREB2) which plays a major role in plant adaptation to water stress. The purpose of the next Chapter is to assess the impact of salt,

osmotic and mix treatments on the expression of genes coding for these transporters in the two considered cultivars of *Oryza glaberrima*.

## What is our strategy ?

*Oryza glaberrima* is close to the Asian rice species *Oryza sativa* : both species share the A genome of the genus *Oryza*. We will take advantage of this proximity to design adapted specific primers in order to analyze the expression of specific genes through an RT-PCR approach. Plants will be exposed to iso-osmotic solution ( $\Psi s$ = -0.47 MPa) containing PEG 12.9%, NaCl 75 mM or mix treatment (PEG 6.4% + NaCl 37.5 mM). Stress intensity will be slightly increased comparatively to Chapter V in order to allow a direct comparison with Chapter IV where specific inhibitors were used to reduce CCC and $Na^+/H^+$ antiporters activities. Plants will be harvested after 1 day and one week to assess the impact of stress duration on plant behaviour. Physiological parameters (plant growth, water content, osmotic potential, mineral nutrition, gas exchange, proline and sugar synthesis) will again be recorded to allow a comparison between gene expression and the whole plant behaviour for each cultivar.




**Overview of questions addressed during this thesis and their relationships with chapters.**

# Chapter VI. Comparison of the impact of osmotic, NaCl and mix treatments on the expression of genes coding for ion transporters in the African rice species *Oryza glaberrima* Steud.

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## Abstract

*Aims* Our goal was to analyze the expression of genes coding for $Na^+$ transporters, $Cl^-$ transporters and one gene coding for the transcription factor involved in plant response to desiccation in two cultivars of *O. glaberrrima* differing in salt-resistance and exposed to NaCl, PEG and to both agents present simultaneously.

*Methods* Seedlings from salt-resistant cultivar (TOG5307) and salt-sensitive (TOG5949) were grown in iso-osmotic nutrient solution ($\Psi s$ = -0.47±0.02 MPa) containing PEG6000 12.9% (water stress), NaCl 50 mM (salt stress) and PEG 6.4% + NaCl 37.5 mM (MIX-treatment) during 1 and 7 days. Plants were analyzed for molecular expressions, mineral nutrients and photosynthetic parameters. Studied genes were *OsHKT1.5*, *OsHKT4*, *OsSOS1*, *OsSOS2*, *OsNHX1*, *OsNHX2*, *OsNRT1*, *OsCLC*, *OsCCC1* and *OsDREB2*.

*Results* Treatments did not reduce root water content and the relative growth rate of number of leaves for the two tested cultivars. The photosynthetic parameters were not affected by stresses after 1 day. Chloride and sodium contents increased in response to NaCl and Mix treatment for both roots and shoots. The NaCl and MIX treatment induced a decrease in $K^+$ concentration. The shoot MDA, proline and soluble sugar concentration did not change after 1 day of treatment but they drastically increased in stressed plants after 7 days. *OsDREB2* was highly induced in both shoot and roots after 7 days. *OsCCC1* and *OsCLC* were highly induced in roots. *OsNRT1* was induced to a higher extent in shoots than in roots. *OsHKT1.5* was slightly induced the shoot. *OsHKT4* was recorded in the shoots but remained undetectable in the roots. *OsNHX1* and *OsNHX2* were obviously more expressed in the shoots than in the roots. *OsSOS1* and *OsSOS2* expression were increased in both shoots and roots.

*Conclusion* Overall, the present study indicated that there is a significant variation in salinity tolerance in *Oryza glaberrima*. Both studied cultivars greatly differed by the quantitative aspect of genes expression.


**Keyword:** Salinity, *Oryza glaberrrima*, transporters, RT-PCR.

# Introduction

Salinity is a major environmental constraint hampering plant growth and development. It has a major deleterious impact on cultivated plant yields and compromises food security in numerous areas of the world (Nagrao et al., 2017). This is especially the case for rice (*Oryza sativa* L.) which is a very salt-sensitive species, mainly at the seedling and at the flowering stage (Lutts et al., 1995). Rice is among the most important plant for human nutrition and it is now well accepted that its culture should be extended to marginal lands which are not currently suitable for rice production. A major portion of these lands are affected by drought and/or by salinity so that the improvement of salinity resistance in this species is a priority (Qadir et al., 2014).

Beside the cultivated rice species *Oryza sativa* L, wild relative may provide useful genes to improve salinity resistance and interspecific crosses and cultivar selection is a long-term task and require a great deal of efforts (Fort el al., 2014). *Oryza glaberrima* Steud. is an African rice species which is marginally cultivated in Africa but exhibits interesting abilities to cope with environmental constraints (Linares 2002; Wambugu et al. 2021). Although some information are currently available for its adaptation to soil drying (Kartika et al., 2020; Kang and Futakuchi 2019) or to iron toxicity (Majerus et al., 2007; Mayabe et al., 2020), its level of resistance to salinity remains poorly documented and require additional investigations.

An excess of soluble salts in the environment may act on almost all aspects of plant physiology: It compromises the plant water status, affects plant mineral nutrition and photosynthesis, modify the hormonal profile and oxidative status (Morton et al., 2019). It mainly acts through an osmotic component related to a decrease in the external water potential and through the progressive accumulation of toxic ions (Munns and Tester 2008). Nevertheless, the respective importance of the two types of constraints is still a matter of debate (Munns and Tester 2008; Morton et al., 2019; Nagrao et al., 2017). Any attempt of generalization should however be considered with caution. The relative importance of ionic and osmotic components depends on the plant species, the used NaCl dose, the plant developmental stage and the kinetics of salt application (Almansouri et al., 1999).

Moreover, osmotic and ionic components should not necessarily be regarded as independent additive constraints even if they act on similar properties

through distinct mechanisms. It is often considered that only halophyte plant species are able to accumulate $Na^+$ and $Cl^-$ ions in their tissue while salt-resistant glycophyte adopt an excluding strategy limiting $Na^+$ and $Cl^-$ absorption and avoiding root-to-shoot translocation of these toxic ions in order to preserve photosynthetic tissues (Maathuis et al., 2014). It has however been hypothesized that on a short term basis, these ions may help the plant to perform osmotic adjustment before the plant acquire the capacity to synthesize enough osmocompatible solutes like proline or soluble sugars (Munns and Gilliham 2015; Munns et al. 2020). This property also partly depends on ion distribution at the cell level since $Na^+$ is considered to be highly toxic to numerous cytosolic enzymes and needs to be stored within vacuoles (Almeida et al., 2017).

Ion absorption and distribution are regulated by a complex network of membrane transporters, channels and pump mediating $Na^+$ and $K^+$ homeostasis. $Na^+$ enters via non selective cation channels (NSCC) and possibly via other cation transporters (Hasegawa 2013). The $Na^+/H^+$ antiporter SOS1 extrudes $Na^+$ at the root soil interface (Brini and Masmoudi 2012; Almeida et al. 2017). The corresponding gene is expressed on xylem/parenchyma cell interface and SOS1 is also reported to be involved in $Na^+$ loading at the xylem. More recently, however, Zhao et al. (2020) suggested that it could act in the opposite direction and plays a role in $Na^+$ retrieval from xylem sap. SOS1 itself is regulated in close interaction with SOS2 and SOS3 proteins and the SOS system plays a key role in salt tolerance in several angiosperm plant species (Roy and Chakraborty 2018). The vacuolar sequestration of $Na^+$ is under the control of NHX transporters which mediate the electroneutral $Na^+/H^+$ exchange, driving the excess of cytosolic $Na^+$ into the vacuole (Zeng et al., 2018). Numerous NHX exist in plant species and their functions cannot be solely explained by $Na^+$ accumulation in vacuoles (Reguera et al., 2014).

Other transporters called HKT are involved in $Na^+$ unloading from the xylem vessels. This transporter family may act not only at the xylem loading site itself, but may also transfer $Na^+$ from the xylem to the phloem, thus avoiding $Na^+$ accumulation in aerial parts through $Na^+$ cycling (Davenport et al., 2007; Jaime-Pérez et al., 2017). Ren et al. (2005) showed that *OsHKT1;5* plays a key role in the salt resistance of *O. sativa* cv Nona Bokra and code for a plasma membrane transporter. Platten et al. (2013) demonstrated that the similar gene *HKT1;5* also plays a key role in *O. glaberrima* and contributes to reduce $Na^+$ concentration in the leaf blades of the African rice species.

Chloride accumulation received far less attention than $Na^+$ content in studies devoted to salt-resistance in plants. Chloride is an essential element required for photosynthesis, act as a counter anion to stabilize membrane potential and is involved in turgor and pH regulation but becomes rapidly toxic if it accumulated at high concentration (Chen et al., 2016; Flowers, 1988; Kobayashi et al., 2006; Marschner, 2011; Teakle and Tyerman, 2010).

Prodjinoto et al. (2021a) demonstrated that $Na^+$ is more toxic than $Cl^-$ for the African rice species. The pattern of modification induced by both types of ions was similar but the quantitative aspect of the response strongly differs between a salt-resistant cv TOG5307 and a salt-sensitive one (TOG5949). In an additional study, Prodjinoto et al. (2021b) used inhibitors of $Na^+$-$H^+$ antiporter (amiloride 100 µM) and cation-chloride-cotransporter (CCC) (bumetanide 200 µM): the salt-resistant cv TOG5307 more efficiently regulate the $Na^+$ uptake while bumetanide reduced $Cl^-$ accumulation in both cultivars, although the accompanying cation may differ between them. Moreover, Prodjinoto et al. (2021c) compared the response of these cultivars when exposed to NaCl and PEG (a non-penetrating osmotic solute): when both agents were simultaneously present, osmotic and ionic component of salt stress acted in an additive way on the salt-sensitive cv but not in the salt-resistant one where an antagonist action between the two stressor was recorded for several parameters.

These observations prompted us to analyze the expression of genes coding for $Na^+$ transporters (*OsHKT1.5*, *OsHKT4*, *OsSOS1*, *OsSOS2*, Os*NHX1*, *OsNHX2*), $Cl^-$ transporters (*OsNRT1*, *OsCLC*, *OsCCC1*) and one gene coding for the transcription factor DREB which is thought to be involved in plant response to desiccation in two cultivars of *O. glaberrima* differing in salt-resistance and exposed to NaCl, PEG and to both agents present simultaneously.

## Material and Methods

### *Plant culture and growing conditions*

Seeds of African rice cultivars, TOG5307 (salt-tolerant, accession number WAB0021855) and TOG5949 (salt-sensitive; accession number

WAB0020144), were obtained from AfricaRice (Bouaké, Ivoiry coast). Non-dehusked seeds were germinated on two layers of Whatman papers (85 mm, Grade 1) moistened with sterile deionized water in a growth chamber at 25 °C and under a 12 hr daylight period. Ten-days-old equally grown seedlings were transferred to hydroponic culture system into phytotron at 29/26 °C under 16/8 h day/night period. Light intensity was 300 µmol $m^{-2}$ $s^{-1}$ (HPIT/400W from PHILIPS metal iodide lamp) and relative humidity was 70±5%. Seedlings were fixed on polystyrene plates floating on 16 L of Yoshida nutritive solution (Yoshida et al., 1976). After 7 days of acclimatization in control conditions, the seedlings were randomly divided into four groups:(1) control: plants grown in Yoshida nutritive solution (EC = 0.71±0.009 $mS.cm^{-1}$ ; $\Psi s$= -0.08±0.003 MPa), (2) PEG: plants grown in Yoshida nutritive solution + 12.9% of PEG 6,000 (EC = 0.73±0.003 $mS.cm^{-1}$ ; $\Psi s$= -0.47±0.02 MPa), (3) NaCl: plants grown in Yoshida nutritive solution containing 75 mM NaCl, ((EC = 7.72±0.08 $mS.cm^{-1}$ ; $\Psi s$= -0.47±0.02 MPa) (4) Mix: plants grown in Yoshida nutritive solution containing 37.5 mM NaCl + 6.4% of PEG 6,000 (EC = 4.10±0.02 $mS.cm^{-1}$; $\Psi s$= -0.45±0.06 MPa). This implies that all stressing solution have the same osmotic potential but exhibit different electrical conductivities. The second actively growing leaf (numbering from the top of the plant), present at the moment of treatment application, was tagged for subsequent physiological measurements. After 1 day and 7 days of treatment, the plants were harvested and divided into shoots and roots for physiological, biochemical et molecular analysis. Roots were rinsed for 30 s in deionized water to remove ions from the free spaces.

***Plant growth, water content, osmotic potential, and ions determination***

On the one hand, plant growth was determined on the basis of relative growth rate (RGR) of the number of leaves, and the length of shoot and root basis according to the formula; $RGR = [(X_f - X_i)/X_i]$, where $X_f$ and $X_i$ are the final and initial X measurement. On the other hand, shoot and root fresh weight (FW) and dry weight (DW) were estimated on six individual plants per treatment. For DW determination, roots and shoots were incubated in an oven at 70 °C for 72 h until reaching constant dry weight. Water content (WC) was calculated as WC=(FW–DW)/FW * 100.

Osmotic potential ($\Psi s$) was estimated on the extracted sap using a Wescor 5600 vapor pressure osmometer as previously described (Lutts et al. 1999). For the determination of $Cl^-$ concentration, 20 mg DW were digested with nitric acid (0.5%) and the mixture was stirred for 48 hours. The supernatant recovered after centrifugation at 10,000 g for 10 minutes at 25 °C was subsequently filtered with Wathman paper and then quantified via liquid chromatography (HPLC-Dionex ICS2000). For $Na^+$ and $K^+$ quantification, 50 mg of DW were incubated in 4ml of 35% $HNO_3$ at 80 °C. The residue was redissolved with aqua regia (HCl 37%: HNO3 65%, 3:1) and filtered Whatman paper. Elements were quantified by flame atomic absorption spectrophotometry (ICE 3300; Thermo Scientific, Waltham, MA). All measurements were performed using technical triplicates per sample.

***Gas-exchange parameters, stomatal conductance***

The second actively growing leaf (numbering from the top of the plant) was used for photosynthesis-related parameters estimation. The instantaneous $CO_2$ assimilation under ambient conditions (400 ppm $CO_2$) (*A*), the instantaneous transpiration (*E*) and inter-cellular $CO_2$ concentration (*C*i) were taken from the second fully expanded leaf using an Infrared Gas Analyzer (LCA4 8.7 ADC, Bioscience, Hertfordshire, UK). The gas exchange was measured using a Parkinson leaf cuvette, on intact leaves for 1 min (20 records $min^{-1}$) with an air flow of 300 mL $min^{-1}$. The chlorophyll fluorescence was determined using a portable pulse-modulated chlorophyll fluorimeter (FMS2, Hansatech, King's Lynn, UK). Using both light and dark fluorescence parameters, the actual PSII efficiency ($\Phi_{PSII}$), the photochemical quenching coefficient ($q_P$), the non-photochemical quenching (NPQ) and the maximal efficiency of PSII photochemistry in the dark-adapted state ($F_v/F_m$) were assessed as previously described (Maxwell and Johnson 2000). Leaf stomatal conductance ($g_s$) was measured on the second fully expanded leaf using an AP4 diffusion porometer (Delta-TDevices Ltd., Cambridge, UK). All measurements were performed on leaves of 8 plants per treatment between 10 a.m. and 2 p.m.

***Malondialdehyde, proline, and total sugars quantification***

Malonyldialdehyde was extracted from shoot by the thiobarbituric acid reaction as reported in Heath and Packer (1968). For each sample, c.a. 250 mg of FW were ground in liquid nitrogen. A solution of 1.25% glycerol and 5% trichloroacetic acid was added to the ground. Then, samples were centrifugated at 4 °C during 10 min at 6,700 g. Solutions were filtered with one layer of Whatman paper (8 mm, Grade 1) and 2 mL of the filtrate were mixed with 2 mL of 0.67% thiobarbituric acid. Samples were incubated in at 100 °C during 30 min and then 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 $mM^{-1}$ $cm^{-1}$ used to calculate MDA concentration.

Proline was quantified spectrophotometrically on shoot using the ninhydrin method according to Bates et al. (1973). For each sample, c. a. 200 mg FW of shoots were ground in liquid nitrogen in a mortar containing 10 mL of 3% sulfosalicylic acid. Samples were then 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 (sigmaAldrich) as controls.

Total soluble sugars were quantified in shoot using anthrone method as previously described by Yemm and Willis (1954): c.a. 300 mg FW of leaves ground in liquid nitrogen. The obtained powder were mixed with 7 mL of ethanol 70% (w/v) for 5 min on ice and centrifugated at 4 °C at 8000 g during 20 min. Then, 200 µL of the supernatant were put in reaction with 1 mL of anthrone solution (0.5 anthrone, 250 mL 95% $H_2SO_4$ and 12.5 mL distilled water). A calibration curve was established using glucose as standard and the absorbance was read at 625 nm.

***RNA Extraction, Reverse transcription-PCR (RT-PCR), and gene expression analysis***

At harvest time, shoot and root of both African rice cultivars were separated and quickly frozen in liquid nitrogen (3 plants were pooled per organ, cultivar and treatment). Total RNA was isolated from 100mg of frozen material per sample using Trizol reagent protocol (Roche, Germany). The RNA quality and concentration were estimated using NanoDrop ND-1000 (Isogen Life Science, De Meern, The Netherlands). DNase treatments were realized using RQ1 RNase-free DNase (Promega, Leiden, The Netherlands) according to the manufacturer's instructions. Reverse transcription was performed with 1 µg of total RNA using the GoScriptTM Reverse Transcription Mix, Oligo (dT) Protocol Kit (Promega Benelux b.v., Leiden, The Netherlands) by following the manufacturer's instructions.

Then, six independent PCR amplifications were conducted for each gene using primer pairs, annealing temperatures, and number of cycles presented in table 1. Na transporter genes were selected from the list used by Irakoze (2020) while chloride transporter genes were identified based on the work of Nakamura et al. (2006) and Teakle and Tyerman (2010). Primers of each studied gene were designed using Primer 3 software (Rozen and Skaletsky 2000). Amplifications were conducted using GoTaq DNA polymerase (Promega Benelux b.v., Leiden, The Netherlands). The PCR products were resolved on agarose gels and expression differences were analyzed by gel densitometry using ImageJ software and expressed as relative values compared to *OsEF1α* expression (peak size of target gene/peak size of *OsEF1α*) (Quinet et al., 2014). For a given sample, gene expression analyses were repeated six times and gave similar results.

**Table 1.** List of primers of *OsHKT1.5*, *OsHKT4*, *OsSOS1*, *OsSOS2*, OsNHX1, *OsNHX2*, *OsNRT1*, *OsCLC*, *OsCCC1*, *OsDREB2*, *OsEF1α* and amplification conditions used for semi-quantitative RT-PCR expression analysis.

| Gene | NCBI accession | Primer | sequence | Tm (°C) | Size (bp) | number of cycle |
|---|---|---|---|---|---|---|
| *OsHKT1.5* | | Forward | 5’-CCTGCCACCTTACACCACTT-3’ | 57 | 198 | 38 |
| | | Reverse | 5’-GCTGTAGTTGATGGGGTCGT-3’ | | | |
| *OsHKT4* | | Forward | 5’-GGAAGTCAATTTCTTCTGATCCA-3’ | 58 | 239 | 39 |
| | | Reverse | 5’-TCATTTCAGGATGAACTCCTTG-3’ | | | |
| *OsSOS1* | | Forward | 5'-CTCCGTGCTCATAGAATCGC-3' | 58 | 207 | 38 |
| | | Reverse | 5'-ATACTCACTCAAGTGGGTCAATACC-3' | | | |
| *OsSOS2* | | Forward | 5′-ATGAAGGTGCTCGACAAGGA-3’ | 58 | 168 | 38 |
| | | Reverse | 5′-GCCTCCAGTGATAAGCTCCA-3′ | | | |
| *OsNHX1* | | Forward | 5'-GTTCAAGAGTTACAACAAAGCACG-3' | 58 | 200 | 38 |
| | | Reverse | 5'-CAGCGGGAATACAAAAGCAG-3' | | | |
| *OsNHX2* | | Forward | 5'-ACCAAGACGAAACACCCCTAC-3' | 57 | 196 | 38 |
| | | Reverse | 5'-AACCCAGCAACTACTCCAAGAA-3' | | | |
| *OsNRT1* | | Forward | 5’-TGGAGAAACACATCGGCTCT-3’ | 58 | 213 | 37 |
| | | Reverse | 5’-CCACCAATGCTGCTGATACC-3’ | | | |
| *OsCLC* | | Forward | 5'-AGTTCGTCGTCACCTCCAAT-3' | 57 | 174 | 36 |

| | | | | | |
|---|---|---|---|---|---|
| | Reverse | 5'-CAACCCCGTTCAAGTAAGCC-3' | | | |
| *OsCCC1* | Forward | 5’-TCTATTTGGAGCCTTGGCCA-3’ | 57 | 162 | 37 |
| | Reverse | 5’-GCTGCCAGTAATCTTGGAGC-3’ | | | |
| *OsDREB2* | Forward | 5'-GGTGGAAGGAGCAAAACCAG-3' | 57 | 181 | 38 |
| | Reverse | 5'-CGGTTTGGTTCACGGATCTC-3' | | | |
| *OsEF1α* | Forward | 5'-TCCTCAAGAACGGTGATGCT-3' | 56 | 164 | 33 |
| | Reverse | 5'-GGTTGGGTCCTTCTTCTCCA-3' | | | |

***Statistical treatment***

Each experiment was repeated three times and led to statistically similar results. All the data in this research were calculated with JMP Pro 14 software. Normality distributions and homoscedasticity were verified using Shapiro-Wilk and Levene's tests respectively and data were transformed when required. Data were analyzed using two-way or three-away analysis of variance (three-way ANOVA). At $P < 0.05$ a post-hoc test Tukey's HSD all-pairwise comparisons) were used to compare means.

## Results

***Plant growth, water status and photosynthetic parameters***

Ten-days-old equally grown seedlings were exposed to isosmotic solutions ($\Psi s$ = -0.47±0.02 MPa) of NaCl, PEG or MIX treatment (PEG+NaCl) for 1 day and 7 days. As show in Table 2, after 7 days of stress, treatments did not reduce root water content and the relative growth rate (RGR) of number of leaves for the two tested cultivars. NaCl stress however significantly decreased the shoot WC in TOG5307. The RGR of length and the dry weight for both shoot and root were significantly decreased in the presence of stresses. The lowest values were recorded during NaCl treatment followed by Mix and PEG. As far as cultivars are concerned, TOG5949 was more affected than TOG5307 (Table 5).

**Table 2.** Impact of iso-osmotic solutions of PEG 12.9%, NaCl 75 mM or mix treatment (PEG 6.4% + NaCl 37.5 mM) applied for 1 or 7 days on relative growth rate of number of leaves, relative growth of length of root and shoot, dry weight of root and shoot, and water content of root and shoot of two contrasting cultivars of African rice (*Oryza glaberrima* Steud. cv TOG5307 (salt-resistant) and TOG5949 (salt-sensitive)). For a particular cultivar, values followed by similar lower case (1 day) or uppercase (7 days) letters are not statistically different according to Tukey post hoc test.

| | | | Number of leaves-RGR | Length -RGR | | Dry weight | | Water content | |
|---|---|---|---|---|---|---|---|---|---|
| Varieties | Treatment | Moment | | Shoot | Root | Shoot | Root | Shoot | Root |
| TOG5307 | Control | 7 days | 0.40 ± 0.06 a | 0.88 ± 0.05 a | 1.18 ± 0.04 a | 0.05 ± 0.003 a | 0.02 ± 0.001 a | 89.07 ± 0.94 a | 81.32 ± 1.57 a |
| TOG5949 | | | 0.17 ± 0.07 a | 0.91 ± 0.01 a | 1.09 ± 0.07 a | 0.05 ± 0.002 a | 0.01 ± 0.001 a | 84.86 ± 0.82 a | 75.29 ± 6.52 a |
| TOG5307 | PEG | | 0.33 ± 0.07 a | 0.89 ± 0.04 a | 0.64 ± 0.09 b | 0.04 ± 0.002 b | 0.02 ± 0.001 a | 90.14 ± 0.52 a | 75.32 ± 0.68 a |
| TOG5949 | | | 0.17 ± 0.07 a | 0.86 ± 0.04 a | 0.34 ± 0.09 b | 0.03 ± 0.003 b | 0.01 ± 0.001 a | 87.24 ± 1.25 a | 73.74 ± 1.55 a |
| TOG5307 | NaCl | | 0.27 ± 0.03 a | 0.31 ± 0.04 b | 1.22 ± 0.06 a | 0.03 ± 0.001 b | 0.01 ± 0.001 b | 75.82 ± 1.81 b | 87.75 ± 8.99 a |
| TOG5949 | | | 0.13 ± 0.03 a | 0.18 ± 0.03 b | 0.40 ± 0.09 b | 0.02 ± 0.002 c | 0.01 ± 0.001 b | 83.64 ± 0.92 a | 84.65 ± 7.94 a |
| TOG5307 | MIX | | 0.33 ± 0.07 a | 0.75 ± 0.04 a | 0.90 ± 0.1 ab | 0.03 ± 0.003 b | 0.01 ± 0.001 b | 85 ± 1.26 a | 85.28 ± 1.39 a |
| TOG5949 | | | 0.13 ± 0.03 a | 0.27 ± 0.05 b | 0.65 ± 0.05 b | 0.02 ± 0.002 c | 0.01 ± 0.001 b | 85.72 ± 0.97 a | 83.25 ± 2.37 a |

After one day of treatment, the maximal efficiency of PSII photochemistry in the dark-adapted state ($F_v/F_m$) (Table 3), the actual PSII efficiency ($\Phi_{PSII}$) and the photochemical quenching coefficient (qP) were not affected by stresses while the non-photochemical quenching (NPQ) was higher in PEG treatment than in controls. After 7 days, however, $F_v/F_m$, was significantly reduced in the two cultivars exposed to NaCl, and to a higher extent in TOG5949 than in TOG5307. Photochemical quenching (qP) was reduced in response to all stresses in TOG5949 and in response to NaCl only in TOG5307 (Table 3). Table 5 confirms that photosynthetic-related parameters ($A$, $E$, $g_s$, $\Phi_{PSII}$, $F_v/F_m$, $q_P$, and NPQ) varied significantly according to cultivars, to treatments and to duration.

The $\Psi$s was lower in shoots than in roots whatever the duration (Table 4). In the absence of stress, both cvs exhibited similar $\Psi$s values. The $\Psi$s was also lower after 7 days than after 1 day whatever the treatment and cultivar. After 1 day, the $\Psi$s of shoots decreased in NaCl-stressed plants for TOG5307, whereas it decreased in response to all stresses in plants of TOG5949. After 7 days, the $\Psi_s$ of stressed shoots of TOG5949 was lower than the $\Psi_s$ of stressed shoot of TOG5307. The $\Psi_s$ varied significantly according to cultivars, to treatments and to duration (Table 5). For both cvs, the lowest $\Psi$s value was recorded in NaCl-stressed plants. As far as roots are concerned the $\Psi$s only slightly decreased in response to the stress treatment was slightly but significantly (Table 5) lower in TOG5949 than in TOG5307.

Net photosynthesis ($A$) increased in TOG5307 plants submitted at PEG treatment after 1 day, it significantly decreased in response to NaCl and MIX treatment in both cvs. after this duration. At this moment, the MIX treatment appeared as the most deleterious one. $A$ values markedly decreased after 7 days of stress and exhibited the lowest value in response to NaCl stress: for this treatment as well as for the MIX treatment, $A$ was obviously higher in TOG5307 than in TOG5949.

Instantaneous transpiration rate ($E$) was marginally reduced in NaCl and MIX stressed plants after 1 day. In contrast, after 7 days it was dramatically reduced in all stressed plants (Table 4), reaching once again the minimal value in response to NaCl; $E$ values were also significantly higher in TOG5307 than in TOG5949.

Stomatal conductance was strongly reduced in TOG5949 already after 1 day of exposure (Table 4). As far as TOG5307 is concerned, the recorded decrease was important in salt-treated plants, while for PEG and MIX treatments,

values remained clearly higher than for TOG5949. A similar trend was observed after 7 days, but the $g_s$ value was lower than after 1 day, even in control plants.

**Table 3.** Impact of iso-osmotic solutions of PEG 12.9%, NaCl 75 mM or mix treatment (PEG 6.4% + NaCl 37.5 mM) applied for 1 or 7 days on maximal efficiency of PSII photochemistry in the dark-adapted state ($F_v/F_m$), actual PSII efficiency ($\Phi_{PSII}$), photochemical quenching ($q_P$) and non-photochemical quenching (NPQ) of two contrasting cultivars of African rice (*Oryza glaberrima* Steud. cv TOG5307 (salt-resistant) and TOG5949 (salt-sensitive)). For a particular cultivar, values followed by similar lower case (1 day) or uppercase (7 days) letters are not statistically different according to Tukey post hoc test.

| Varieties | Treatment | Duration | $F_v/F_m$ | $\Phi_{PSII}$ | qP | NPQ |
|---|---|---|---|---|---|---|
| TOG5307 | Control | 1 day | 0.87 ± 0.004 a | 0.78 ± 0.007 a | 0.92 ± 0.014 a | 0.25 ± 0.008 b |
| TOG5949 | | | 0.85 ± 0.008 a | 0.78 ± 0.006 a | 0.92 ± 0.009 a | 0.25 ± 0.007 b |
| TOG5307 | PEG | | 0.86 ± 0.002 a | 0.82 ± 0.010 a | 0.96 ± 0.009 a | 0.28 ± 0.008 a |
| TOG5949 | | | 0.84 ± 0.004 a | 0.79 ± 0.011 a | 0.93 ± 0.015 a | 0.34 ± 0.007 a |
| TOG5307 | NaCl | | 0.87 ± 0.005 a | 0.81 ± 0.005 a | 0.92 ± 0.010 a | 0.21 ± 0.009 bc |
| TOG5949 | | | 0.87 ± 0.006 a | 0.78 ± 0.004 a | 0.92 ± 0.006 a | 0.24 ± 0.005 b |
| TOG5307 | MIX | | 0.87 ± 0.006 a | 0.81 ± 0.003 a | 0.93 ± 0.005 a | 0.20 ± 0.004 c |
| TOG5949 | | | 0.87 ± 0.005 a | 0.81 ± 0.004 a | 0.92 ± 0.013 a | 0.26 ± 0.010 b |
| TOG5307 | Control | 7 days | 0.86 ± 0.003 A | 0.78 ± 0.009 A | 0.92 ± 0.006 A | 0.21 ± 0.012 C |

| | | | | | | |
|---|---|---|---|---|---|---|
| TOG5949 | | | 0.86 ± 0.005 A | 0.78 ± 0.008 A | 0.92 ± 0.003 A | 0.21 ± 0.010 D |
| TOG5307 | PEG | | 0.84 ± 0.005 A | 0.76 ± 0.011 AB | 0.89 ± 0.006 A | 0.25 ± 0.008 C |
| TOG5949 | | | 0.81 ± 0.004 B | 0.73 ± 0.015 B | 0.88 ± 0.006 B | 0.32 ± 0.007 C |
| TOG5307 | NaCl | | 0.79 ± 0.002 C | 0.71 ± 0.004 B | 0.85 ± 0.016 B | 0.44 ± 0.009 A |
| TOG5949 | | | 0.77 ± 0.004 C | 0.69 ± 0.002 B | 0.81 ± 0.003 C | 0.53 ± 0.013 A |
| TOG5307 | MIX | | 0.81 ± 0.003 B | 0.75 ± 0.012 AB | 0.87 ± 0.004 AB | 0.36 ± 0.009 B |
| TOG5949 | | | 0.77 ± 0.002 C | 0.73 ± 0.010 B | 0.82 ± 0.008 C | 0.42 ± 0.013 B |

***Mineral contents***

Chloride contents (Figure. 1A) increased in response to NaCl and Mix treatment for both roots and shoots. TOG5949 exhibited a higher $Cl^-$ concentration than TOG5307. Moreover, it has to be noticed that $Cl^-$ accumulation was already significant after 1 day of stress exposure and that $Cl^-$ accumulation recorded for the MIX treatment remained limited: after 7 days of exposure, root and shoot $Cl^-$ concentration recorded for the MIX treatment was similar to control plants for both *cvs*.

The NaCl and MIX treatments also induced a strong increase in shoot and root $Na^+$ content (Figure 1.B) and Na:K ratio (Figure 1. D). Throughout the experiment, TOG5307 accumulated less sodium than TOG5949. Once again,

$Na^+$ accumulation after 7 days of treatment was not significantly affected by the MIX treatment at the root level.

The NaCl and MIX treatment induced a decrease in $K^+$ concentration (Figure 1.C). After 1 day, the recorded decrease was similar in NaCl and MIX treatments for both roots and shoots and for the two tested cultivars. A similar trend was recorded after 7 days, except that the $K^+$ concentration significantly decreased in response to PEG in TOG5307 and that the $K^+$ decrease recorded at the shoot level for this cv was higher for MIX than for NaCl treatment.

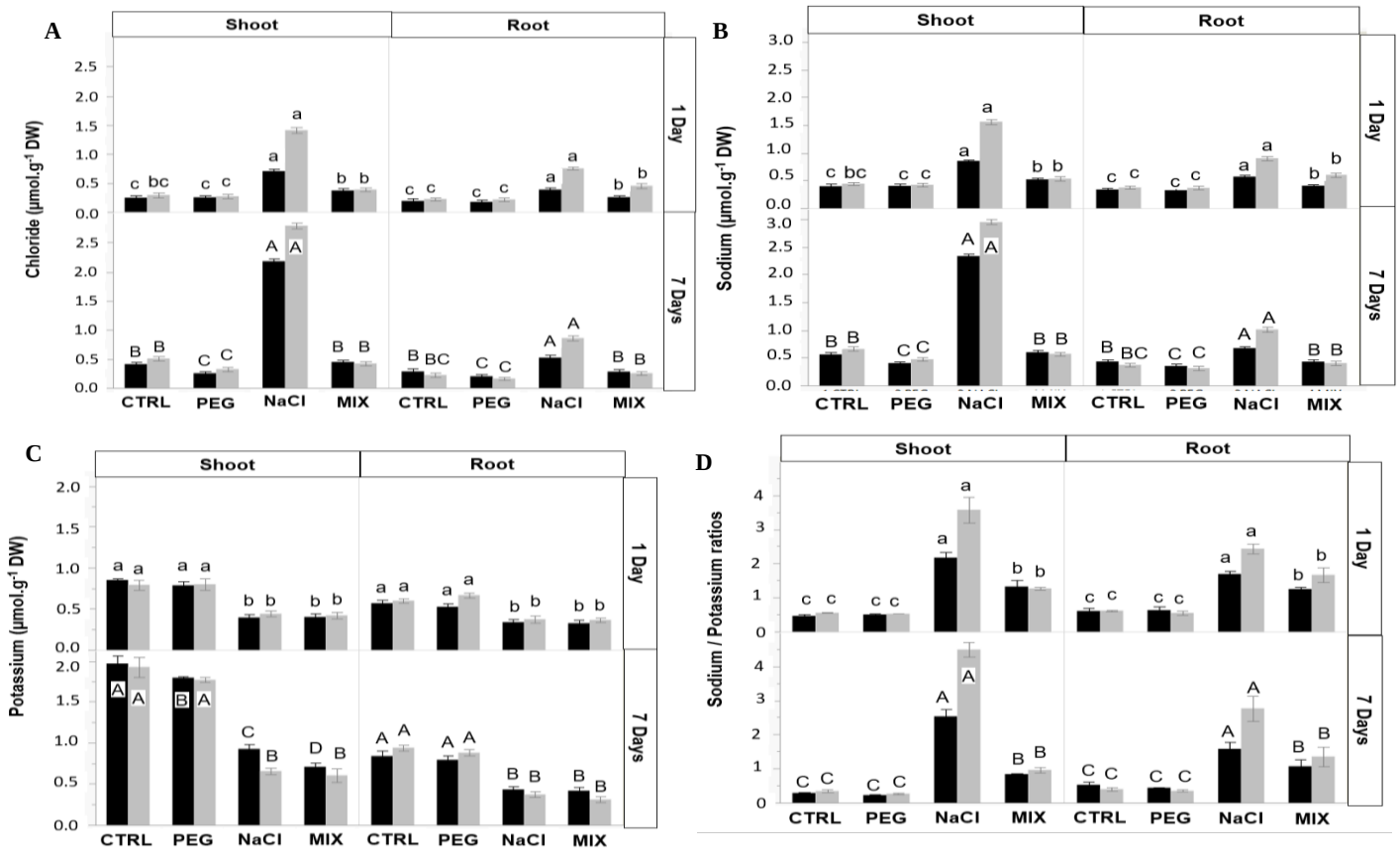


**Figure 1.** (A) chloride, (B) sodium, (C) potassium concentrations, and (D) Na:K ratio of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 cultivated during 1 day and 7 days in control conditions (CTRL) or in the presence of 75 mM of either NaCl, PEG-6000 or MIX (PEG +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 latter (1 day) or the same uppercase (7 days) for a particular cultivar do not differ statistically.

**Table 4.** Impact of iso-osmotic solutions of PEG 12.9%, NaCl 75 mM or mix treatment (PEG 6.4% + NaCl 37.5 mM) applied for 1 or 7 days on osmotic potential, net photosynthesis, transpiration, stomatic conductance, malondialdehyde, proline, and total sugars concentration of two contrasting cultivars of African rice (*Oryza glaberrima* Steud. cv TOG5307 (salt-resistant) and TOG5949 (salt-sensitive)). For a particular cultivar, values followed by similar lower case (1 day) or uppercase (7 days) letters are not statistically different according to Tukey post hoc test.

| Varieties | Treatment | Duration | A (µmol $CO_2$ $m^{-2}$ $s^{-1}$) | E (mmol $m^{-2}$ $s^{-1}$) | $g_s$ (mmol $H_2O$ $m^{-2}$ $s^{-1}$) | Ψs.shoot (MPa) | Ψs.root (MPa) | MDA (mmol $g^{-1}$ FW) | Proline (µmol $g^{-1}$ FW) | Total sugars (mg $g^{-1}$ FW) |
|---|---|---|---|---|---|---|---|---|---|---|
| TOG5307 | Control | 1 day | 3.87 ± 0.07 b | 3.50 ± 0.10 a | 697.00 ± 11.55 a | -1.26 ± 0.03 a | -0.65 ± 0.03 a | 1.54 ± 0.07 a | 1.83 ± 0.08 d | 9.43 ± 0.30 ab |
| TOG5949 | | | 3.98 ± 0.12 a | 3.43 ± 0.09 a | 688.00 ± 13.20 a | -1.26 ± 0.03 a | -0.66 ± 0.02 a | 1.47 ± 0.09 b | 1.95 ± 0.11 c | 9.58 ± 0.27 c |
| TOG5307 | PEG | | 4.93 ± 0.12 a | 3.49 ± 0.10 a | 580.33 ± 6.67 c | -1.30 ± 0.01 a | -0.82 ± 0.02 b | 1.64 ± 0.08 a | 3.85 ± 0.08 a | 9.63 ± 0.16 ab |
| TOG5949 | | | 3.89 ± 0.09 a | 3.30 ± 0.11 ab | 237.33 ± 8.19 c | -1.61 ± 0.03 b | -0.89 ± 0.02 c | 1.71 ± 0.05 a | 2.64 ± 0.18 b | 10.51 ± 0.58 c |
| TOG5307 | NaCl | | 3.06 ± 0.11 c | 3.18 ± 0.09 b | 317.00 ± 5.00 d | -1.52 ± 0.04 b | -0.81 ± 0.03 b | 1.63 ± 0.04 a | 3.18 ± 0.06 c | 9.89 ± 0.15 a |
| TOG5949 | | | 2.94 ± 0.08 b | 2.99 ± 0.09 bc | 282.33 ± 6.67 b | -1.66 ± 0.03 b | -0.90 ± 0.01 c | 1.65 ± 0.07 a | 3.14 ± 0.08 a | 11.32 ± 0.52 a |
| TOG5949 | MIX | | 2.27 ± 0.08 d | 2.87 ± 0.14 ab | 255.67 ± 16.33 b | -1.29 ± 0.04 a | -0.78 ± 0.01 b | 1.70 ± 0.08 a | 3.02 ± 0.10 b | 10.23 ± 0.37 b |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| TOG5307 | | | 2.46 ± 0.12 c | 3.34 ± 0.08 c | 638.67 ± 8.82 bc | -1.56 ± 0.03 b | -0.79 ± 0.03 b | 1.58 ± 0.07 a | 3.52 ± 0.08 a | 8.96 ± 0.27 bc |
| TOG5307 | Control | 7 days | 5.59 ± 0.16 A | 3.35 ± 0.08 A | 389.00 ± 13.57 A | -1.55 ± 0.01 A | -0.71 ± 0.01 A | 3.12 ± 0.26 C | 2.93 ± 0.10 D | 9.79 ± 0.35 B |
| TOG5949 | | | 5.55 ± 0.09 A | 3.36 ± 0.12 A | 383.33 ± 8.29 A | -1.58 ± 0.02 A | -0.70 ± 0.01 A | 3.25 ± 0.18 C | 3.01 ± 0.24 D | 10.10 ± 0.91 B |
| TOG5307 | PEG | | 3.49 ± 0.09 B | 1.97 ± 0.12 B | 291.33 ± 8.84 B | -1.91 ± 0.09 B | -0.81 ± 0.02 B | 5.49 ± 0.41 B | 8.46 ± 0.32 C | 14.18 ± 0.56 A |
| TOG5949 | | | 3.15 ± 0.05 B | 1.70 ± 0.10 B | 225.67 ± 9.06 B | -2.42 ± 0.05 B | -0.84 ± 0.01 B | 11.97 ± 0.40 B | 6.42 ± 0.59 C | 15.85 ± 0.80 A |
| TOG5307 | NaCl | | 1.82 ± 0.13 C | 0.90 ± 0.09 B | 154.67 ± 6.36 D | -2.88 ± 0.03 D | -0.87 ± 0.01 C | 15.84 ± 0.76 A | 21.29 ± 1.12 A | 14.23 ± 0.64 A |
| TOG5949 | | | 0.90 ± 0.11 D | 0.67 ± 0.03 D | 123.67 ± 6.01 C | -3.42 ± 0.06 D | -0.88 ± 0.01 B | 19.20 ± 0.88 A | 16.56 ± 1.04 A | 16.23 ± 0.64 A |
| TOG5307 | MIX | | 3.29 ± 0.10 B | 1.80 ± 0.10 C | 253.67 ± 8.33 C | -2.22 ± 0.09 C | -0.83 ± 0.01 BC | 4.77 ± 0.75 B | 13.96 ± 0.74 B | 13.69 ± 0.68 A |
| TOG5949 | | | 2.22 ± 0.08 C | 1.26 ± 0.03 C | 236.67 ± 7.97 B | -2.79 ± 0.10 C | -0.85 ± 0.01 B | 10.93 ± 0.72 B | 11.34 ± 0.66 B | 15.89 ± 0.61 A |

***Malondialdehyde, proline, and total sugars concentrations***

The shoot MDA concentration did not change after 1 day of treatment but it drastically increased in stressed plants after 7 days (Table 5). NaCl-stressed plants accumulated higher concentrations of MDA (Table 4). The shoot MDA concentration of plants exposed to PEG and MIX treatments for 7 days were similar. In all cases, TOG5949 accumulated higher MDA concentration than TOG5307.

The shoot proline concentration was slightly increased in all stressed plants (Table 5) after 1 day of treatment: although being significant in all cases (Table 4), such an increase remained extremely limited from a relative point of view. In contrast, shoot proline significantly accumulated after 7 days of stress exposure, with the highest accumulation recorded in NaCl-treated plants. The MIX treatment presented intermediate value while the PEG-treated plants accumulated the lowest amount of proline among stressed plants. In all cases, shoot proline concentration was higher in TOG5307 than in TOG5949.

After 1 day of treatment, total soluble sugar remained unaffected in TOG5307 and slightly increased in NaCl-treated plants of TOG5949, only. Accumulation was more marked after 7 days and appeared similar in all stressed plants, with a higher concentration in TOG5949 than in TOG5307 (Table 4).

***Relative expression of genes: Semi-quantitative RT-PCR expression analysis***

*OsDREB2* was highly induced in both shoot and roots after 7 days (Figure 2A). In contrast, it was not induced in roots and only marginally increased in shoots after 1 day of stress. Between 1 and 7 days, *OsDREB2* expression increased by more than 3-fold in shoot and 4-fold in root. The expression of *OsDREB2* was similar for all considered stress treatments in both shoots and roots after 7 days, and it was slightly higher in TOG5307 than in TOG5949.

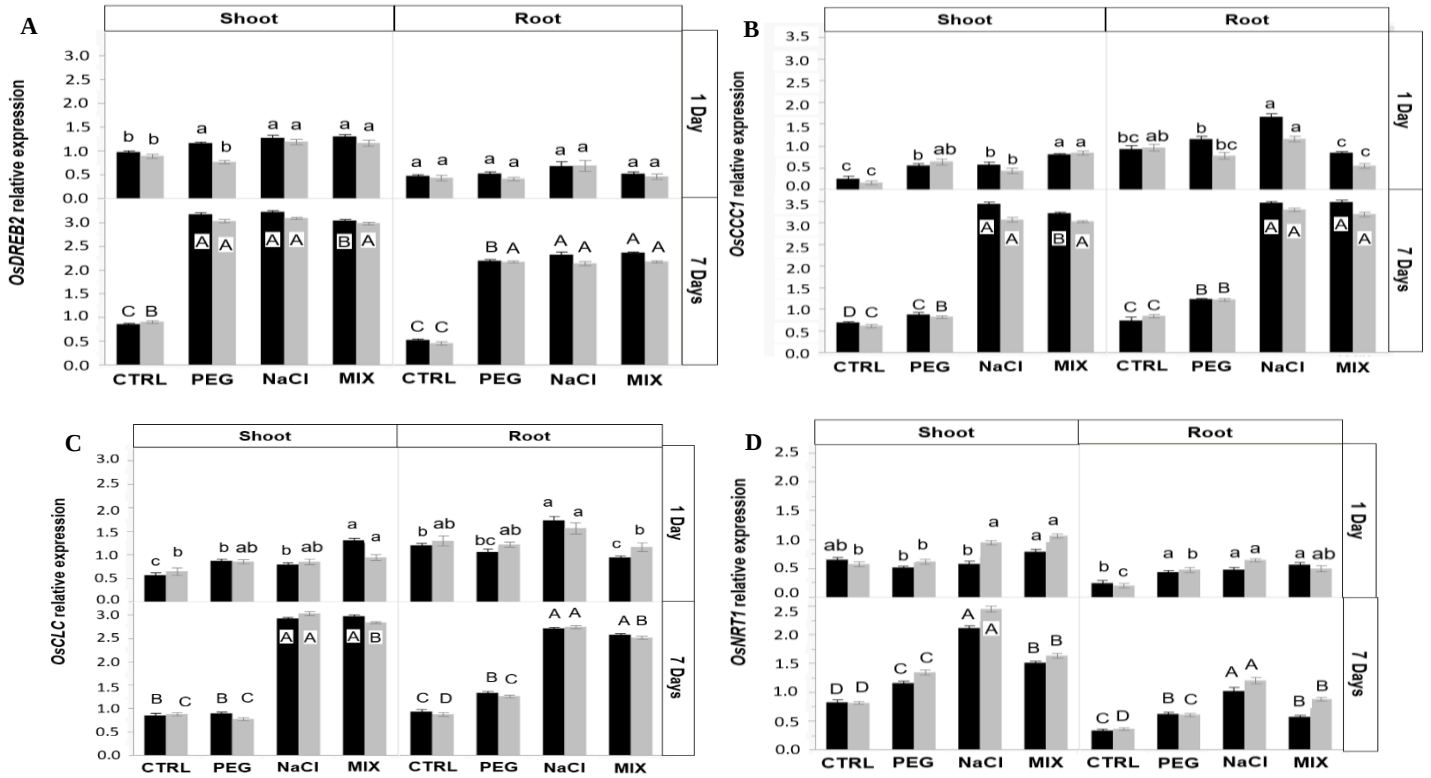


**Figure 2.** Semi-quantitative RT-PCR expression analysis of (A) *OsDREB2*, (B) *OsCCC1*, (C) *OsCLC*, and (D) *OsNRT1* genes in African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 cultivated during 1 day and 7 days in control conditions (CTRL) or in the presence of 75 mM of either NaCl, PEG-6000 or MIX (PEG +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 latter (1 day) or the same uppercase (7 days) for a particular cultivar do not differ statistically.

After 1 day exposure, *OsCCC1* coding for a cation-channel-cotransporter was highly induced in roots (Fig. 2B) and, to a lower extent in shoots of both cultivars. As far as roots are concerned, the highest expression was recorded in response to NaCl treatment, while it was recorded in MIX treatment at the shoot level. An important increase in *OsCCC1* expression was recorded after 7 days in roots and shoots exposure in response to NaCl and MIX treatment: it is interesting to notice that although these treatments did not involve the same concentration of exogenous $Cl^-$, the recorded expression of the gene was similar. Moreover, a slight although significant expression was also reported for non-ionic PEG stress (Fig. 2B). The expression was always slightly higher in TOG5307 than in TOG5949.

*OsCLC* is coding for another $Cl^-$ transporter (Fig. 2C) and was induced after 1 day of stress in the roots of NaCl-treated plants and in the shoots of plants exposed to MIX treatment. As previously noticed for *OsCCC1*, the expression of this gene was especially increased in response to NaCl- and to MIX-treated plants after 7 days in both shoots and roots.

*OsNRT1* was induced to a higher extent in shoots than in roots (Figure 2.D). TOG5307 exhibited a lower expression than TOG5949 after 1and 7 days exposure. There was a significant difference between treatments and between duration of stress exposure (Table 5). NaCl-stressed plants had higher value followed by MIX-stressed plants. Once again, the non-ionic stress PEG stimulated *OsNRT1* expression in roots after 1 and 7 days of treatment, as well as in shoots after 7 days of treatment, although to a lower extent than ionic stresses.

As far as *OsHKT1.5* is concerned, we did not observe any expression at the shoot level after 1 day exposure (Figure 3A). After 7 days, *OsHKT1.5* was slightly induced the shoot level in response to the MIX treatment. This gene was more expressed in the roots than in the shoots: its expression was however reduced by NaCl stress after 1 day of exposure. After 7 days of treatment, it was reduced in response to PEG but it was increased in the salt-sensitive cultivar TOG5949 in response to NaCl and to MIX treatment.

The expression of *OsHKT4* was recorded in the shoots but remained undetectable in the roots (Fig. 3B) After 1 day of treatment, the expression increased in the salt-sensitive cultivar in response to all stress treatments, the highest expression being recorded in NaCl-treated plants. In the salt-resistant cv TOG5307, it was increased in response to PEG and NaCl stress, but not in

response to MIX treatment. After 7 days of exposure, the expression of *OsHKT4* was increased in all cultivars and in response to all treatments. The expression was still the highest in NaCl-treated plants and was higher in TOG5949 than in TOG5307.

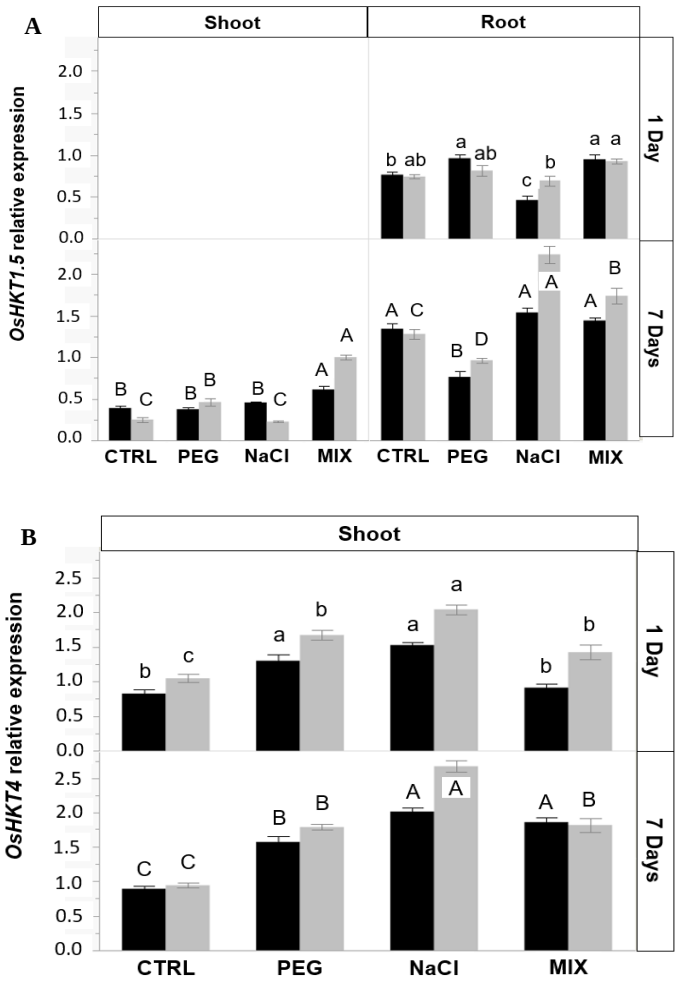


**Figure 3.** Semi-quantitative RT-PCR expression analysis of (A) *OsHKT1*.5, and (B) *OsHKT4* genes in African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 cultivated during 1 day and 7 days in control conditions (CTRL) or in the presence of 75 mM of either NaCl, PEG-6000 or MIX (PEG +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 latter (1 day) or the same uppercase (7 days) for a particular cultivar do not differ statistically.

*OsNHX1* was obviously more expressed in the shoots than in the roots (Fig. 4A). After both 1 and 7 days of treatment, the highest expression was observed in the shoots of NaCl-treated plants and was higher in TOG5307 than in TOG949. PEG treatment had no impact on OsNHX1 expression, while the MIX treatment increased *OsNHX1* expression in the shoot after 7 days of stress. At the root level treated during 1 day, *OsNHX1* was induced by PEG stress only, while it was slightly increased in response to all treatments after 7 days of treatment, the highest value being recorded for the MIX treatment.

After one day of treatment, *OsNHX2* expression (Fig 4B) was reduced in the shoot of plant exposed to NaCl stress while it was clearly induced in both plants exposed to PEG and to MIX treatment. After 7 days of treatment, expression of *OsNHX2* exhibited a clear stimulation in response to NaCl and to PEG, and to a lower extent in response to MIX treatment. At the root level, *OsNHX2* was stimulated in NaCl-treated plants after both 1 and 7 days of stress, as well as in MIX-treatment. A significant increase in *OsNHX2* expression was also recorded in roots exposed to PEG treatment after 7 days.

*OsSOS1* expression (Fig. 4C) was increased in both shoots and roots after 1 day of stress. Stimulation was recorded in response to all treatments at the shoot level and mainly in response to NaCl at the root level. After 7 days of treatment, *OsSOS1* was strongly increased in the shoots and roots of NaCl-treated plants but was only slightly higher in TOG5307 than in TOG5949. *OsSOS1* was also overexpressed in response to the MIX-treatment but it remained unaffected in response to PEG.

As far as *OsSOS2* is concerned (Fig 4D), the gene expression could not have been accurately quantified in control and PEG-treated plants after one day of stress. This is probably due to the fact that gene expression is very low in Control plants, and was not induced by the PEG stress treatment. After 7 days of treatment, *OsSOS2* expression was only recorded in NaCl- and in MIX-treated plants. The highest expression was found for NaCl-treated shoots and roots and was slightly higher for TOG5307 comparatively to TOG5949.

**Table 5.** Statistical effects of cultivar (V), treatment (T), duration (D) and their interactions as determined by 3-way analysis of variance of all parameters in of African rice seedlings (*Oryza glaberrima* Steud.) from salt-resistant (TOG5307) and salt-sensitive (TOG5949) cultivars. Symbols: *, **, *** and **** represent statistical significance at $P < 0.05$, 0.01, 0.001 and 0.0001, respectively; and NS = not significant at $P = 0.05$.

| Parameters | | Analyse of Variance (ANOVA 3) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | V | T | D | VxT | VxD | TxD | VxTxD |
| Number of leaves-RGR | | *** | NS | - | NS | - | - | - |
| Fv/Fm | | **** | **** | **** | NS | NS | **** | *** |
| PS2 | | **** | **** | **** | * | NS | * | NS |
| qP | | *** | **** | **** | * | NS | **** | NS |
| NPQ | | **** | **** | **** | **** | ** | **** | * |
| A | | **** | **** | *** | **** | *** | **** | NS |
| E | | **** | **** | **** | * | NS | **** | NS |
| gs | | **** | **** | **** | **** | **** | **** | **** |
| MDA | | **** | **** | **** | **** | **** | **** | **** |
| Proline | | **** | **** | **** | ** | *** | **** | * |
| Total Sugars | | **** | **** | **** | NS | NS | **** | NS |
| Ψs (MPa) | Root | ** | **** | * | NS | NS | * | NS |
| | Shoot | **** | **** | **** | **** | **** | **** | NS |
| Sodium | Root | **** | **** | NS | **** | ** | ** | NS |
| | Shoot | **** | **** | **** | **** | NS | **** | NS |
| Potassium | Root | NS | **** | **** | * | NS | **** | NS |
| | Shoot | NS | **** | **** | NS | NS | **** | NS |
| Na : K ratio | Root | *** | **** | NS | **** | NS | NS | NS |
| | Shoot | **** | **** | NS | **** | NS | **** | NS |
| Chloride | Root | **** | **** | NS | **** | ** | ** | NS |
| | Shoot | **** | **** | **** | **** | NS | **** | NS |
| Length-RGR | Root | **** | **** | - | *** | - | - | - |
| | Shoot | **** | **** | - | **** | - | - | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Fresh Weight | Root | **** | **** | - | * | - | - | - |
| | Shoot | **** | **** | - | NS | - | - | - |
| Dry Weight | Root | *** | **** | - | NS | - | - | - |
| | Shoot | ** | **** | - | NS | - | - | - |
| Water content | Root | NS | * | - | NS | - | - | - |
| | Shoot | NS | **** | - | *** | - | - | - |
| *OsHKT1.5* | Root | **** | **** | **** | **** | **** | **** | * |
| | Shoot | **** | **** | **** | **** | *** | **** | NS |
| *OsHKT4* | Root | - | - | - | - | - | - | - |
| | Shoot | **** | **** | **** | *** | * | **** | ** |
| *OsHKT8* | Root | **** | **** | **** | **** | NS | **** | * |
| | Shoot | *** | **** | NS | NS | NS | **** | *** |
| *OsNHX1* | Root | **** | **** | **** | **** | NS | **** | * |
| | Shoot | **** | **** | **** | **** | NS | **** | NS |
| *OsNHX2* | Root | **** | **** | **** | **** | NS | **** | NS |
| | Shoot | *** | **** | **** | *** | NS | **** | ** |
| *OsNHX2* | Root | **** | **** | **** | **** | NS | **** | NS |
| | Shoot | *** | **** | **** | *** | NS | **** | ** |
| *OsSOS1* | Root | **** | **** | **** | *** | NS | **** | NS |
| | Shoot | **** | **** | **** | *** | NS | **** | * |
| *OsSOS2* | Root | * | **** | - | NS | - | - | - |
| | Shoot | NS | **** | - | NS | - | - | - |
| *OsNRT1* | Root | *** | **** | **** | ** | * | **** | ** |
| | Shoot | **** | **** | **** | **** | NS | **** | NS |
| *OsCLC* | Root | NS | **** | **** | **** | NS | **** | * |
| | Shoot | NS | **** | **** | **** | NS | **** | NS |
| *OsCCC1* | Root | **** | **** | **** | **** | **** | **** | NS |
| | Shoot | **** | **** | **** | **** | ** | *** | NS |
| *OsDREB2* | Root | ** | **** | **** | NS | NS | **** | NS |
| | Shoot | **** | **** | **** | **** | * | *** | NS |

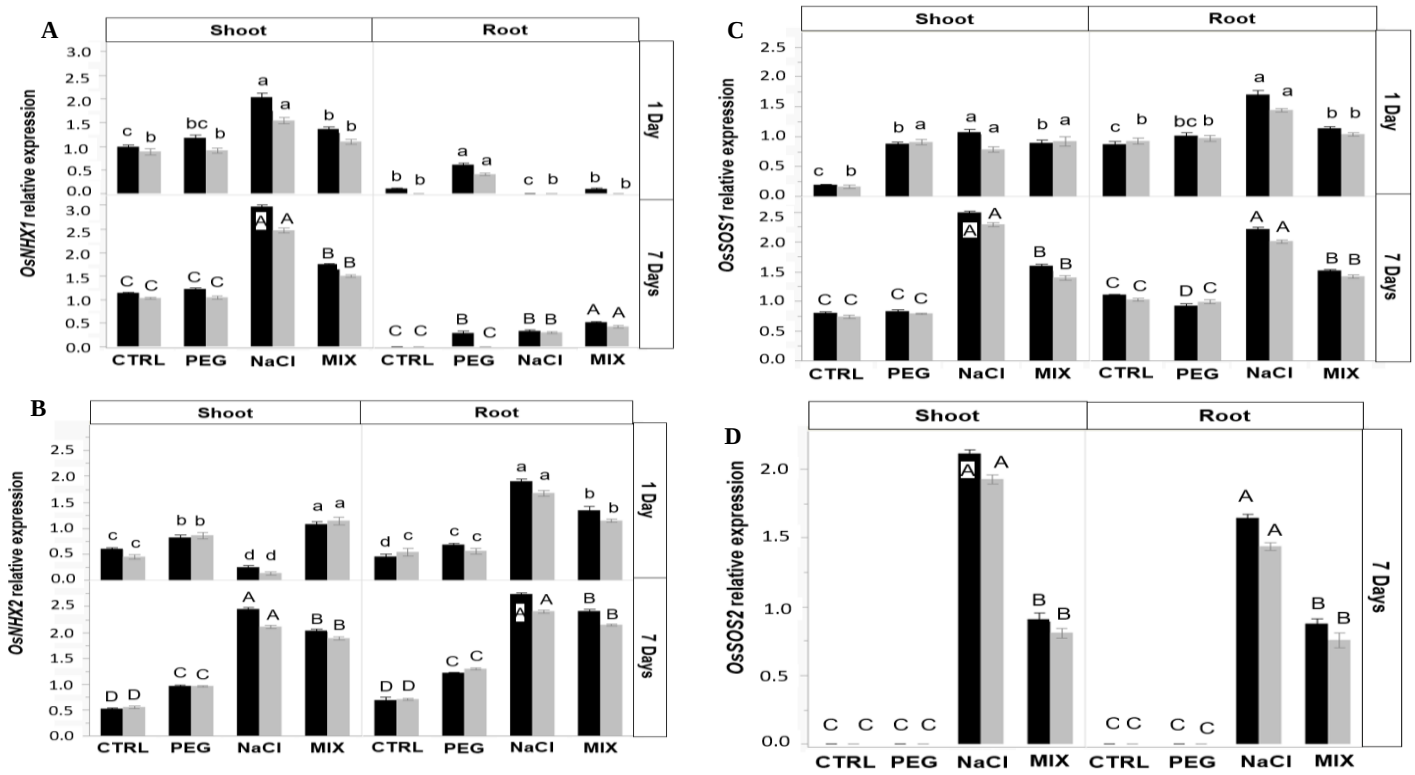


**Figure 4.** Semi-quantitative RT-PCR expression analysis of (A) *OsNHX1*, (B) *OsNHX2*, (C) *OsSOS1*, and (D) *OsSOS2* genes in African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 cultivated during 1 day and 7 days in control conditions (CTRL) or in the presence of 75 mM of either NaCl, PEG-6000 or MIX (PEG +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 latter (1 day) or the same uppercase (7 days) for a particular cultivar do not differ statistically.

## Discussion

In the present experiment, plants were exposed to iso-osmotic concentration (mean $\Psi$s = -0.047 MPa) but encountered different electrical conductivity in the nutrient solution. The PEG treatment reduced WC in the roots but not in the leaves. Unexpectedly, the NaCl treatment applied for 7 days decreased the leaf WC of the salt-resistant cv TOG5307 (75.82%) while the WC of the salt-sensitive TOG5949 remained similar to the control (83.64%). This could be related to the fact that $\Psi$s of NaCl-treated shoots was higher less negative in TOG5307 than in TOG5949 and that the transpiration rate leading to water loss was higher in the former than in the latter. Nevertheless, $\Psi$s values in both roots and shoots of the two cultivars remained lower than the external $\Psi$s of nutrient solution, and this suggests that TOG5307 is, to some extent, tolerant to partial desiccation: indeed, *A* values remained higher in the leaves of the dehydrated TOG5307 than in TOG5949, as it was also the case for growing parameters. Our data indicated that the light phase of photosynthesis in terms of chlorophyll-fluorescence related parameters was affected in those leaves, but less than in the hydrated leaves of TOG5949, supporting the view of Rane et al. (2021) that stability of PSII is an important property to desiccation tolerance.

Desiccation tolerance of vegetative organs is a complex trait in plants (Oliver et al., 2020). In the extreme case of resurrection plants, it relies on a complex network of interacting molecular targets which are protecting the plant not from dehydration but also during rehydration as well, when a massive flow of water induces a brutal rehydration leading to cellular disturbances (Xu et al., 2021). The situation is quite different in the present case since dehydration remained limited but the data indicated that the salt-resistant cv. is, to some extent, tolerant to mild desiccation. The maintenance of leaf elongation, despite a decrease in WC and $\Psi$s may involve an increase in the cell wall extensibility (Wu and Cosgrove, 2000). The mechanisms of desiccation tolerance were reported to be triggered by the DREB (Dehydration-responsive-element-binding) proteins (Yuqing et al., 2021). As far as *OsDREB2* is concerned, the expression of the corresponding gene was induced to a same extent in response to all treatments after 7 days of stress, suggesting that the parameter triggering overexpression was the external water potential rather than the internal water content. DREB is however a complex

subfamily divided into six subgroups and overexpression of other members of this family could be involved in plant response to water status.

Plants exposed to PEG only slightly decreased their root osmotic potential, and after 1 day of exposure a shoot $\Psi$s decrease was recorded in the salt-sensitive cultivar only. After 7 days, shoot $\Psi$s was lower in salt-sensitive than in salt-resistant one. This trend could not be explained by proline accumulation which was always higher in the salt-resistant genotype. It was expected that ion accumulation, mainly $K^+$ may be specifically absorbed for osmotic adjustment of PEG-treated plants (Castillo et al. 2007). However, in our case, PEG did not stimulate $K^+$ concentration, and even tended to slightly decreased it after 7 days in shoots of TOG5307. Chloride was also reported to be involved in turgor regulation, through its role in $\Psi$s decreasing. Teodero et al. (1998) reported that in response to osmotic stress induced by mannitol, a stimulation of $Cl^-$ influx may rapidly occur in Arabidopsis cells. However, we did not record any increase in $Cl^-$ content in PEG-treated plants and a small decrease was even recorded in the shoots of both cultivars after 7 days of exposure. Nevertheless, some genes coding for Cl-transporter were overexpressed already after 1 day of treatment in PEG-treated plants. This was the case for the gene coding for OsNRT1, which is a low-affinity $Cl^-$ transporter (Teakle and Tyerman 2010). It has however to be mentioned that this poorly selective transporter also transports $NO_3^-$ anions through plasma membrane (Segonzac et al. 2007) so that its stimulation may also reflect the need to supply nitrogen to stresses plants rather than an attempt to regulate osmotic potential by $Cl^-$ influx; the same gene was also overexpressed at the shoot level and in both cultivars after 7 days.

The situation was easier to analyze in NaCl-treated plants since this treatment had a higher impact on mineral nutrition parameters. Chloride significantly accumulated in both roots and shoots already after 1 day and this trend even increased after 7 days. A similar observation is valid for $Na^+$. This is in opposition with previous data (Prodjinoto et al., 2021a) which demonstrated that the roots of TOG5307 are able to efficiently sequester $Cl^-$ ions.
The ion transporters and channels play a key role not only for ion absorption, but also for long-distance translocation, distribution between organs and compartmentation within the cells. For the two studied cultivars considered in the present work, Prodjinoto et al. (2021b) reported that inhibitors of $Na^+$-$H^+$ antiporters in roots induced an increase in $Na^+$ in all organs, with a higher impact in the salt-resistant cultivar than in the salt-sensitive one, suggesting

an important role of SOS1 in salinity resistance of *Oryza glaberrima*. It is indeed well established that this $Na^+$:$H^+$ antiporter transports $Na^+$ accumulated in the roots back to the external medium, thus preventing $Na^+$ xylem sap loading (Zhu et al. 2003). Moreover, SOS1 has also been identified in xylem parenchyma where it could be involved in $Na^+$ xylem retrieval (Zhao et al., 2020) but the situation is not clear regarding this point since other authors consider that SOS1 might be involved in xylem $Na^+$ loading rather than $Na^+$ retrieval. The corresponding gene was induced in NaCl-treated plants in both cultivars already after 1 day of treatment: although its expression was higher in salt-resistant TOG5307 than in salt-sensitive TOG5949, the difference between the two cultivars remained low from a relative point of view. The highest stimulation was observed in the shoots and this corroborates the hypothesis of Prodjinoto et al. (2021b) that SOS1 in leaves is involved in $Na^+$ extrusion to the apoplasm, especially in the salt-resistant cultivar. It is interesting to notice that *SOS1* gene expression was recorded in all tested plants while the expression of *SOS2*, coding for a dikinase required for SOS1 activation, was detected only after 7 days of exposure to NaCl only.

Beside SOS1, NHX1 and NHX2 are involved in vacuolar sequestration of toxic $Na^+$ and thus protect $Na^+$-sensitive enzymes located in cytosol (Almeida et al., 2017; Zhu, 2003). *OsNHX1* was only slightly expressed in roots but it was more clearly overexpressed in the shoots, and to a higher extent in the salt-resistant cultivar than in the salt-sensitive one. In contrast, *OsNHX2* was strongly induced in the roots of NaCl-treated plants, already after 1 day of treatment, while overexpression in the shoots was recorded after 7 days of stress only. In all cases, expression was higher in TOG5307 than in TOG5949, which supports the view that vacuolar sequestration of $Na^+$ indeed plays a role in salinity resistance. This also supports the view of Zeng et al. (2018) that different members of this large family may be differently regulated in the various organs of adult rice plant.

Since several years, high-affinity $K^+$ transporter (HKT1) receive considerable attention because it encodes an $Na^+$-preferential transporter which principally control the withdrawal of $Na^+$ from the xylem sap to the phloem (Davenport et al., 2007; Ren et al., 2005; Hasegawa, 2013; Almeida et al., 2017). Zhang et al. (2018) demonstrated that a *ZmHKT1* loss-of-function in maize causes salt hypersensitivity. The sodium transporter OsHKT1;5 has been identified as a major component of salt tolerance in rice (*Oryza sativa* L.) at both vegetative and reproductive stages (Kobayashi et al., 2017; Jayabalan et al.,

2019). We were however unable to detect expression of *OsHKT1;5* in the shoot after 1 day of treatment and it was not overexpressed in the roots at this period in response to NaCl. After 7 days, overexpression in the roots was detected for the NaCl-sensitive cultivar only, and the gene expression was not stimulated in shoots. On the basis of these observations, it seems difficult to attribute a major role of this gene in the response to NaCl for the two tested cultivars. This is puzzling, considering that, according to Platten et al. (2013), *HKT1;5* plays a major role in *O. glaberrima.* However, according to these authors, different alleles exist for this gene which strongly influence its impact on salt resistance in *O. glaberrima*. Similarly in *O. sativa*, Jayabalan et al. (2019) demonstrated that only 4 amino acid changes occurred between the highly salt resistant cultivar Nona Bokra and the highly salt-sensitive cultivar Koshihikari and that one single V395 in the vicinity of the sodium selective filter residue strongly enhance $Na^+$ transport rates by reducing pore rigidity. *OsHKT4* was detected in the shoot only, with the highest level of expression in response to NaCl, but also a higher level of expression in the salt-sensitive cultivar: it might thus be regarded as an unsuccessful attempt of this genotype to limit $Na^+$ accumulation in photosynthetic tissues.

Three genes coding for chloride transporters were considered in the present study. CCCs are secondary active electroneutral transporters which are functioning as $Na^+$:$K^+$:2$Cl^-$ co-transporters. In the studied cultivars TOG5307 and TOG5949, Prodjinoto et al. (2021b) used bumetamide as an inhibitor of CCC in *O. glaberrima* and concluded that it is a major contributor of $Cl^-$ transport, although the counterions appeared to be preferentially $K^+$ in the salt-resistant cultivar and $Na^+$ in the salt-sensitive one. In the present study, gene coding for CCC1 was very rapidly induced at the root level, and to a higher extent in the salt-resistant genotypes. It is surprising that a gene coding for a transporter of a toxic ion could be stimulated by the exogenous presence of this ion! According to Teakle and Tyermann (2010), however, this transporter is also involved in $Cl^-$ xylem retrieval and its higher expression in TOG5307 may thus explain that this cultivar was more able to retain $Cl^-$ at the root level (Prodjinoto et al., 2021a). According to Brumos et al. (2009), *AtCCC* ortholog in Citrus $Cl^-$ accumulating genotype had higher expression than $Cl^-$ excluding one. We found a different result here and gene was more expressed in TOG5307 than in TOG5949. The high level of gene expression in the shoot suggests that this transporter may have other functions than a simple retrieval of $Cl^-$ at the xylem loading site. Even in the absence of salt stress, CCC is

required for cell elongation and osmoregulation (Kong et al., 2011; Chen et al., 2016). Overexpression at the shoot level thus requires further studies.

Gene coding for CLC1 displayed a similar pattern of expression than gene coding for CCC: it was stimulated in response to NaCl in the roots already after 1 day of exposure, and after 7 days in both organs. CLC is supposed to act as a voltage-gated chloride channel located at the tonoplast (Subba et al., 2020). It is involved in $Cl^-$ vacuolar sequestration. According to Diédhiou and Golldack (2006), *OsCLC1* expression is higher in roots and shoots in the salt-resistant cultivar Pokkali while it was repressed in the salt-sensitive IR29 in *O. sativa*. Such difference was not recorded in our study on *O. glaberrima* and both TOG5307 and TOG5949 had almost similar levels of expression. It has to be mentioned that CLC may also be involved in $NO_3^-$ nutrition and, according to De Angeli et al. (2006), $Cl^-$ transport may even be much less than $NO_3^-$. Gene coding for CLC was also up-regulated in response to PEG and Li et al. (2006) reported that this gene may be induced by dehydration, suggesting a possible role of this gene in osmoregulation through $NO_3^-$ accumulation besides its role in vacuolar sequestration.

The simultaneous presence of NaCl and PEG allowed us to quantify the impact of non-ionic osmotic stress on ion accumulation and expression of genes coding for transporters. Chloride and sodium accumulations were strongly reduced in the shoots when PEG was present. Even if the exogenous dose of NaCl used in the MIX treatment was reduced by 50%, the level of accumulation in response to MIX treatment was unexpectedly low (and even in some case similar to control) and this could not be explained by stomatal closure or decrease in transpiration rates. PEG is known to induce an increase in the viscosity of nutrient solution, but it is hard to believe that this process may alter $Na^+$ and $Cl^-$ diffusion to such an extent, especially considering that the PEG-treated plants did not suffer from major elements deficiencies (detailed data not shown). Hence, these observations suggest that the non-ionic water stress may drastically alter accumulation of toxic ions, although this did not lead to any significant improvement of plant growth or water status. Despite this strong difference in $Cl^-$ accumulation between NaCl and MIX treatment, genes coding for $Cl^-$ transporters were regulated exactly in the same way. In contrast, most genes involved in $Na^+$ transport exhibited a lower expression in response to MIX treatment than in NaCl-treated plants. Additional molecular studies focusing on involved transcription factors and transduction signals are required to explain these unexpected data.

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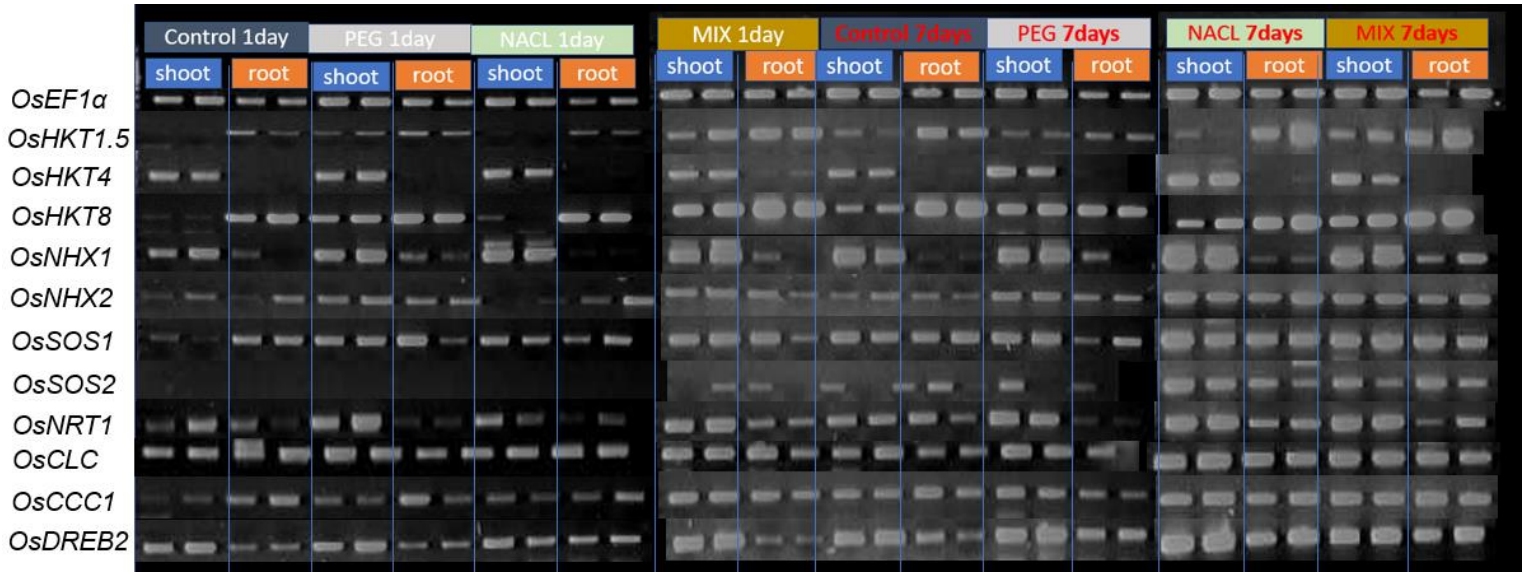
**Supplemental data :** Expression analysis of genes *OsEF1α*, *OsHKT1.5*, *OsHKT4*, *OsSOS1*, *OsSOS2*, OsNHX1, *OsNHX2*, *OsNRT1*, *OsCLC*, *OsCCC1*, and *OsDREB2* in African rice *Oryza glaberrima* varieties shoot and root after 1 day and 7 days under control, PEG, NaCl and Mix conditions. At the level of each organ, the first and the second band correspond respectively to the varieties TOG5307 and TOG5949.



# PART III
# General discussion

# General discussion

## 1. Preliminary remark.... some terminology.

The terminology that applies to the behaviour of plants under abiotic stress remains ambiguous. This is particularly true for the notions of "resistance" and "tolerance": both terms are used in the literature and there is no consensus within the scientific community on the criteria that distinguish them. The distinction between these two notions is however important to establish, as it directly conditions the formulation of explanatory hypotheses resulting from our observations, and the understanding of the strategies implemented by the plant confronted with a stress. In view of the sometimes contradictories opinions of specialists, any distinction has its share of arbitrariness. In the general discussion that follows, we have decided to use the terminology of Levitt (1980). According to this approach, stress is linked to the existence of an environmental constraint likely to induce one or more metabolic disturbances. A plant will be qualified as **resistant** if it is capable of completing its entire cycle and producing fertile offspring in the presence of this external constraint. To do this, it can use two ranges of strategies: in an **avoidance** strategy, it prevents the stress from taking hold inside the plant. In the case of **tolerance**, the stress settles within the tissues, but the tissues implement a strategy that allows the metabolism to continue in a "normal" way. It should be noted that a third category, defined as **avoidance**, refers to phenological adaptations of plants that develop when the stress is not present (such as the case of ephemeral plants in semi-desert environments that develop when water is available) but this last category does not concern us in the case of this thesis.

If we apply these notions to the salt stress that concerns our work, we can say that a plant that prevents absorbing $Na^+$ and $Cl^-$ is avoiding, whereas a plant that absorbs these elements while continuing its metabolic activity is tolerant. In both cases, it is resistance. If we refer to the water component of salt stress, we can say that a plant that maintains its water supply and keeps its tissues hydrated is avoidant, while a plant that continues its metabolism while partially dehydrated is tolerant.

However, these conceptual distinctions remain theoretical, for several reasons. The first reason is that it is difficult to establish when tolerance begins: at what level of toxic ions, at what level of dehydration can we speak of tolerance? The second reason is that these two strategies are not mutually exclusive; faced with a given situation, the plant can simultaneously implement both types of strategies: a plant exposed to salinity can both limit the absorption of toxic ions, and set up protection strategies with regard to the toxic ions whose absorption was unable to avoid. The general notion of resistance then takes on its full meaning, and the best performing plant will be the one whose overall strategy is the least costly from an energy point of view. The third reason is that despite its immobility, the plant is a complex organism, and this complexity is marked in time and space. In time, because it is phenologically evolving and strategies that may be useful at the seedling stage may no longer be useful at the reproductive stage. In space, because it is morphologically complex and what is effective for some organs may be ineffective or even counterproductive for other organs with a different function. The fourth reason lies not in the complexity of the plant, but in the complexity of the constraint: it can be of variable intensity, of variable duration, it can fluctuate in time, it can appear suddenly or, on the contrary, gradually. The plant "perceives" the present situation with an acuity that depends on multiple molecular relays, but it cannot "predict" the future evolution of this situation. It must therefore, as part of its adaptation to the environment, have a physiological plasticity that allows it to change its strategy when necessary. The speed and efficiency of such changes are guarantees of resistance... but these aspects are difficult to grasp in the context of an experimental approach, since the scientist often work in fully-controlled environmental conditions that do not take account of such environmental complexity. This reality cannot be ignored, particularly in the case of salt stress, which induces several types of constraints at the plant level.

## 2. A little reminder

African rice, *Oryza glaberrima*, is known to be resistant to many abiotic and biotic stresses. The present study aimed to identify contrasting cultivars of *Oryza glaberrima* with respect to their resistance to salt stress based on physiological and yield parameters. It also aimed at discriminating the ionic and osmotic components of salt stress. For the identification of salinity

resistant cultivars, our experimental strategy was based on hydroponic cultivation under controlled conditions and soil cultivation under semi-controlled conditions. The discrimination of the components of salt stress, which is at the heart of our thesis, was mainly carried out through experiments on hydroponic crops involving different cocktails of iso-osmotic solutions under controlled conditions.

### 3. Does exploitable variability exist within *O. glaberrima* for salt stress resistance?

Less studied than *Oryza sativa* L., *Oryza glaberrima* is sometimes considered to have less variability than the Asian species (Bello et al., 2018). According to Li et al. (2011), this low variability is the result of the domestication process itself, and *O. glaberrima* is thought to have emerged from a limited population of *O. barthii*, which is itself characterized by low diversity. In a recent comprehensive study in Benin, Loko et al. (2021) pointed out that the diversity of *O. glaberrima* may be greatly underestimated: the success of the NERICA varieties (see below) has contributed to the marginalization of many *O. glaberrima* cultivars, which are now only present in limited geographical areas, mainly in the north of the country, where this material remains due to local socio-cultural considerations. Problems of local nomenclature do not facilitate the constitution of reliable collections either, as the names attributed to the same variety may vary according to ethnic groups. According to Barry et al. (2008), the maintenance of a high proportion of local varieties of *O. glaberrima* in a region reflects the predominance of subsistence production systems with low intensification.

However, African rice is of considerable interest due to its resistance to several abiotic stresses such as drought (Efisue and Derera, 2012; Maji, 2001; Ndjiondjop et al., 2012; Shaibu et al., 2018), iron toxicity (Sahrawat and Sika, 2002; Sikirou et al., 2018), soil acidity (Sano et al., 1984), and submersion (Aliyu et al., 2015; Futakuchi et al., 2001; Sakagami et al., 2013). The interest of the species cannot therefore be denied and it is surprising that it has not received more attention for the study of its response to salinity, even though *Oryza sativa* is a crop particularly sensitive to this constraint (Chen et al., 2021; Singh et al., 2008; Hoang et al., 2016). In a study published in 2010, Watanabe et al. estimated that *O. glaberrima* is globally more sensitive to salt

stress than *O. sativa*. However, these authors consider the Pokkali variety as a reference for the estimation of salt resistance. This is a tall indica landrace which, like Nona Bokra, is not a material likely to be grown in the Beninese agricultural context. The resistance potential of these cultivars may be linked to dilution phenomena of accumulated toxic ions leading to a lower concentration in the aerial parts with a consequent reduction in the harvest index (Yeo et al., 1990). It should be noted that Kartika et al. (2020) pointed out that *O. glaberrima* performs better than *O. sativa* in response to progressive drought and that this advantage could be related to the average stomatal size. Any comparison of the two species is therefore biased by the limited number of genotypes that are humanly possible to compare in the same experiment and no definitive conclusions can be drawn.

In this thesis, we tested 25 cultivars of *O. glaberrima* and compared them with a single cultivar of *O. sativa* I Kong Pao (IKP). The level of resistance of IKP can be considered as intermediate and remains largely inferior to that of Pokkali or Nona Bokra, but its characteristics seem to us to be more in line with the context in which our research is taking place. It is of course not our intention to compare the two species on such an unequal basis, and the number of 25 cultivars is in itself too limited to make a statement on the level of resistance of *O. glaberrima* as a whole. However, it was possible to consider a division into three distinct groups, reflecting the existence of variability within the material tested, and to select two cultivars with contrasting properties (Chapter I). Although the screening was carried out in the vegetative phase, this difference in behaviour persists in the reproductive phase (Chapter II). It is clear that the behaviour of the plants at the two stages responds to distinct parameters and genetic information specific to each of these stages (Singh et al., 2021), but in our case, this correspondence has the merit to exist. Our analysis agrees to some extent with the findings of Lakshim et al. (2019) that certain morpho-physiological and phenological parameters may be of interest to breeders aiming to improve *O. glaberrima*. Furthermore, the difference in behaviour between the two cultivars selected is marked by a highly significant effect of the 'cultivar' factor for almost all the parameters analyzed (Chapter V; Table 1; Chapter VI; Table 5). The interaction between the cultivar factor and the treatment factor was also very often highly significant, underlining the fact that the stress response strategy differs.

We have pointed out that the cultivar TOG5307 had a good tolerance index (in the sense defined above) with respect to accumulated $Na^+$ (Chapter I). However, it should be noted that this tolerance index remains lower than that

of IKP. If the quantitative estimation of this index pleads in favour of a better behaviour of IKP, nothing indicates in this approach that the properties which underlie this index are identical and it is not impossible that the combination of complementary cellular or molecular properties does not allow to obtain an even better result in terms of resistance. Moreover, the index is calculated only in relation to accumulated $Na^+$ and neither chloride nor water contents have been taken into account in this chapter. However, Chapter V indicates that TOG5307 also shows good tolerance to partial dehydration, which was not analyzed for IKP.

The main interest in *O. glaberrima* lies in obtaining NERICA varieties from crosses between *O. sativa* and *O. glaberrima*. The fact that both species share the same A genome makes this hybridization possible, although an anther culture and chromosome doubling step is required to restore the fertility of the hybrids (Reyes et al., 2021). Subsequent backcrosses using the *O. sativa cv* as a recurrent parent allow the yield potential of the latter to be retained and only the genes of interest from *O. glaberrima* to be retained (Hoang et al., 2016). It is not too much to say that these NERICA varieties have, in many places, revolutionized rice cultivation in Africa, and that in this sense, *O. glaberrima* supports significant improvement (Yelome et al., 2018) and an increase in the diversity of plant material that can be grown (Bah et al. 2018). There has been a plethora of studies on the properties of these NERICA varieties in relation to drought tolerance (Kang and Futakuchi 2019; Menge et al. 2019), lodging resistance (Olagunjulu et al., 2021), root elongation kinetics (Obra et al., 2019; Miyazakio and Arita 2020), nitrogen fertilization efficiency (Inubushi et al., 2020), and resistance to high temperatures (Ravikiran et al., 2020). This underlines the interest of the African species in a rice cultivation policy better adapted to the needs of the populations, and the integration of the TOG5307 variety in such improvement schemes could be considered for the improvement of salt resistance.

However, the adoption of NERICA varieties by local populations is not as automatic as one might think and multiple reluctances persist (Akinnagbe and Akinbobola 2021) as will be explained latter. Loko et al (2021) report on the abandonment of NERICA varieties in many areas in southern Benin. Several causes can be mentioned, among which Yokouchi and Saito (2017) mention the fragility of the harvested grain, a difficulty in accessing financial means to properly manage more productive but more demanding varieties and especially organoleptic parameters associated with the aroma of the products obtained. This important criterion for consumers should not be neglected in

the context of salt stress, given the effects of salinity on the biochemical properties of rice grain (Razzaq et al. 2020). In addition to *Oryza glaberrima*, there is now interest in non-crop species of the genus *Oryza* that may have interesting genes and traits for the improvement of the Asian species *Oryza sativa* (Lei et al., 2013; Atwell et al., 2014; Fang et al., 2011; Solis et al., 2020).

## 4. Is the ionic component more toxic than the osmotic component of salt stress?

The osmotic component compromises water uptake by the plant and is therefore comparable to what is observed in drought situations as it induces water stress (Liu et al., 202; Kaur and Pati, 2017; Morton et al., 2019). However, a soil solution always contains soluble salts. Applying a soil-based protocol to simulate water stress will certainly lower the water content, but on the other hand it will increase the electrical conductivity of the soil. It is therefore almost utopian to be able to really compare the osmotic and ionic components on the basis of such a protocol. Goharrizi et al. (2020) evaluated the evolution of physiological and biochemical traits of four pistachio rootstocks under drought, salinity and drought + salinity stress. These authors were able to select the most resistant pistachio rootstocks under drought + salt stress. Unfortunately, they could not clearly discriminate between osmotic and ionic components of salt stress because their work was carried out in pots.

In our work, we chose to use a much more static system: hydroponics. To truly mimic osmotic stress without fear of modifying the ionic component, we used polyethylene glycol (PEG) which is a non-ionic and non-penetrating agent. Although the PEG treatment was compared to the NaCl treatment, we also applied a MIX treatment (NaCl + PEG) to the plants in order to evaluate the simultaneous effect of salinity and drought (see Chapter V and VI).

In this experimental strategy, we applied a single stress intensity and used a sudden exposure of the plants to the stress in the vegetative phase only. These two strategic choices may influence the answer to the question asked. For example, it could be assumed that with a higher salt dose, the osmotic component is relatively more important than the ionic component: an abrupt closure of the stomata may slow down the transpiratory flux more rapidly and

thus contribute to limit the accumulation of toxic ions. The kinetics of stress imposition are also important: Almansouri et al (1999) clearly demonstrated in durum wheat that a progressive application makes it possible, to a certain extent, for the plant to set up adaptation strategies and that the speed of this set up is not identical for the maintenance of water status and for the regulation of mineral nutrition. According to these authors, the relationships between the kinetic aspects of imposition and the kinetic aspects of response vary considerably depending on the cultivar. The following discussion is therefore only valid within the limits of our self-imposed experimental choices.

The results obtained in our study suggest that *O. glaberrima* has a good capacity to adapt to osmotic stress. Indeed, the water content of the aerial parts was only slightly influenced by the treatments used. The rapidity of stomatal closure may explain this process, but it is interesting to note that the stomatal conductance of the resistant cv. TOG5307 is much less reduced after two days in response to PEG than in response to NaCl (Chapter V; Fig. 3). Numerous biochemical players condition stomatal regulation (Chen et al., 2021; Zhao et al., 2020). The role of abscisic acid is widely discussed and has been the subject of many studies (Liu et al., 2021 for review). Ben Hassine and Lutts (2010) demonstrated that the kinetics of ABA synthesis were not identical in plants exposed to PEG and in plants exposed to NaCl.

Despite the differences in the effects of PEG and NaCl on the $g_s$ value of TOG5307, it should be noted that such differences do not translate into differences in leaf water content. One hypothesis would be that the water losses through the stomata in the presence of PEG are compensated by a better osmotic adjustment of this cultivar, which could constitute a guarantee of water supply. While it is true that leaf $\Psi_s$ was slightly lower in plants exposed to NaCl for two days than in those exposed to PEG, the differences are not at all comparable to what is observed for stomatal conductance.

To answer the question posed above regarding the relative toxicity of the two stresses, it must be possible to determine, for each modified parameter, whether it is a symptom of damage or an attempt of adaptation. This is easy to assess for some parameters such as MDA levels: the more secondary oxidative stress the plant encounters, the more MDA it will produce (Billah et al. 2021). In this respect, things are clear in our study: after 15 days, plants exposed to NaCl produce much more MDA than plants exposed to PEG, and the sensitive cultivar contains more than the resistant cv. For other compounds, which are not symptoms of damage, things are sometimes more

difficult to understand. If such a compound accumulates, a comparison of the accumulation rates in a sensitive and a resistant cv. can provide an answer. However, it should be noted that the fact that an adaptation attempt has been made does not mean that it has been successful.

We can clearly illustrate this duality in the case of proline. Considered as the major compatible osmotic compound in rice (which is unable to synthesize glycinebetaine), we observed contradictions in our work: in Chapter I, proline accumulated little in TOG5307, whereas in Chapter V and VI, it accumulated more in the resistant *cv* TOG5307 than in the sensitive *cv* TOG5949. However, the leaf $\Psi s$ was lower in TOG5949 than in TOG5307, which is contradictory. In fact, proline has multiple functions in the plant besides osmotic adjustment. Lutts et al. (1999b) showed that proline accumulated in sensitive *O. sativa* cvs. due to a higher concentration of potentially toxic ammonium, which proline synthesis consumes via activation of glutamate dehydrogenase. In this perspective, proline would be both a symptom of damage (it results from an accumulation of toxic $NH_4^+$ and can also have negative effects on the activity of certain enzymes (Lutts and Guerrier 1995a)) AND an adaptation strategy (as it contributes to osmotic adjustment).

Castillo et al (2007) stated that *Oryza sativa* exposed to salt stress is mainly sensitive to the osmotic component. This view is shared, less peremptorily, by other researchers (Singh et al., 2008; Ganie et al., 2021; Qin et al., 2020; Bhatt et al., 2020). However, our observations suggest that the situation may be different in *Oryza glaberrima*. Considering the NaCl toxicity index ($TI_{NaCl}$) and the PEG toxicity index ($TI_{PEG}$) (Chapter V), we found that $TI_{NaCl}$ was always higher than $TI_{PEG}$ and that this was true for both cultivars and for both durations. This means that ionic toxicity is a determining factor in the behaviour of the plant.

On the other hand, the values of the toxicity indices for the mixed treatment (NaCl+PEG) underline an interesting difference between the two cultivars as to the additive character of the disturbances induced by the two types of constraints: in the case of the sensitive cv. TOG5949, $TI_{MIX\ OBS}$ was comparable to $TI_{MIX\ PRED}$ and the two constraints thus act in an additive way for the considered salt doses. On the other hand, $TI_{MIX\ OBS}$ was lower than $TI_{MIX\ PRED}$ for TOG5307: the observed toxicity of the mixed treatment was therefore lower than the expected value, which tends to suggest that the presence of one of the constraints helps the plant to overcome the other constraint. This highlights the variability in the behaviour of distinct *cvs*. in *O. glaberrima*

already discussed above but also raises interesting questions about the molecular targets and mechanisms that may explain the antagonistic effect of multiple stresses. The same variability between *cvs* was highlighted by Lutts et al (2004) on the comparative effects of PEG and NaCl on cell viability of *Triticum durum* cultivars. Similarly, a positive role of NaCl in salt stress resistance in *Atriplex halimus* has been reported (Martinez et al., 2005), but the latter is a halophyte species that has probably adopted very different strategies than a sensitive glycophyte such as rice. Further statistical analysis, combined with ad hoc corrections such as Bonferroni's, will certainly allow us to better value these observations.

## 5. Validity of the biphasic model: does osmotic stress appear before ionic stress?

In order to understand the salinity resistance of plants to salt stress, Munns and Tester (2008) developed a model based on temporal considerations: the biphasic model. This model is a leading authority on the physiology of salt stress. From a conceptual point of view, it undoubtedly responds to logical considerations. According to this model, the presence of NaCl reduces the osmotic potential of the external environment. Consequently, even if the plant is in hydroponics or in soil where the presence of water has reached the field capacity level, the low osmotic potential makes this water unavailable for the roots (Rasool et al., 2013). This is the first phase of the model, also called the "osmotic component". The second phase of the model, called "the ionic component", results from the progressive accumulation of potentially toxic ions (Munns 2005; Munns and Gilliham 2015).

A number of questions, however, remain unanswered. Do these two phases occur sequentially, one after the other, or can they 'overlap'? If so, how long does this overlap last? Are the two components independent in their actions or can they influence each other? Finally, it is easy to define when there is salt stress, i.e. when the saturated soil paste extract is higher than 4 dS/m. This is an arbitrary definition that does not fully respond to a biological consideration. Munns' model follows a logic where the plant perceives the external osmotic potential and the internal toxic ion levels. Is it that simple? While we have a lot of knowledge about the transduction pathways (Acosta-Motos et al., 2017; Mansuri et al., 2019; Morton et al., 2019; Zhao et al., 2020), our knowledge

about the initial 'sensing' steps is much more fragmentary (Wang et al., 2018; Ponce et al., 2021).

At what point does a plant perceive a "water stress component"? Shouldn't "sensing" in relation to osmotic stress imply a change in the water status of root cells? And conversely, is a root unable to perceive the electrical conductivity of its environment, or even to obtain information on the nature of the ions responsible for this conductivity via epidermal information relays? These considerations, which consider that the osmotic stress of salt stress is external whereas the stress of ions is internal, would be useful to complete by a better knowledge of the perception signals: this does not call into question the validity of the model but validates it on a theoretical level without applying it directly to a concrete biological reality.

The question is relevant in rice in relation to the kinetics of osmotic adjustment that may allow the plant to cope with this osmotic component, which is assumed to be the first to act. Compatible organic solutes are expensive to produce and their synthesis is time consuming as it requires the activation of metabolic pathways that are only marginally active in the absence of stress (Alzahrani, 2021). The use of excess ions is an immediate and cheap solution for osmotic adjustment and Munns herself has recognized the validity of this hypothesis (Munns et al. 2020). The plant must then find a compromise between maintaining a water status by accumulating these ions and the risks of ion toxicity resulting from this maintenance: we are therefore no longer in the logic of components that act sequentially.

In rice, the absorption of mineral elements can in fact take place very quickly, and probably much more easily than in many other species. Twenty years ago, Lefèvre et al. (2001) already reported that highly toxic $Cl^-$ levels could accumulate in the tissues of *Oryza sativa* exposed to KCl even before the slightest significant disturbance in water status was observed. A similar observation was reported by Roshandel and Flowers (2009) for plants exposed to NaCl. In our work, an accumulation of ions was observed at the root level after 24 hours ($Na^+$ and $Cl^-$) but this was counterbalanced by a significant loss of $K^+$ (Chapter VI). At the aerial level, the accumulation of $Na^+$ and $Cl^-$ was marked after one day of treatment: there was indeed a reduction of osmotic potential of 0.2 MPa but it was also observed in the roots of PEG-treated plants that did not have excess ions at their disposal. In Chapter V, high rates of $Na^+$ accumulation at the root and leaf level after 2 days also resulted in only a moderate reduction of $\Psi_s$ at this time, and the value provided does not differ

from that measured on PEG-exposed plants. Our observations therefore do not support the hypothesis of an intervention of ions in the early osmotic adjustment processes. However, osmotic adjustment is not necessarily the only strategy for resisting the osmotic component of salt stress and an increase in parietal extensibility can intervene to maintain root elongation independently of this adjustment (Martinez et al., 2004).

The fact that the gene coding for DREB2 was not activated after 24 h of stress, while many genes coding for ion transporters are activated, both at the root and leaf level (Chapter VI), again suggests that a sequential intervention of the osmotic component followed by the ionic component is not a reality in *Oryza glaberrima*. It should also be noted that in this same species, other ionic toxicities such as iron toxicity, which does not induce a significant reduction in osmotic potential in nutrient solution, considerably alters the water status of the plant more than the salt stress that we are discussing here (Majerus et al., 2007a; 2007b).

## 6. What is the importance and nature of ionic toxicity?

Salinity is defined on the basis of the electrical conductivity of the substrate and does not prejudge the nature of the soluble salts present in the soil solution. While the majority of saline soils are indeed characterized by the presence of an excess of NaCl, it would be inaccurate to believe that other salts do not influence the electrical conductivity of the substrates concerned. Recent work has shown the prevalence of $Na_2SO_4$ in some regions and has highlighted that sodium sulphate can induce distinct effects of NaCl in rice (Irakoze et al. 2019, 2020) and exert a specific influence on soil properties (Irakoze et al. 2021a). In a recent study, Irakoze et al. (2021b) showed using a transcriptomic approach that only a very small proportion of genes were regulated identically when rice was subjected to NaCl and $Na_2SO_4$ salinity. However, almost all studies on the physiology of salt stress in rice have focused on NaCl, and it is this salt that we have studied in *Oryza glaberrima*.

Almost half of the world's arable land will be degraded by salinity by 2050 (Butcher et al., 2016; Cuevas et al., 2019; Ivushkin et al., 2019). Moreover, due to climate change and anthropogenic actions, most crop plants are already subject to salt stress or at best will be within a few years. Since a soil always

contains a certain amount of soluble salts, there is an urgent need to understand how the ionic component impacts the physiological and molecular mechanisms of *Oryza glaberrima* plants. However, the ionic component of salinity is a complex constraint in itself as it involves the accumulation of cation ($Na^+$) and anion ($Cl^-$). While most studies focus solely on the impact of $Na^+$ to elucidate the ionic component of NaCl, in this thesis we used a simple but effective protocol to discriminate between sodium and chloride toxicity on *Oryza glaberrima* cultivars (Chapter III). This protocol has been previously tested on Asian rice and barley (Kumar and Khare, 2016; Khare et al., 2015, 2020; Tavakkoli et al., 2010, 2011). It is based on the use of a sodium-rich ($Na^+$-dominant) solution cocktail, a chloride-rich ($Cl^-$-dominant) solution cocktail, and a NaCl solution (Chapter III).

Our results on *Oryza glaberrima* suggest that the $Na^+$-dominant solution is generally more toxic than the $Cl^-$-dominant solution. However, the highest toxicity was recorded in *Oryza glaberrima* plants treated with NaCl. We also noticed that the impacts of $Na^+$ and $Cl^-$ on the studied parameters sere additive, except for mineral nutrition where synergistic interactions were recorded for $Na^+$ and S accumulation. The behaviour of TOG5949 was more affected than the behaviour of TOG5307 for most parameters and for all types of ionic toxicity (NaCl, $Na^+$-dominant, $Cl^-$-dominant). Indeed, TOG5307 showed a higher level of resistance than TOG5949 in terms of maintaining growth and photosynthesis and controlling $Cl^-$ and $Na^+$ accumulation. Our data confirm 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 calculation of specific ion toxicity indices for $Na^+$ and for $Cl^-$ (Chapter III) shows that both ions acted globally on the same parameters but that the effect of $Na^+$ was quantitatively much more important than the effect of $Cl^-$.

Under saline conditions, several ion transporters and channels play a key role not only for ion uptake, but also for long-distance translocation, distribution between organs and compartmentalization within cells (Teakle and Tyerman 2010; Zhao et al., 2020). Using inhibitors of certain $Na^+$ transporters (amiloride), we recorded an increase in $Na^+$ in all organs, with a greater impact in the salt-resistant cultivar (TOG5307) than in the salt-sensitive cultivar (TOG5949). This suggests that the $Na^+/H^+$ antiporter (SOS1) plays an important role in the salinity resistance of *Oryza glaberrima*. The use of bumetanide, an inhibitor of certain $Cl^-$ transporters, reduced $Cl^-$ accumulation in both cultivars as well as $K^+$ accumulation in TOG5307 and $Na^+$ accumulation in TOG5949. This suggests that the resistant cultivar would

preferentially use $K^+$ as a $Cl^-$ cotransporter while the sensitive cultivar would preferentially use $Na^+$ for this purpose (see chapter IV). However, there is still ambiguity as to the role of SOS1 at the level of the vascular blades, since it is not yet clear whether this transporter is involved in the loading or unloading of $Na^+$ at the xylem level. The fact that the use of bumetanide increased the cell viability of TOG5949 leaf cells and reduces the senescence processes that are one of the major manifestations of salt stress in rice (Lutts et al. 1996b) suggested that $Cl^-$ ions play a role in the induction of senescence processes in the sensitive *cv* but not in the resistant cv. It should also be noted that in the two *O. glaberrima* cultivars tested in the present study, the roots were not retention sites for $Na^+$ and that this element therefore easily reaches the aerial parts, whereas a much higher root sequestration is generally reported for this element in *Oryza sativa* (Lutts et al. 1996a; 1996c).

The stimulation by salinity of the expression of the *SOS1* gene was expected since this gene limits $Na^+$ uptake (Chen et al., 2021; Liu et al., 2021). While this gene is expressed in plants that are not exposed to salinity and therefore has a basic constitutive expression, it is noteworthy that the expression of *SOS2* was only observed in the presence of salt: as *SOS2* is required for the activation of *SOS1*, this would tend to suggest that the SOS1 protein was present but not active in plants that were not exposed to salinity, and that the activation of the SOS system was based on the expression of the activating gene *SOS2* by NaCl. Similarly, stimulation of the expression of the *NHX1* and *NHX2* genes was consistent with the implementation of a resistance strategy since these transporters are involved in the vacuolar sequestration of the toxic $Na^+$ ion. However, our results show that *NHX1* was only expressed in the aerial parts, whereas *NHX2* is expressed in both the aerial and root parts.

The *OsHKT1;5* gene plays a key role in $Na^+$ exclusion mechanisms: *HKT1;5* allows $Na^+$ retrieval from the xylem to the phloem, thereby preventing its accumulation in photosynthetically active leaf blades (Kobayasi et al., 2017). In *O. sativa*, this gene was expressed in both roots and leaf sheaths. *In O. glaberrima*, we found that this gene was not expressed in the aerial parts after 1 day of treatment, and that it was expressed only very weakly and comparably to the control, after 7 days of treatment. A low level of expression of this gene means that there is little recycling of $Na^+$ from the aerial parts to the roots and would therefore fit in well with the low capacity for root sequestration of $Na^+$ that we have just mentioned in *O. glaberrima*. Nevertheless, the fact that this gene was not expressed in the control plants after 1 day, whereas it was after 7 days when these plants had not received any treatment, does not exclude that

an artefact has occurred in our manipulations. This result must therefore be confirmed.

A transcriptomic approach certainly provides interesting information but must be qualified. It is not because the expression of a gene is activated that the biochemical or physiological processes it controls are stimulated. Kubala et al. (2015) conducted a parallel transcriptomic and proteomic approach in oilseed rape exposed to salinity: the overexpression of many genes did not result in the accumulation of the corresponding protein. It is a simplification to equate gene expression with the activation of a metabolic process. It is not uninteresting in this context to note that the differences between our two cultivars were much larger for $Na^+$ and $Cl^-$ contents than the differences recorded for the genes coding for transporters. Ganie (2020) points out that post-transcriptional regulation of a gene involves multiple proteins (RNA-binding proteins (RBPs)) that have critical functions in splicing, nucleoplasmic translocation and translation control. If one of these players is missing, an RNA can accumulate without any metabolic change, leading an unwary experimenter to assume that a metabolic pathway is activated when it is in fact repressed.

In the present study, ion concentrations were measured at the level of the entire root system, or at the level of all leaves, or at the level of a single leaf of a certain order. This strategy provides useful information, but is not intended to be exhaustive. For example, it has been shown in *Oryza sativa* that the preferential sequestration of toxic ions such as $Na^+$ in the oldest leaves is an effective resistance strategy in that it aims to sacrifice metabolically inactive older leaves to preserve younger leaves whose photosynthetic activity is important for the survival and growth of the stressed plant (Yeo et al., 1990; Lutts et al., 1999a).

In addition to this differential distribution between organs, a differential distribution within organs is particularly interesting to consider. Estimating $Na^+$ or $Cl^-$ contents at the level of a whole leaf does not provide any information on where these elements are located and the same content may correspond to very different distributions. The physiological consequences of a toxic element depend on both its content AND its location. Thus, an accumulation in the epidermis will not have the same consequences as an accumulation in a chlorophyll parenchyma. The same reasoning can be applied at the cellular level: while a parietal or vacuolar accumulation may have no effect on cellular metabolism, a cytosolic, chloroplastic or

mitochondrial accumulation may have disastrous consequences for cellular functioning. It is therefore particularly useful to have reliable techniques that can account for this distribution. In *Oryza glaberrima*, Fernandes et al. (2020) have successfully applied RBS (Rutherford backscattering spectrometry), ERDA (elastic recoil detection analysis), PIXE (particle induced X-ray emission spectroscopy) and PIGE (proton induced gamma-ray emission) techniques that allow them to obtain precise information on the distribution of a wide range of elements, including Na and Cl. The application of such techniques to plants exposed to salinity could prove particularly interesting, although the cost of these techniques still limits their application to a small number of samples.

## 7. How does the duration of the stress influence the nature of the salinity response?

As mentioned in point 1 of this discussion, the plant is not able to predict how long the stress will last in the future. It “manages the present situation”, but may have to change its strategy if the stress continues for a long period. Quinet et al. (2012) compared the behaviour of an *Oryza sativa* cultivar subjected to iron toxicity for 3 days and 3 weeks: less than 10% of the genes were regulated in the same way when comparing the two durations. The plant therefore 'adapts' its behaviour over time and this physiological plasticity is a valuable asset under stress conditions. The choices made by the experimenters do not always help to understand this phenological plasticity. In molecular studies, the stress intensities are often very high and the stress durations are very short. Conversely, in a physiological study context, the durations are longer and the stress intensities are more realistic, but a kinetic approach involving analysis after several durations is not always envisaged.

Two durations were considered in Chapters V and VI: "short" durations (1 or 2 days) and "longer" durations (one or two weeks). In both chapters the duration factor had a very high significant effect. Of course, this does not imply that the response strategy of the plant changes, but it does indicate that the expression of damage symptoms increases and/or the adaptive response intensifies as the stress is prolonged. This is indeed the case for leaf $\Psi$s, $Na^+$

and $Cl^-$ accumulation, instantaneous transpiration and stomatal conductance. Other parameters, which were not affected after two days of exposure, were modified after two weeks: this was the case for biochemical parameters such as MDA, proline and flavonoid contents. It is possible that a delay is required for a change in metabolism to materialize in the accumulation of these compounds. The only parameter for which a trend reversal was observed concerns the net photosynthesis of TOG5307 plants exposed to PEG, which increased after 2 days and decreased significantly after 15 days (Chapter V). The difference in duration between "short" and "long" treatments was therefore not sufficient to induce a net change in strategy.

While the "duration" factor had a significant impact on all the parameters measured, this was much less the case for the duration x cultivar interaction, which was non-significant for several parameters, particularly for parameters related to the light phase of photosynthesis. Moreover, these interactions were also rarely significant for the expression levels of the genes studied, which shows that the two cultivars selected were influenced in a comparable way by the duration of the stress.

We have pointed out above (see point 4) that the two cultivars studied appeared to be more sensitive to the ionic component of salt stress than to the osmotic component. To see if the relative importance of these two components varies over time, we compared the $TI_{PEG}$, $TI_{NaCl}$ and $TI_{MIX\ OBS}$ of the two cultivars after 2 days and after 15 days of treatment. As expected the values increased for both cultivars studied but do they increase in the same way? The increase in $TI_{PEG}$ was 2.15 times for TOG5307 and 2.47 for TOG5949. The increase in $TI_{NaCl}$ is 3.85 times for TOG5307 and 3.65 times for TOG5949. The increase in $TI_{MIX\ OBS}$ was 2.24 in TOG5307 and 2.79 in TOG5949. In other words, the osmotic component remained the lowest constraint and its increase was greater in the sensitive cv. than in the resistant cv. The ionic component was still the most important and showed the highest increase, but the increase was lower in the sensitive *cv* than in the resistant one.

## 8. What is the level of resistance to salt stress during the reproductive phase and its influence on yield parameters?

Besides the vegetative phase, which has received much attention, flowering is also considered to be one of the most salinity-sensitive phases in rice (Khatun and Flowers 1995; Lutts et al., 1995). The experiment to determine the yield parameters of the two *O. glaberrima* cultivars studied was carried out in Benin, with plants grown on artificially salinized soil (Chapter II). The growing conditions were therefore significantly different, even though the plant material was identical. Studying a range of *Oryza sativa* cultivars, Lutts et al. (1995) established that resistance to salinity in the germination phase, or in the vegetative growth phase, does not necessarily imply significant resistance in the flowering and grain maturation phase. For the two *Oryza glaberrima* cultivars in our study, however, this correspondence exists and the variety TOG5307 that accumulated the least $Na^+$ in the vegetative phase was also the one that accumulated the least in the grain and glumes. In *Oryza sativa*, Zhu et al (2001; 2004) have already hypothesized that $Na^+$ levels quantified in the vegetative phase could be used as a predictive factor for yield parameters. A comparable approach should be carried out on a wide range of *O. glaberrima* cultivars in order to make a sound statement in this respect.

In *Oryza sativa*, salinity induces many panicle exertion problems (Lutts et al. 1995) that we did not find in our study. The salinity-induced yield reduction in the Asian species is associated with a reduction in the number of spikelets per panicle and the percentage of fertile tillers (Zhu et al., 2000; 2004; Lutts et al., 1995). These observations suggest a deleterious effect of salt stress during panicle formation well before panicle exsertion. While comparable effects were observed in our two *Oryza glaberrima* cultivars, they appeared to be less significant than those reported by Zhu et al. (2000; 2004) who worked at equivalent doses of 30 mM in *O. sativa*. *O. glaberrima* differs from the Asian species by a higher tillering and a less branched panicle and one could wonder whether these morphological differences could be related to the impact of salt on these parameters.

Another possible cause of salt-induced yield loss in rice is a reduction in pollen viability, which is thought to lead to increased spikelet sterility. According to Khatun and Flowers (1995), a reduction in pollen viability is directly related to the accumulation of $Na^+$ and $Cl^-$ in mature pollen. We did not take pollen viability into account in our work. It should be noted that

pollen accumulation of toxic ions is not necessarily the only parameter to be considered: using a laser ablation technique in tomato, Ghanem et al. (2009) showed no accumulation of these ions in the pollen and attributed the alteration of pollen viability to tapetum dysfunction and altered sugar supply. Abdullah et al. (2001) confirmed that such a difficulty in sugar supply can cause sterility in rice and Yang et al (2002) highlighted the importance of phytohormones such as abscisic acid and cytokinins in controlling this process in plants exposed to salinity.

An accumulation of $Na^+$ and $Cl^-$ has also been reported at the level of stigmas and styles (Ghanem et al., 2009) and could be at the origin of a reduction in the receptivity of stigmas and malformations at the level of flowers (Ekanayake et al., 1989). According to the latter authors, salinity could induce in rice, a highly cleistogamous species, an early opening of the flowers generating a significant risk of desiccation. Other hormonal compounds, the polyamines (and mainly putrescine) play an essential role in floral morphogenesis. Ndayiragije and Lutts (2006; 2007) showed that an application of putrescine to rice plants under salinity can increase pollen viability and the number of spikelets per panicle.

A priority in *Oryza glaberrima* would therefore be to study the flowering stages that precede fertilization and grain filling by paying particular attention to the hormonal status of the floral structures and by studying (e.g. using the techniques discussed in point 6) the precise distribution of $Na^+$ and $Cl^-$ within these structures.

## 9. *Oryza glaberrima*: a boon for Beninese rice cultivation?

Benin is located in West Africa in the tropical zone between the equator and the Tropic of Cancer. The entire 125 km of the south of the country faces the Atlantic Ocean. In addition, Benin's coastal strip is low and sandy. This exposes the country to problems of marine intrusion and capillary rise of certain water tables. As a result, most of Benin's coastal land is subject to soil salinization. This makes it difficult to grow certain species. For example, Ezin et al. (2012) showed that salinity has severely affected the growth and development of tomatoes grown in coastal areas of the Republic of Benin. In addition to soil salinity, these authors demonstrated that the wet breeze of high

tide and the watering of crops with salty water are significant contributors to salinity on the Beninese coast. Mc Innis et al. (2013) used electrical resistivity tomography (ETR) techniques to demonstrate that salinization by seawater is not limited to the coastal region and that particular hydrological conditions can lead to salinization of groundwater in the interior of the country. Salinity has a very heterogeneous distribution that makes it difficult to take into account the whole problem of soil salinization, but there is reason to believe that these problems have been greatly underestimated (Darboux-Afoudat et al., 1997; Silliman et al., 2010). Many areas in central Benin that are suitable for lowland rice cultivation have been experiencing increasing salinization problems for several years (Danvi et al., 2016). Given the sensitivity of rice to salinity, it is now important to pay particular attention to this issue.

Current rice production in Benin is 406,000 tons, or a yield of 4 to 5 tons per hectare (Faostat, 2020). However, this quantity is very insufficient for the 11 million Beninese. Thus, Benin had to invest nearly 500 billion CFA francs in 2018 to be able to import rice and thus meet consumer demand. To compensate for this loss of capital, the Beninese government has launched an emergency program to accelerate rice production in order to reach 1 million tons by 2022. The country's goal is to reach a yield of 7 tons per hectare. Although rice can be grown on five of Benin's seven agricultural development poles (Zannou et al., 2018), it is also crucial to take into account the potential of the saline lands located along the 125 km of Benin's coast (from Sèmè-kpodji through Ouidha to Grand-Popo). In our doctoral research we have shown that the African rice *Oryza glaberrima* has the potential to cope with the problem of soil salinization. Indeed, we have clearly demonstrated that some cultivars of *Oryza glaberrima* are resistant to salinity. For example, the cultivar TOG5307 is able to maintain high net photosynthesis under salinity conditions, to exhibit a high level of tolerance to accumulated $Na^+$ ions and to effectively regulate chloride accumulation in its aerial parts (Chapter I). In addition, the cultivar TOG5307 has the highest resistance at the reproductive stage and is able to produce rice grains with low $Na^+$ content, which is sequestered in the glumes (Chapter II). This *Oryza glaberrima* cultivar could therefore be grown on saline soils where *Oryza sativa* can no longer be grown and where the soil, already showing excessive electrical conductivity, is no longer recoverable.

However, the use of hardy material on marginal areas does not obviate the need for effective measures to reduce soil salinization in the future. Numerous studies show that the poorest rice farmers in Benin are those who do not use

irrigation techniques (Nonvide 2018; 2019). Several initiatives are advocated to ensure the development of irrigation in rice farming, notably through the granting of micro-credits (Dossou et al., 2020). These projects have the merit of aiming to increase yields in the short term but have the disadvantage of not taking into consideration the risks of salinization in the long term: it is however well established that there is a link between irrigation and soil salinization, especially when irrigation is carried out with poor quality water, and it will therefore be crucial, in the future, to define irrigation policies that allow for a maximum reduction in the risks of salinization by taking into account all local climatic and agro-hydrological parameters (Singh 2018).

*Oryza glaberrima* is not unknown to the Beninese. It is found in the north of the country where it persists due to local socio-cultural considerations (Loko et al., 2021). It is therefore imperative that the Beninese authorities consider the development of *Oryza glaberrima* in Beninese rice production. Its interest is undeniable for its resistance to numerous biotic constraints, particularly viral diseases (Anatao et al. 2021). It has been little studied for its response to salinity and our thesis shows that interesting characteristics exist within this species for salinity resistance. Since 2006, the qualities of *O. glaberrima* have mainly been exploited in the framework of interspecific hybridization with O. *sativa* and the selection of NERICA varieties. The undoubted qualities of the latter have generated much excitement, but some hopes have been dashed and many farmers have stopped growing these varieties (Yokouchi and Saito, 2017). This is because these improved varieties require high-performance cultivation conditions that farmers (especially the poorest ones) are unable to implement due to lack of adequate technology and information on their specificities. Another problem for the use of NERICA varieties is access to good quality seed in sufficient quantities. The private sector is not interested in the issue, and the establishment of participatory farmer networks to ensure community-wide seed production has not yet been proven to work (Yokouchi and Saito 2016).

Salinity is a complex stress. Therefore, we also proposed simple but efficient protocols to discriminate between water and ionic components of salt stress (Chapter III and V). Our work thus opens the way to understanding the mechanisms of salinity tolerance in African rice. The adoption of hardy resistant cultivars, such as TOG5307, will not be a universal panacea to the problem of soil salinization, but it may be part of the solution, especially for the poorest farmers with limited access to land and credit. However, data on the actual level of salinization of these Beninese soils is still rather limited. It

is therefore important in the future to carry out structural studies to assess the level of salinity of land and water on the Beninese coast but also in the more arid regions of the country.

# Conclusions and perspectives

Our thesis has shown that there is sufficient variability within the African rice species *Oryza glaberrima* to identify cultivars with contrasting levels of salinity resistance. A comparative study was performed in the presence of NaCl with the salt-resistant cultivar TOG5307 and the salt-sensitive cultivar TOG5949 It showed that the resistant cultivar accumulated less $Na^+$ in all its organs and less $Cl^-$ in the aerial parts than the sensitive cultivar. Salt resistance is therefore mainly based on an avoidance strategy, but concomitant tolerance mechanisms may exist in the resistant cultivar TOG5307 for $Na^+$ accumulation probably in relation to an apoplastic localization of this element.

For the salt doses used, both cultivars are more affected by the ionic component of salt stress than by its osmotic component. *Oryza glaberrima* therefore has adapted strategies to ensure optimal regulation of its water status under stress conditions, although tolerance to partial dehydration of the leaf blades can be envisaged in the resistant cultivar TOG5307.

For both the sensitive and the resistant cultivar, $Na^+$ is more toxic than $Cl^-$. Both ions act on the same parameters and the toxicity of NaCl corresponds to an additive action of the toxicities of each ions, except for some parameters such as S contents for which a synergistic action is demonstrated. The application of amiloride (inhibitor of transporters involved in $Na^+$-$H^+$ antiport) underlines the importance of root exudation and vacuolar sequestration strategies of this element in the resistance mechanisms. The effects of bumetanide, an inhibitor of CCC (cation-chloride-cotransporters) suggest that this co-transportation preferentially involves $K^+$ in resistant *cv* and $Na^+$ in sensitive *cv*.

The use of a non-penetrating osmotic agent (PEG) confirms that the osmotic component of salt stress is less important than the ionic component. Regulation of water status is based on a reduction in stomatal conductance to limit water loss through transpiration and an osmotic adjustment process to ensure water supply. The accumulation of $Na^+$ and $Cl^-$ ions is rapid and is

accompanied by a reduction in $K^+$ levels. This ion accumulation appears to be faster than the synthesis of compatible organic compounds such as proline and Munns' biphasic model may therefore not involve the osmotic and ionic components sequentially, with a likely temporal overlap of the two phases in *Oryza glaberrima*. Comparison of the effects of PEG, NaCl and a mixed treatment (PEG+NaCl) showed that the two agents acted additively in the sensitive cultivar and partially antagonistically in the resistant one.

The study of the expression of genes coding for $Na^+$ transporters shows, in both cultivars, a stimulation of the expression of *NHX1* and *NHX2* genes involved in the vacuolar sequestration of this toxic element, and of the SOS1 gene involved in the rejection of $Na^+$ to the surrounding medium at the root meristem level. However, the latter gene is also expressed in unstressed plants, while the gene coding for the SOS2 protein that activates SOS1 is only expressed in the presence of NaCl. The *CCC1* gene involved in the retention of chloride ions in the vascular tissues of the central cylinder, as well as the *CLC1* gene involved in the vacuolar sequestration of chloride ions, are also overexpressed in the presence of NaCl. On the other hand, two *HKT* genes (*HKT1;5* and *HKT4*) that play a major role in the recirculation of $Na^+$ through the phloem in order to preserve photosynthetic tissues in the Asian species *Oryza sativa* play only a minor role in *Oryza glaberrima*, which could explain the low root $Na^+$ retention capacity observed in this species. Moreover, the fact that the differences between the two cultivars are more marked for the levels of accumulated toxic ions than for the expression levels of the genes coding for the transporters suggests the existence of post-transcriptional regulation mechanisms, the importance and nature of which remain to be specified.

The distinct levels of salinity resistance observed in the vegetative phase in the two cultivars are also observed in the reproductive phase in plants grown under semi-controlled conditions on artificially salinized soil. The resistant cultivar TOG5307 accumulates less $Na^+$ in glumes and degloved grains than the sensitive cultivar TOG5949. The $Na^+$ levels observed in the vegetative phase could thus possibly serve as an early predictor of subsequent behavior during the reproductive phase. Salt delayed panicle exertion and reduced yield parameters in both cultivars by significantly decreasing the number of grains per panicle, the percentage of full grains, the weight of grain per plant, the weight of 1000 grains, the number of fertile tillers per plant and the length of the panicle. For all these parameters, the effect of salt was more pronounced in the sensitive cultivar than in the resistant cultivar.

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*The data collected in the framework of this thesis constitute an initial knowledge base on which further work could be based.*

*By exploiting the tools available for* Oryza sativa*, it would be very interesting to complete the present study with omic approaches (transcriptomics, proteomics and metabolomics) in order to have a more complete overview of the effects of salinity on the material studied. Hormonal profiling of this material could also be of great interest, both in the vegetative phase and during reproduction. A complete follow-up of the material throughout its cycle (germination, seedling, tillering, heading, anthesis, grain maturation) would allow us to refine our initial information and to better target the kinetic aspects of the strategies implemented by the plant.*

*It would be useful to complete our approach of the reproductive phase by studying the progamic phase that precedes fertilization. Consideration of pollen viability, analysis of flower structure, estimation of stigmatic receptivity and study of pollen tube progression would help us to better understand the causes of salt-induced spikelet sterility.*

*Ion distribution analyses (ion mapping by LA-ICP or micro-PIXE) should be undertaken in order to identify the precise location of toxic ions within tissues and even cells in cultivars with different levels of salinity resistance. As far as possible, the study should be applied to different organs (roots, leaves of different ages, flowers) and could also integrate the distribution of elements such as K and Mg whose levels are reduced by the application of salt stress.*

*On the other hand, it would be useful to extend the study to a much wider range of cultivars in order to better understand the variability within this species. The effect of salts other than NaCl, which can lead to salinity problems in the field, should also be taken into consideration.*

*In the context of climate change, where abiotic constraints will become increasingly important, a hardy plant such as* Oryza glaberrima *is worthy of interest and this ancient species, domesticated long ago, could have a bright future.*

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*« Il paraît toujours impossible, jusqu'à ce qu'il devienne réalité. »*

*Nelson Rolihlahla MANDELA*

*« Quand on veut une chose, tout l'Univers conspire à nous permettre de réaliser notre rêve. »*

*Paulo Coelho*