Discriminating the effects of the anionic components of salt-stress in rice (*Oryza sativa* L.): comparison between NaCl and Na_2SO_4 toxicities

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

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“It always seems impossible until it is done.”

Nelson Rolihlahla Mandela

List of abbreviations

ABA: Abscisic acid
ANOVA: Analysis of variance
AOAD: antioxidant activity measured in dichloromethane extract
AOAM: antioxidant activity measured in methanol extract
cDNA: complementary deoxyribonucleic acid
CEC: Cation Exchange Capacity
cv.: cultivar
DNA: Deoxyribonucleic acid
DPPH: α, α-diphenyl-2-picrylhydrazyl
DTT: dithiotreitol
EC: Electrical Conductivity
ESP: Exchangeable Sodium Percentage
FAO: Food and Agriculture Organization
FAOSTAT: Food and Agriculture Organization Statistics
GSH: reduced glutathione
GSSG: oxidized glutathione
HKT: High-affinity K^+ transporter
HPLC: High-Performance Liquid Chromatography
HSD: Honestly Significance Difference
IGEBU: Institut Géographique du Burundi
MDA: Malondialdehyde
mRNA: messenger ribonucleic acid
NHX: Na^+/H^+ exchanger
OPA: orthophthalaldehyde
PCR: Polymerase Chain Reaction
pH: hydrogen potential
PSII: Photosystem II
qPCR: quantitative Polymerase Chain Reaction
QTL : Quantitative Trait Loci
RNA: Ribonucleic acid
ROS: Reactive Oxygen Species
RT-PCR: Reverse Transcriptase- Polymerase Chain Reaction
SAR: Sodium Adsorption Ratio

SOS: Salt Overly Sensitive
TDS: Total Dissolved Salts
USDA: United States Department of Agriculture
v/v: volume/volume
w/v: weight/volume

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General introduction and objectives

According to the FAO estimates, by 2050, the world food supply would have to increase by up to 70% - nearly 100% in developing countries to feed the world population of over nine billion people globally (Dubois 2011). Unlike these growing food needs, a number of abiotic stresses including soil salinity globally threatens staple crop production. Global agricultural production is negatively affected by the gradual salinization of arable land, especially in arid and semi-arid regions where salts from basal rocks come into the upper layer of soil due to evapotranspiration mechanism and where unsuitable irrigation procedures are commonly used. According to Kosová et al. (2013), about 7% of world agricultural land is affected by salinity and this number could increase up to 20% in the future due to land salinization as a consequence of an artificial irrigation and an unsuitable land management.

Rice (*Oryza sativa* L.*)* is recognized as one of the most important crops in the world since it provides the main source of energy for more than half of the world population (Gnanamanickam 2009). Rice production indeed represents 30% of the world cereal production and, hence, rice is the second largest produced cereal in the world. In Burundi, rice is the second most consumed cereal food after maize. Thus, the average level of consumption per capita reaches high levels of 50 kgs per year in urban centers (Gahiro 2011).

This species is extremely sensitive to salt stress. Current guidelines indicate that rice yields decrease by 12% for every unit (dS/m) increase in electrical conductivity of saturated soil extract above 3.0 dS/m (Grattan et al. 2002). Munns and Tester (2008) showed that soil salinity stresses plants in two ways. Firstly, high accumulation of salt ions in the soil solution makes it harder for roots to extract water; this is referred to as the osmotic effect of salinity. Secondly, high concentrations of salt ions within the plant can be detrimental to cell metabolism in the transpiring leaves; this is called the ion-excess effect of salinity. These salt effects not only reduce water potential but also cause ions imbalance and toxicity inside the plant; resulting in reduction of plant growth and development as well as in decrease of crop production (Parihar et al. 2015). The detrimental effect is observed at the whole plant stages, from germination to grain maturation, although the seedling stage and flowering phases are commonly considered as

the most sensitive steps in rice (Lutts et al. 1995). Salt stress affects all the major processes in plants, including plant growth, photosynthesis, hormonal and plant water status, ion nutrition and oxidative status.

Remaining fixed on the substrate, plants have developed a whole set of biochemical and molecular mechanisms as an attempt to cope with salt stress. According to Parida and Das (2005), biochemical pathways leading to products and processes that improve salt tolerance are likely to act additively or even synergistically. The author showed that the biochemical protecting strategies include selective accumulation or exclusion of ions, control of ions uptake by root and transport into leaves, compartmentalization of ions at the cellular and whole plant levels, synthesis of compatible solutes, change in photosynthetic pathway, stabilization of membrane structure, induction of antioxidative enzymes and antioxidant compounds as well as modification in plant hormones metabolism.

NaCl represents the major soluble salt causing soil salinity in the world and the vast majority of studies dealing with plant response to salinity stress have been conducted using NaCl salinity. An excess of sulfate salts, however, may also affect some areas and this is especially the case in Burundi where the lower valley of the Rusizi plain is affected by sodium sulfate from volcanic origin aggravated by poor management of irrigation and drainage (Nijimbere 2014b). Na_2SO_4-based salinity has already affected more than 15% of the arable land in the lower valley of the Rusizi plain and 182 fields of at least 0.5 ha each have been completely abandoned in 2010 (Kabwa 2010). The lower valley of the Rusizi plain being considered as the breadbasket of rice in Burundi, the reduction in rice production caused by salt stress led to a dramatic negative effect on the rice farmer’s well-being and the country's economy. The use of several land-improvement techniques has not been able to recover the part affected by salinity and the main route currently being proposed to solve the problem is the selection of rice cultivars tolerant to Na_2SO_4 salinity; this procedure requiring in-depth knowledge of the rice specific plant's tolerance mechanisms to sulfate salinity.

Despite its economic importance which has been evidenced by several authors (Reginato et al. 2019; Reich et al. 2017; Samson et al. 2016; Thapa et al. 2017; Yokas et al. 2008), rice responses to Na_2SO_4 salinity only received minor attention. **The basic scientific reasoning that**

inspired us to conduct this research work is that Na_2SO_4 salinity could negatively affect the physiological processes of rice crop and that, in response to stress, the rice plant could establish Na_2SO_4-specific tolerance mechanisms.

It remains uncertain whether a higher toxicity of either NaCl or Na_2SO_4 in plants is due to an altered toxicity of sodium ion or an additive effect of both cation and anion. **The general objective of our thesis is therefore to elucidate the effects of anionic components of salt stress on normal functional status of rice (*Oryza sativa* L.) plant.**

Two specific objectives are considered:

- ✓ **Comparison between NaCl and Na_2SO_4 effects on rice physiological, biochemical and molecular parameters.**
- ✓ **Assessment of the tolerance mechanisms adopted by the rice plant exposed to chloride or sulfate salinity.**

This PhD thesis intends to provide an answer to the five following questions:

- ✓ Does the anionic component of salt stress have a specific impact on rice behavior for a same Na^+ external concentrations?
- ✓ Are the differences between the NaCl and Na_2SO_4 toxicities on rice similar at the vegetative and reproductive phase of development from both a quantitative and a qualitative point of view?
- ✓ Could the "NaCl-resistant" rice cultivars be considered as "Na_2SO_4-resistant" as well?
- ✓ Do the differential responses to chloride and sulfate salinities partly rely on specific gene expression?
- ✓ Are the data obtained for the two types of salinity in controlled conditions and hydroponic culture comparable to those obtained in field conditions on a soil substrate?

This document consists in two main parts. The first part presents a literature review summarizing the description of the rice plant as well as current knowledge on the effects of salinity on cultivated plants. We then describe the tolerance mechanisms to saline stress adopted by some plants in relation to mineral nutrition, oxidative stress management and osmotic adjustment processes. We also talk about the

soil salinization process and a brief description of Rusizi plain close this part.

In the second part, we present the results of different experimental studies conducted throughout our thesis and organized in five separate chapters. The first chapter reports on a study comparing the effects of NaCl and Na_2SO_4 salinities on two contrasting rice cultivars (*Oryza sativa* L.), one sensitive to saline stress (I Kong Pao) and the other tolerant to saline stress (Pokkali). The analyzed parameters concern the growth and water status of the stressed seedlings. Subsequently, the responses to salinity of the two cultivars have been analyzed in relation to osmolytes and antioxidants synthesis. This study aims to better understand the mechanisms adopted by the rice seedlings to overcome osmotic and oxidative stresses.

The second chapter shows the results of a study that highlighted the effects of NaCl and Na_2SO_4 on photosynthesis and yield-related parameters in rice. First, the effect of both types of salinity was compared on gas exchanges at leaf level during the seedling and tillering stages. Secondly, the effect of salinity on the synthesis of photosynthetic pigments was evaluated. Thirdly, the effect of salinity was analyzed on different yield components and finally, the concentration of salt ions was quantified in the floral organs namely hulls and endosperm.

The third chapter concerns a combined transcriptomic and physiological study, which was performed on rice salt-sensitive cultivar (IKP) in order to elucidate the genome-level responses to chloride and sulfate salinities. The results of this study offer the first comprehensive picture of genome expression modulation in response to sulfate salinity in two distinct rice organs (shoots and roots) and could be very useful in improving rice plant tolerance to Na_2SO_4.

The fourth chapter focuses on the differential effects of salt stress on Na^+-regulatory mechanisms. As attempt to explore the difference between the mechanisms underlying NaCl and Na_2SO_4 tolerance in rice via excess Na^+ exclusion or sequestration, the expression profile of twelve genes encoding Na^+ transport was analyzed and the salt effect on some physiological parameters was evaluated. The gene expression profiles were determined depending on plant organ and stress duration. We also compared the results of our transcriptomic study (Chapter 3)

with the results obtained in this chapter in order to highlight the influence of the analysis method on the gene expression profile.

For these four chapters, the experiments were conducted under hydroponic conditions. However, the interactions between root-zone environments and plant responses to increased osmotic pressure or specific ion concentrations in the field are complicated by many soil processes, which have been far less studied for Na_2SO_4 than for NaCl salinity. Thus, the fifth chapter presents the results of a study conducted in soil conditions in order to elucidate the effect of chloride and sulfate salinities on the soil chemical properties as well as on rice physiological- and yield-related parameters.

As presented in Fig. 1, chapter 1 and 2 should allow us to give an answer to questions 1, 2 and 3. Chapter 3 and 4 should help us to answer to question 4. Finally, chapter 5 will provide a partial answer to question 5.

At the end of this research work, a general discussion will allow us to integrate all experimental achievements in relation to the problems pointed out. We will briefly and clearly outline the differential effects of chloride and sulfate salinities on the physiological, biochemical and molecular parameters of rice plant. In this part, we will also describe the tolerance mechanisms to salt stress adopted by the different rice cultivars. The outlooks of the future studies will then be presented.

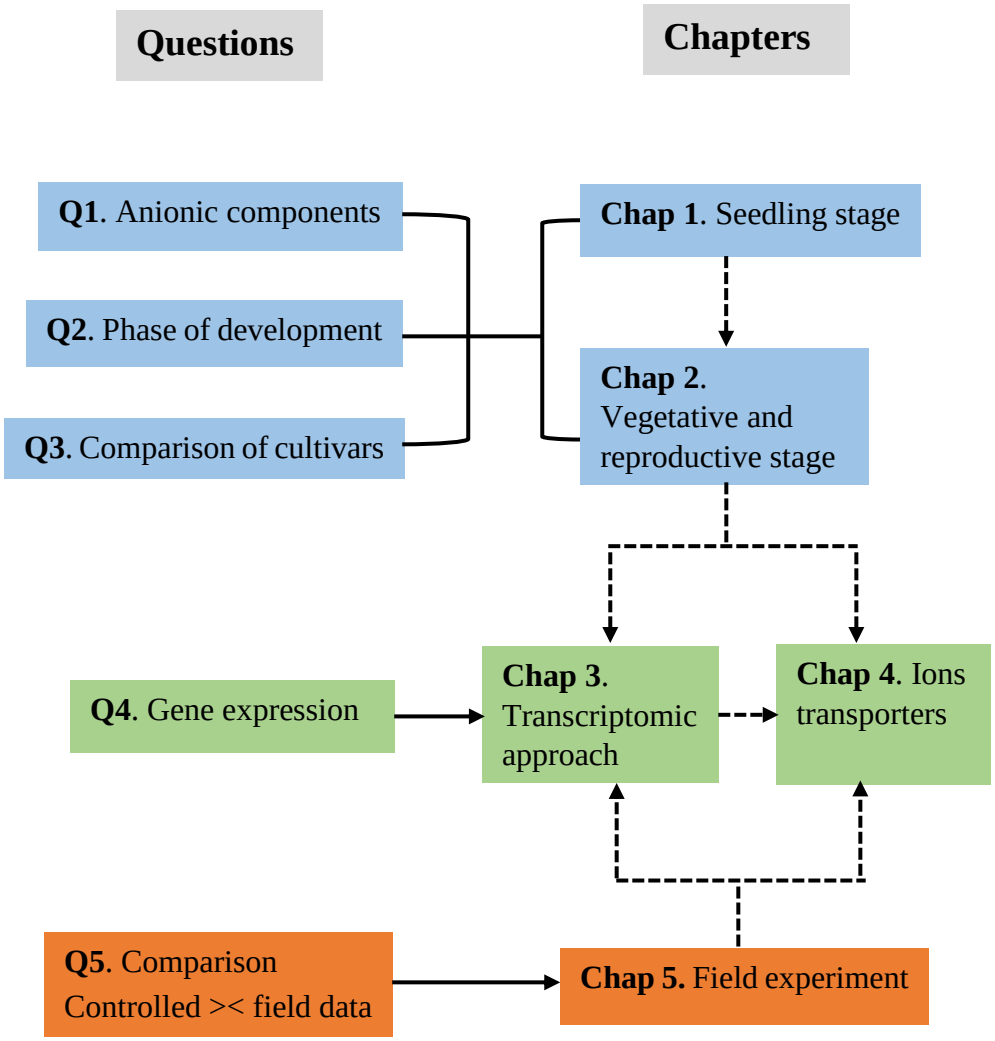

Figure 1. Synthetic overview of the five research questions addressed during our thesis project and the specific source of their answers in the different chapters of the thesis.

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Bibliographical revue

Bibliographical review

1.1 Rice plant

1.1.1 Taxonomy

Rice, a member of the family Gramineae or Poaceae ("true grass"), belongs to the genus *Oryza* which includes species among which 22 are wild and only 2 are cultivated (Vaughan et al. 2003). The species across the whole genus have been analyzed on the basis of studies carried out in the world's main herbaria and in the field in Asia and Africa. Tateoka (1963) clarified the basic groups of species within the genus, and he called these groups species complexes (Table 1).

Cultivated species are *Oryza sativa* and *Oryza glaberrima* (African rice), of which the first is grown worldwide, while the latter is only grown on a limited scale in West Africa. The evolutionary processes of the two cultigen has been diagrammatically described in Fig 1 by Chang (1976).

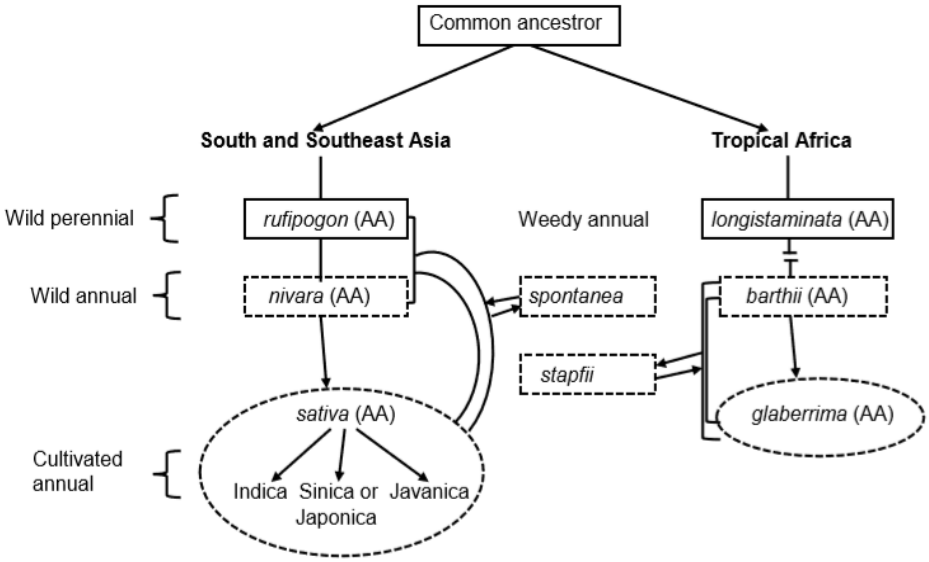

Figure.1. Evolutionary pathway of the two cultivated species of rice. Taxa boxed by solid lines are wild perennials. Taxa boxed by broken lines are annuals. Arrow with solid line indicates direct descent. Arrow with broken line indicates indirect descent. Double arrows indicate introgressive hybridization (Chang 1976).

Table.1. *Oryza* species (Tateoka 1963)

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. rufipognon sensu lacto (*syn: *O. nivara* for the annual form *O. rufipognon sensu stricto*	24 (0.95)	AA	(Annual) seasonally dry; open (Perennial) seasonally deepwater and wet round; open for the perennial form*)*
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 ; 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

Table 1. *Continued*

Section Complex Species	Chromosome number (DNA content [pg/2C])	Genome group	Usual habitat
O. punctate 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 (1.99)	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
Brachyantha B.R. Lu			
O. brachyantha Chev. et Roehr	24	FF	Rock pools ; open

1.1.2 Botanical description of cultivated rice

The rice plant consists of round and hallow stems, the flat leaves and terminal panicles. The whole plant includes vegetative organs: roots, stems, leaves and the reproductive organs namely the panicles consisting of a set of spikelets (Fig.2) (Ikisan 2018).

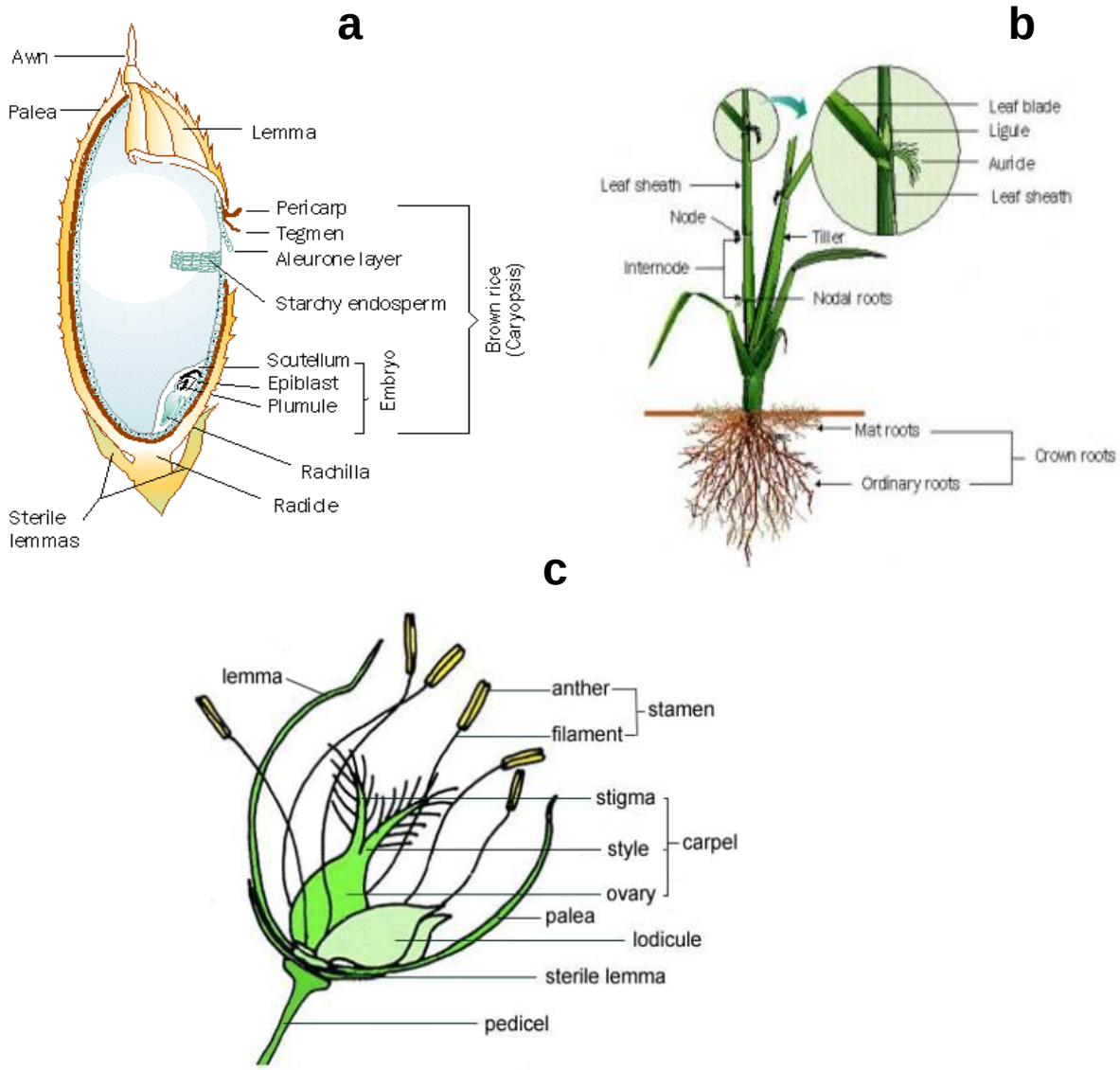

Figure.2. Morphology of rice plant: seed (a), plant at vegetative stage (b) and spikelet (c) (Ikisan 2018)

Seeds

The rice grain consists in three essential parts (Fig.2a):

- the rice hull comprising: the glumes (large portions above the pedicels that link the spikelets with secondary branches) and the two lemmas called palea (upper lemma with three veins) and lemma (lemma inferior with five veins). The beard is an extension of the ventral vein of the lemma. The lemmas, which envelop the grain of the rice (caryopsis), constitute the rice hull;
- the endosperm that serves as a food source for the embryo;
- the embryo located on the ventral part of the spikelet.

Seedlings

After dormancy, germination and seedling development begin. At this stage, the seed must absorb the optimal quantity of water and must be exposed to a temperature ranging from 10 to 40°C. The physiological definition of germination is usually the time when the radicle or coleoptile (embryonic shoot) emerges from the ruptured seed coat (Yoshida 1981).
The young seedling stage is the period following the germination, during which the young shoot feeds mainly on the reserves of the seed (about 14 days). It continues to produce leaves at the rate of one leaf every 3 or 4 days. The seedling stage corresponds to the period between the emergence of the first leaf and the appearance of the fifth leaf. During this stage, the seedling also produces roots. This is a critical stage in which the plant is very fragile.

Tillering plants

The tillering stage begins when the seedling no longer depends on the seed reserves and ends at the panicular initiation stage. Tillering usually begins with the emergence of the first tiller when seedlings have five leaves (Fig.2b). This first tiller develops between the main stem and the second leaf from the base of the plant. Subsequently when the 6th leaf emerges the second tiller develops between the main stem and the 3d leaf from the base (Ikisan 2018).

Tillers growing from the main stem are called primary tillers. These may generate secondary tillers, which may in turn generate tertiary

tillers. These are produced in a synchronous manner. Over time, the developed tillers become independent by producing their own roots. Varieties and landraces of rice differ in tillering ability. The tillering is influenced by environmental factors such as spacing, light, nutrient supply, and cultural practices.

Panicle and spikelets

According to Yoshida (1981), the rice's panicle is essentially made up of base, axis, primary and secondary branches, pedicel, rudimentary glumes, and the spikelets. The panicle axis extends from the panicle base to the apex; it has 8-10 nodes at 2- to 4-cm intervals from which primary branches develop. Secondary branches develop from the primary branches. Pedicels develop from the nodes of the primary and secondary branches; the spikelets are positioned above them.

The author explained that the flower is enclosed in the lemma and palea, which may be either awned or awnless (Fig.2c). The flower consists of the pistil and stamens, and the components of the pistil are the stigmas, styles, and ovary.

1.1.3 Production and economic importance of cultivated rice

Rice is the most consumed cereal grain on the planet and is grown in 9% of the world's arable land which accounts for third highest worldwide production of food, after maize and wheat (FAOSTAT 2010). In terms of global human nutrition and calorific intake, 21% of per capita energy and 15% of per capita protein are provided by rice (with the percentage in developping countries being 27% and 20%, respectively) ranking it as the most important among the crop that feed the world (Khush and Virk 2000).

In Burundi, the national production of paddy rice and the harvested area were respectively 146 633 tonnes and 63 751 hectares in 2016 (FAOSTAT 2016). The average level of per capita consumption, although low for the whole country (8.5 kg / year), reached high level (50 kg / year) in urban centers (Gahiro 2015).
Rice has become a culture of great interest in Burundi because its development could, on the one hand, contribute to reducing the deficit of the domestic supply of cereals. In fact, the production of conventional cereals (wheat, maize, finger corn and sorghum) is

declining due to the varietal degeneration and the attack of endemic viral and fungal diseases in the country. On the other hand, the promotion and the increase in domestic rice production would reduce the country's scarce foreign exchange for the import of rice to offset the small but structural production gap.

1.1.4 Impact of salt stress on global agricultural production

Regarding global agricultural production, the losses caused by salinity are estimated to be substantial and could increase with time. The problem of soil salinization is prevalent at a varying degree in all continents of the world. In some regions, soil salinization is referred to as “Silent killer” of natural production since it usually kills plants and soil organisms in the affected areas or as “White death” since it conjures up white images of lifeless shining lands studded with death trees (Hossain 2014).

Salinity has already affected a land area of more than 800 million hectares worldwide (FAO 2008); This equates to more than 6% of the land area covering the world.. At least 20% of all irrigated lands are salt-affected, with some estimates being as high as 50% (Pitman and Läuchli 2002). Ghassemi et al. (1995) estimated that the global income loss due to salinity was about $US 11.4 billion per year in irrigated and $US 1.2 billion per year in non-irrigated areas. The rice plant is one of the main crops threatened by salt stress in the world for two main reasons: on the one hand, it is a plant mainly grown in irrigated areas and on the other hand, it is the third highest worldwide production of food which means that it occupies a large arable area in the world.

1.2 Salinity stress effects on morpho-physiological traits of plant and mechanisms of salinity tolerance

1.2.1 Salinity stress effects on morpho-physiological traits of plant

Soil salinity stresses plants in two ways. Firstly, the accumulation of a large amount of salt ions in the soil solution prevents the roots from easily absorbing the water needed for the proper growth of the plant; this is referred to as the osmotic or water-deficit effect of salinity. Secondly, a high concentration of salt ions in the plant's organs cause ions imbalance or disturbances in ion homeostasis and toxicity; this is called the salt-specific or ion-excess effect of salinity (Munns and Tester 2008). This altered water status leads to initial growth reduction and limitation of plant productivity. Since salt stress consists of the osmotic and ionic components, the reduction in plant growth is mainly due to the osmotic potential and the amount of dissolved salts in the soil solution (Parida and Das 2005). Although the change in water status is the initial cause of growth suppression, the contribution of subsequent processes to inhibition of cell division and expansion and acceleration of cell death has not been well documented (Hasegawa et al. 2000). Salt stress affects all the major processes such as germination, growth, photosynthetic pigments and photosynthesis, water relation, nutrient imbalance and yield. These are discussed under separate headings.

1.2.1.1 Germination

Seed germination is one of the most fundamental and vital phase in the growth cycle of a plant that determines the yield. Seed germination (radical emergence) is the result of embryo-axis growth and depends on water uptake, storage reserve mobilizations and transport of the reserve of hydrolysis products to the embryo-axis (Gomes-Filho et al. 2008). The germination percentage decreases greatly when the seeds are in saline conditions (Prisco and O'LEARY 1970) and the inhibition of seedling gowth would be related to the decrease in seed reserve mobilization. The ability of seed to germinate under salt stress conditions is an indication of salt tolerance of that crop, at least, at the early stage of growth and for many plants, salt stress is more inhibitory during seed germination than at any other stage of growth (Bewley and Black 2012) but this should not be regarded as a general rule. Läuchli

and Grattan (2007) proposed a generalized relationship between percent germination and time after adding water at different salt levels (Fig. 3).

Salinity affects germination process many-folds. It alters the imbibitions of water by seeds due to low osmotic potential of germination media (Khan and Weber 2006), causes toxicity which changes the enzymes activity of nucleic acid metabolism (Gomes-Filho et al. 2008), alters protein metabolism (Dantas et al. 2007), disturbs hormonal balance (Khan and Rizvi 1994) and reduces the utilization of seed reserves (Othman et al. 2006). Salinity stress could alter the aleurone cell layer in monocot seeds, compromising the cell structure as well as perception of gibberellic acid which trigger amylase synthesis.

Under saline conditions, the germination performance varies from one crop to another and this variation is very often observed even between the cultivars of a single species. Studying the effect of salt stress on germination in durum wheat (*Triticum durum* Desf.), Almansouri et al. (2001) found the detrimental effect of NaCl which may be linked to long-term effects of accumulated toxic-ions. These authors concluded that the behaviour of the three cultivars during germination did not fully reflect their mean level of putative stress resistance in field conditions and that germination is, therefore, not recommended as a reliable selection criterion for breeding purposes.

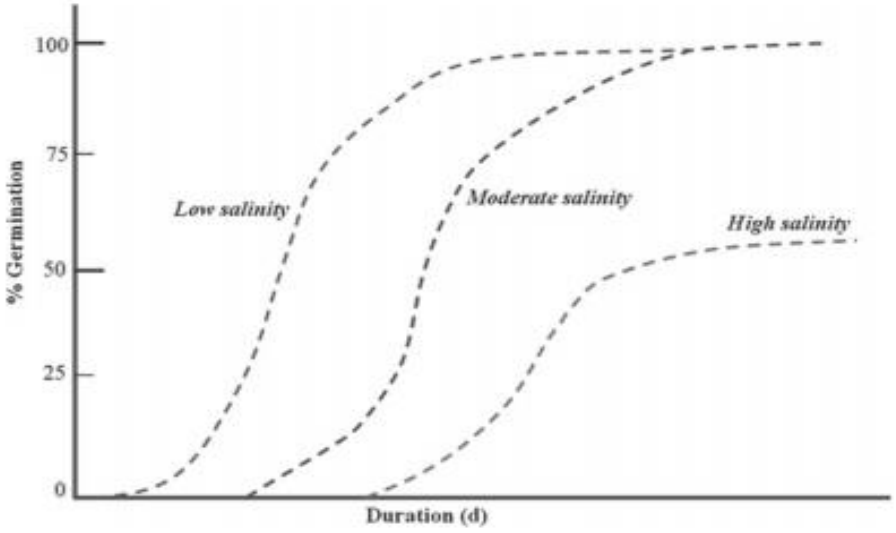

Figure 3. Relationship between rate of germination and time after sowing at different salinity levels (Läuchli and Grattan 2007).

The response of eight maize hybrids against five different salinity levels namely 0, 60, 120, 180 and 240 mM were studied at germination stage (Khodarahmpour et al. 2012). Results indicated that maximum reduction in germination percentage (77.4%), germination rate (32.4%), length of radicle (79.5%) and plumule (78%), seedling length (78.1%) and seed vigor (95%) were obtained in highest level of salinity (240 mM).

Concerning the rice plant, Kaddah (1963) prouved that rice is very tolerant to salt during germination but very sensitive to it during early seedling stage and progressively gains tolerance during the tillering stage, again becomes sensitive when flowering, and is tolerant during the period of grain maturation. The studies that have been conducted on salt stress effects at the rice germination stage showed that the tolerance depended on the level of applied stress and the type of cultivar (Hakim et al. 2010; Khan et al. 1997).

1.2.1.2 Growth

According to Munns and Tester (2008), the growth response to salinity stress occurs in two phases: an immediate response to increased osmotic pressure due to a high concentration of salt ions in the root-zone (known as osmotic phase), and a slower response due to the concentration of salt ions in leaves (known as ionic phase). The authors prove that the salinity stress is highly detrimental to shoot growth than to root growth and they distinguish three categories of plant species based on their tolerance profile (Fig. 4). Considering that the solid green line represents the change in the growth rate after the addition of NaCl, the broken green line in Fig 4a represents the first category of plant species which are characterized by an increased tolerance to the osmotic stress. The broken red line in Fig 4b represents the second category of plant species, which have an increased tolerance to ionic stress while the broken green-and-red lines in Fig 4c represent the third category of plant species characterized by an increased tolerance to both osmotic and ionic stresses.

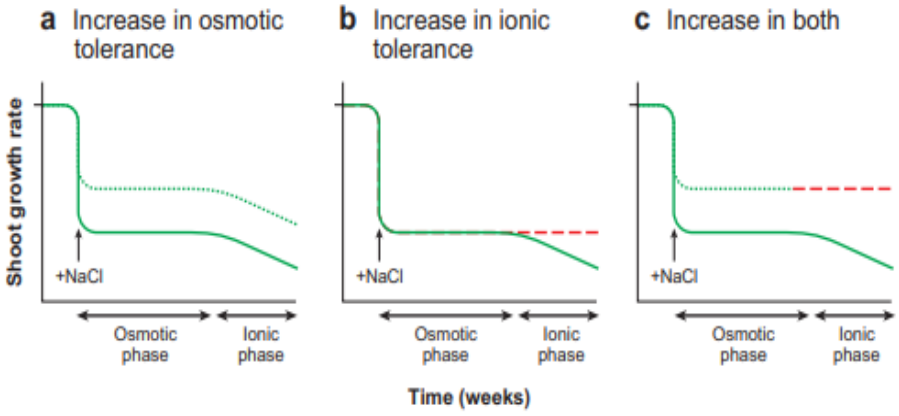

Figure 4. Overview of the two-phase growth response to salinity for plant differing in salt sensitivity (Munns and Tester 2008).

The scientific community widely accepts this theoretical model. There is however no clear evidence that the two phases are temporarily distinctively separated and one may argue that they could partly overlap. It might also be hypothesized that the relative importance of osmotic versus ionic component of salt stress depends on the stress intensity and the kinetics of stress application (Munns 2005). Almansouri et al. (1999) demonstrated that in *Triticum durum*, plant response to salinity was completely different if salinity is applied by small increment or suddenly. The authors found that salt accumulation is not evenly distributed among plant organs and that the various plant tissues are not behaving in a uniform way: accordingly, osmotic component may still prevail in some part of the plants while ionic stress is already present in other parts.

Many studies reported that the seedling stage is the most salt-sensitive stage in *Oryza sativa* (Lutts et al. 1995; Yeo and Flowers 1986; Yeo et al. 1990). Yeo et al. (1991) concluded that the initial growth reduction in rice caused by salinization is due to a limitation of water supply and a clear distinction was made between the initial effects of low salinity which are recoverable and the long-term effects which result from the accumulation of salt within expanded leaves.

Studying the NaCl and $CaCl_2$ (2:1 molar concentration) effects on rice seedling growth at five stress levels, Zeng and Shannon (2000b) showed that seedling growth was significantly reduced by salinity at the lowest

salinity treatment (1.9 $dS.m^{-1}$). Significant reduction of seedling growth occurred at longer cumulative thermal time than at higher salt levels and seedling survival was significantly reduced when salinity was 3.4 $dS.m^{-1}$. It has also been prouved that rice seedling growth was reduced depending on salt stress levels and cultivars type (Lutts et al. 1996a). This is confirmed by Munns and Tester (2008) who show that sensitive species (including rice crop) hardly control the accumulation of toxic ions and that the consequence is the domination of the ionic effect on the osmotic effect early after exposure of the plant to salt stress.

1.2.1.3 Ion levels

1.2.1.3.1 The anionic component of salt stress

In contrast to Na^+, which is not an essential element for higher plants (except C4 plants where traces of Na^+ are required to phosphoenolpyruvate regeneration), both Cl and S are essential elements for all plant species.

Chloride anion

During decades, chloride impact on plant response to salinity received quite less attention than Na^+. In the soil solution, Cl occurs predominantly as chloride ions (Cl^-) and it show little affinity for adsorption on the soil component. It is not involved in oxido-reduction reactions and, by forming outersphere surface complexes, this ion is quite weakly adsorbed by soil particles through electrostatic forces. It has however be reported that Cl could be, to some extent, retained by iron-hydroxide by replacing the hydroxide group but this phenomenon mainly occurs under very low pH (Geilfus, 2019). Chloride is not only present in excess in salt-affected soils but may also be present in Cl-containing dust from volcanic eruptions (Burgos et al., 2017) or may be issued from fertilization with Cl-rich animal wastes or produced by numerous urban, municipal and industrial activities (Ordonez-Barona et al., 2018).

Chloride has long been recognized as an essential element for plants and it is very mobile within the plant, especially in the roots where it is thought to be translocated mainly by the symplastic pathway (White and Broadley, 2001). In photosynthetic tissues, two Cl^- ions are present

in each oxygen-evolving complex of PSII and assume key functions in water splitting (Wege et al., 2017). Chloride also assumes some important roles in regulation of asparagine synthetase and the tonoplast V-ATPase. Reineke et al. (1995) reported that some plant hormones (especially auxin in the form of 4-chloroindole-3-acetic acid) might be associated in planta with chloride ions and assume important role in pericarp differentiation. Chloride ion fluxes were reported during elongation of pollen tube and possibly generate osmotic gradient for cell expansion and SLAH3 protein interacting with Ca^{2+}-dependent protein kinases are involved in Cl^- influx at the tip of the cell tube (Hepler and Winship, 2015). Two others proteins (SLAC1 and ALMT12) mediate Cl^- efflux from guard cell during stomatal closure associated with a decrease in the guard cell water content (Wege et al., 2017). De Angeli et al. (2013) demonstrated that a tonoplast Cl^- channel ALMT9 is directly involved in turgor increase controlling the opposite process of stomatal opening.

The above-mentioned functions requires only small amounts of endogenous Cl^-. It has however been frequently reported that plants actively accumulate more chloride that the amounts expected for micronutrients, even in the absence of salt (Wege et al., 2017 ; Li et al., 2017 ; Colmenero-Flores et al., 2019 ; Bazihizina et al., 2019). One possible explanation is that Cl^- is acting as a « cheap » osmoticum and that its accumulation requires less energy than the synthesis of organic anions such as malate. It has been demonstrated that Cl^- assume important functions for osmoregulation and turgor maintenance (Franco-Navarro et al., 2016 ; Burdach et al., 2014). According to Colmenero-Flores et al. (2019), the importance of Cl^- in turgor maintenance may be due to its role as electrical charge balance of cations (K^+, Ca^{2+},..).

Chloride uptake from a normal soil is an active process requiring energy. Since early report of White and Broadley (2001) almost 20 years ago, considerable progress has been made in the identification of molecular basis of Cl absorption and translocation. However, clear evidences of specific Cl^- transporters at the root epidermis level are still lacking and it is often considered that the bulk of Cl^- enters in cells through NO_3^- transporters, which could also explain that exogenous high Cl^- inhibit nitrate uptake (Wege et al., 2017 ; Li et al., 2017). Consequently, there is a strong impact of Cl^- on nitrogen use efficiency since the net NO_3^- uptake is the sum of NO_3^- influx and efflux. It has

been shown that in some circumstances, root NO_3^- efflux can be also as high as NO_3^- influx (Wege et al., 2017).

Within the roots, the symplastic pathway is the most important for Cl^- uptake. Important proteins such as SLAH1 nd SLAH3 form a heteromeric channel that mediate Cl^- fluxes from the pericycle to the xylem. Other key actors for Cl^- transfer are members of the NPF proteins family. Under conditions of salinity, however, an important proportion of Cl^- is taken up passively by anion channels. Xylem loading may be restricted by down-regulation of the gene coding for NPF7.3, NPF2.4 and SLAH1 and phytohormone such as ABA was shown to be involved in this repression (Wen and Kaiser, 2018 ; Geilfus, 2019).

Since a few years, numerous studies have been conducted to more deeply understand the metabolic functions of chloride in plants maintained under control non-saline conditions. It is surprising that not so many studies consider the specific impact of chloride toxicity in salt-stress conditions. Root cellular concentration may increase rapidly: Lorenzen et al. (2004) demonstrated that, in roots of Arabidopsis treated with 100 mM NaCl, the concentration of Cl^- in the cytosolic segments near the hypocotyls might increase to 50 mM within 2 hours. Beside down-regulation of genes coding for root-to-shoot translocation, some proteins involved in the efflux of Cl^- from the root cells to the soil solution may also be activated, such as NRT1.5 and NF2.5, which may be involved in the avoidance of Cl^- overaccumulation in salt-treated plants (Henderson et al., 2014).

The lack of information regarding chloride toxicity may be partly due to the presence of counterion in NaCl treatment, and it is difficult to discriminate between the effects of the two overaccumulated elements in plant tissues. Some authors used mixed salts or Na^+-humate to study the ionic effect of Na^+ without interference with Cl^- and, by comparison, to quantify Cl^- toxicity. Using those strategies, Genc et al. (2016) concluded that Cl^- was more toxic than Na^+. Tavakkoli et al. (2011) considered that in barley, toxicity of Na^+ and Cl^- may act on similar molecular and cellular targets but according to different modalities so that the presence of the two ions lead to additive deleterious effects. Geilfus et al. (2015) interestingly reported in broad bean that Cl applied in the form of L-cysteinium chloride may lead to apoplasm alkalinisation at the shoot level, then increasing ABA in the surrounding

of guard cells and leading to stomatal closure before Cl reached the shoot, suggesting that Cl may trigger a long-distance root-to-shoot signal very quickly.

Bazihizina et al. (2019) recently argue that under saline conditions, halophyte plant species display a contrasting behaviour for Cl^- uptake and accumulation comparatively to glycophyte. According to these authors, halophytes require a very high internal concentration of Cl^- for regulation of cell turgor and growth and invest metabolic energy for specific uptake of Cl^- by the roots. This specific behaviour partly rely on an inhibition of Cl^- efflux process and a tight regulation of influx. Halophyre reduced energy requirement not only for Cl^- but also for Na^+ transport since Cl^- influx must be coupled with cation influx to maintain charge balance.

Sulfate anion

Sulfur is present in high amounts in most soils and is an essential nutrient for all living organisms (Gallardo et al., 2014; Chan et al., 2019). It is involved in the synthesis of numerous sulfur-containing metabolites which assume crucial functions in the plant metabolism. It is considered that more than 40% of enzymes depend on the presence of sulfhydryl group (-SH) for their catalytic activity (Fuentes-Lara et al., 2019).

It may exist in the environment at different oxidation states (from +6 to -2). The most oxidized form is sulfate (SO_4^{2-}) and constitutes the chemical form absorbed by the plant. In soils, sulfur is present as inorganic forms (mostly S^0 and SO_4^{2-}). For SO_4^{2-}, a dynamic equilibrium occurs between soil solution and ions adsorbed on the soil exchange complex. Anaerobic microorganisms may use sulfate as final acceptor of electrons and produce H_2S which could diffuse in the atmosphere or be reoxidized in the presence of O_2. In agricultural soils, it is important to consider sulfate-containing fertilizers (ammonium sulfate, potassium sulfate, gypsum,…) and sulfur may also be provided as S^0 in organic fertilizers (Ruiz et al., 2003). Another source of sulfate in the environment is rain and snow precipitation and aerosols. Under normal non-saline conditions, a continuous supply of S is required on agricultural soils: as an example, it is considered that one hectare of maize consumes 25 Kg. The amounts to apply should consider the part

of S that is volatilized in the soils on the one hand and consumed by microorganisms on the other hand (Fuentes-Lara et al., 2019).

Sulfate transporters are integral membrane proteins: they control the flux of sulfate across the plasma membrane, but also across endomembrane in numerous organelles. This is a highly regulated process controlling the proportion of sulfate metabolized in the cytosol and in chloroplast on the one hand and the amount of sulfur stored in the vacuoles on the other hand (Smith et al., 2000). Transcriptional and post-transcriptional regulatory mechanisms thus control the expression of genes coding for sulfate transporters in different organs, cell types and subcellular compartments. High-affinity sulfate transporters are localized in the outermost cell layer in close contact in the rhizosphere. These transporters belong to the SULTR family (Takahashi et al., 2012). Sulfate influx occurs through active transport in S-limited conditions and S may be absorbed from the apoplast in the cell from cortical parenchyma using a proton gradient to facilitate co-transport. Mycorrhizal symbiosis may also be important for S acquisition from S-deficient soils and multilevel of coordination may exist between phosphate and sulfate homeostasis in plants (Rouached, 2011). Endogenous glutathione may affect gene expression coding for sulfate transporters. Some recent information also suggests that ethylene acts as a hormonal signal regulating sulfate uptake (Takahasi, 2019). In the presence of an excess of available sulfate, such as it may occur in some salt-affected soils, sulfate uptake needs to be down-regulated and a nuclear-localized serine hydroxymethyltransferase-like protein (MSA1: MORE SULFUR ACCUMULATION) is involved in the repression of some (but not all!) SULTR genes (Huang et al., 2016). SULTR2;1 is localized in pericycle and xylem parenchyma cells and is involved in xylem loading. It has been proven that in case of S overaccumulation in the shoot part, GSH may act as a signal translocated by the phloem to down-regulate S loading. Members of the SULTR3 and SULTR4 subfamily are also known to control root-to-shoot translocation. At the leaf level, mechanism of sulfate unloading from the xylem sap remains poorly documented but the low-affinity sulfate transporters SULTR2;2 is suspected to be involved in this process (Takahashi, 2019). According to Aubry et al. (2014), the distribution of transcripts corresponding to sulfate transporters in the leaves of A. thaliana preferentially occurs in cells where the photosynthetic C fixation remains low.

An important difference between sulfate on the one hand, and chloride and sodium on the other hand, is that S is assimilated by plant cells and that the majority of S issued is found in organic molecules. Several enzymes are involved in sulfur assimilation, most of them being mainly involved in cysteine and glutathione biosynthesis. Absorbed sulfate is converted to adenosine-5'-phosphosulfate (APS) through the action of ATP sulfurylase, and APS is then converted to sulfite by APS reductase. O-acetylserine reacts with sulfide produced from sulfite to ensure the synthesis of cysteine through the action of O-acetylserine sulfhydrylase. Cystein is an important aminoacid in proteins but is also a component of glutathione involved in protection against oxidative stress. These enzymes are now well characterized and molecular tools allowed scientists to perform phylogenic analysis (Kopriva et al., 2007). ATP sulfurylase is encoded by a small multigene family and the different corresponding enzymes are present in numerous organelles (see Jez, 2019 for recent review). One of the most important is the chloroplast isoform, which is crucial for plants since it assumes more than 90% of the reductive sulfate assimilation (Jez 2019; Kopriva et al., 2007). APS reductase and sulfite reductase constitute complex enzymes and their X-ray crystal structures were only recently established. Sulfite reductase catalyzes a critical step in plants for maintaining the flux of the sulfur pathway and it also appears to regulate the synthesis of the phytohormone abscisic acid (Batool et al., 2018). Iron is an important element for sulfite reductase activity which is under the control of a ferredoxin/thioredoxin system (Hirasawa et al., 2004).

Secondary sulfur metabolism produces sulfated compounds such as glucosinolates and sulfated peptides (Chan et al., 2019). Glucosinolate are defense compounds with additional roles in oxidative stress. It has been interestingly reported that foliar and root glucosinolates concentration correlate in *Brassica* species with resistance to drought and salt stress (Del Carlmen Martinez Ballesta et al., 2013) and MYB transcription factors were demonstrated to be involved in this process (Martinez-Ballesta et al., 2015) even if the underlying biochemical and physiological processes involved remain unclear. Sulfotransferases constitute a complex family of enzymes involved in the synthesis of growth-regulating peptides, hormone derivatives and sulfation of ROS-protecting flavonoids (Hashiguchi et al., 2014). Synthesis of those compounds depend on the partitioning of sulfur between primary and secondary metabolism: the regulation of this partitioning still remains

to be elucidated but it is not excluded that abiotic stresses modify this regulating process (Chen et al., 2013).

While there is an enormous amount of literature devoted to sulfur metabolism in plants, there is a surprising paucity of data regarding the impact of an excess of available sulfate on the plants. A research in the Scopus data bank (performed in January 2020) with the term "sulfate toxicity" in the title and "plant" in title, abstract or keywords provided only 31 references (just for comparison, replacing sulfate toxicity by "salinity" provided 7359 references!). It tends to erroneously suggest that sulfate excess is not a problem for plants, while we previously mentioned that a consistent part of salinity may be due to sulfate salts. It may be considered, however, that sulfate itself is not drastically toxic for plants but that some products issued from sulfur assimilation could have deleterious effect if they accumulate at high concentration. According to Geurts et al. (2019), sulfate accumulation may also occur as a consequence of eutrophication, independently of salinity. Performing analysis on mesocosm on wetland plant species, these authors confirm that the main effect of sulfate excess is in fact due to sulphide accumulation. Sulphide may accumulate within plant tissues as a consequence of sulfite reduction catalyzed by sulfite reductase. Sulphide toxicity has mainly been studied in marine and fresh water aquatic environment since it may occur *outside* the plant under anaerobic or hypoxic conditions. It was reported to impair growth, and to induce iron chlorosis and leaf necrosis and to inhibit flowering of semi-aquatic plant species (Smolders and Roeofs 1996; Van der Welle et al., 2007). This situation may occur in rice, at least in permanently irrigated fields (Fort et al., 2017), but this is independent of sulfate involvement in salt stress. The question remains open to determine if, under sulfate salinity, absorption of sulfate may lead to toxic sulphide production and this probably deeply dependent on the ratio between sulfite reductase which produce sulphide, and O-AcetylSerine Sulfhydrylase which integrate sulphide in cysteine. Moreover, the toxicity of sulphide itself is still a matter of debate and some authors demonstrated that small amounts of sulphide may be useful to protect plants against heavy metals toxicity (Guan et al., 2018) and resistance to higher concentrations of sulphide as shown to drastically differ among plant species (Martin and Maricle, 2015). Gallardo et al. (2014) performed an interesting analysis demonstrating that drought and salinity (NaCl) are regulating some specific SULTR genes, which may contribute to adjust sulfur distribution in plants exposed to these

environmental constraints. Cao et al. (2014) also showed that sulfate availability may act on ABA synthesis and influence behaviour of Arabidopsis toward salinity. In all cases, however, salinity is imposed in the form of NaCl and, to the best of our knowledge, no similar studies have never been performed using sodium sulfate as stressing agent.

1.2.1.3.2 Effects of salinity on mineral nutrition

The presence of excessive soluble salts in the soil competes with the uptake and metabolism of mineral nutrient that are essential to plants. Imbalances may result from the effect of salinity on nutrient availability, competitive uptake, transport or partitioning within the plant or may be caused by physiological inactivation of a given nutrient resulting in an increase in the plant's internal requirement for that essential element (Grattan and Grieve 1999). two or more of these processes may affect the plant simultaneously but their impacts on plant growth, development and production depend on the ionic component of salt stress, the concentration of salts in the growing substrate , the crop species or the type of cultivar inside the same crop species, the sudied element as well as other environmental factors.

According to Zhu (2001), increased salt uptake induces specific ion toxicities like that of Na^+, Cl^- or sulfate (SO_4^{2-}) that decrease the uptake of essential nutrients like phosphorus (P), potassium (K), nitrogen (N) and calcium (Ca). Soils contain extreme ratios of Na^+: Ca^{2+}, Na^+: K^+, Na^+: Mg^{2+}, and Cl^-: NO_3^- under saline conditions, which cause reduced plant growth due to specific ion toxicities (e.g., Na^+,Cl^- or SO_4^{2-}) and ionic imbalances acting on biophysical and/or metabolic components of plant growth (Grattan and Grieve 1999). Salinity can also cause a combination of complex interactions that affect plant metabolism, susceptibility to injury or internal nutrient requirement (Chen et al. 2018; Machado and Serralheiro 2017).

Assimilated in large quantities, nitrogen is one of the essential elements of the plant and is a constituent of many plant cell components, including amino and nucleic acids. Therefore, nitrogen deficiency rapidly inhibits plant growth. Concerning the salinity effects on nitrogen absorption, whereas in most cases the total shoot N uptake (mg N per plant) decreases under saline conditions, the N concentration (mg N per kg dry weight) increases or remains unchanged under optimal N conditions (Hu and Schmidhalter 1998; Munns and Termaat 1986).

These findings suggest that the slower growth rate of crop plants under salinity might prevent the dilution effect of nutrient elements and that the N concentration in plants might not be associated with salt injury for plant growth. Knowing that maize and wheat became more sensitive to salinity as the ratio of NH_4^+: NO_3^- increased (Botella et al. 1997; Leidi et al. 1991); the N form might also influence the sensitivity of the plant to salinity. Ammonium NH_4^+ is a putative toxic compound and may be produced by some metabolic pathways such as photorespiration which could be increased in response to salt and there is consequently a need for plant species to activate the GS/GOGAT pathway to stimulate NH_4^+ assimilation in glutamine synthesis (Lutts et al. 1999b).

Phosphorus (P) is a constituent of nucleic acids, phospholipids, phosphoproteins, dinucleotides, and adenosine triphosphate. Hence, P is required for processes including the storage and transfer of energy, photosynthesis, regulation of some enzymes activities (especially through phosphorylation processes), and the transport of carbohydrates (Hu and Schmidhalter 2005). The interaction between salinity and P nutrition in plants is complex and it is highly dependent on the plant species (or cultivar), plant developmental age, the composition and level of salinity and the concentration of P in the substrate (Grattan and Grieve 1999). According to Champagnol (1979), it is unlikely that Cl^- and $H_2PO_4^-$ ions are competitive in terms of plant uptake even if Zhukovskaya (1973) reported that Cl^- and SO_4^{2-} salts reduced P uptake in barley and sunflower. Most studies dealing with salt-induced deacrease in P concentrations in plant tissues were conducted in soils. Phosphate availability is reduced in saline soils not only because of ionic strength effects that reduce the activity of phosphate but also because phosphate concentrations in soil solution are tightly controlled by sorption processes and by the low-solubility of Ca-P minerals (Jiang et al. 2019). Hence, deleterious impact of salinity on P nutrition should be regarded as a consequence of a salt-decrease in P bioavailability rather than an effect on the plant absorption process. Unlike studies conducted in the field, most studies using sand or solution cultures demonstrated that salinity increased the tissue P level (Grattan and Grieve 1999).

K^+ is another essential nutrient for plant life. The cellular roles of K^+ can roughly be summarized as : (1) charge balancing in the cytoplasm, where K^+ is the dominant counterion for the large excess of negative charge on proteins and nucleic acids, (2) activation of crucial enzymatic

reactions and (3) substantial contribution to the osmotic pressure of the vacuole and hence to cell turgor which endows non-lignified plant cells with structural rigidity (Kochian and Lucas 1989; Maathuis and Sanders 1996).

High levels of external Na^+ negatively affect K^+ acquisition by the roots via the disruption of root membranes integrity and alter their selectivity. Potassium nutrition is particularly important to salt-stressed plants since considerable evidence indicates that metabolic damage results from high cytoplasmic Na^+/K^+ ratios and high Na^+ concentrations may interfere with K^+ acquisition (Lynch and Läuchli 1984). Studying the effects of salt stress on mineral nutrition in rice (*Oryza sativa* L.) cultivars differing in salinity resistance, Lutts et al. (1996a) found that the salt stress induced an increase in root and shoot Na^+ and Cl^- contents as well as a decrease in K^+ concentrations. The K^+/Na^+ ratio, which is an indicator of the metabolic status of stressed tissues, was always lower in salt-sensitive than in salt-resistant cultivars and we can deduce that antagonism between K^+ and Na^+ uptake differs according to the sensitivity of rice cultivars to salinity. In fact, the underlying mechanism for maintaining adequate tissue K^+ levels under salt stress seems to be dependent upon selective K^+ uptake and selective cellular K^+ and Na^+ compartmentalization and distribution in the shoots (Carden et al. 2003).

Calcium (Ca^{2+}) stabilizes and confers more structural integrity to biological tissues at scales ranging from intercellular membranes to the cell walls of woody stems. It plays an important regulatory role in phosphorylation of nuclear proteins; cell division; cell wall and membrane synthesis and function; intra- and intercellular signaling; protein synthesis and responses to environmental stimuli (McLaughlin and Wimmer 1999).

However, these functions are disturbed when a plant is subjected to salt stress following a decrease in the calcium content in favor of sodium accumulation. Therefore, high Na^+/Ca^{2+} and Na^+/K^+ ratios in saline growth medium may impair the selectivity and the integrity of cell membrane and result in passive accumulation of Na^+ in the root and shoot (Kramer et al. 1977). Because Na^+ readily displaces Ca^{2+} from its extracellular binding sites, Ca^{2+} availability could be seriously reduced under salinity, especially at low Ca^{2+}: Na^{2+} ratios (CRAMER et al. 1988). In addition, the low Ca^{2+}: Na^+ ratio in saline media plays a

significant role in inhibiting plant growth as well as causing significant changes in morphology and anatomy (Cramer 1992). Analyzing the effect of Na^+/Ca^{2+} ratios in saline culture solution on the growth and mineral nutrition of rice (*Oryza sativa* L.), Muhammed et al. (1987) proved that Na^+/Ca^{2+} ratios of the growth medium significantly influenced the shoot and root growth of rice. Indeed, at a given salinity level, the moisture percentage in shoots increased with increasing Na^+/Ca^+ ratio in culture solution, indicating the role of NaCl in inducing plant succulence and within a given salinity, root weight increased with decreasing Na^+/Ca^{2+} ratio, indicating the role of Ca^{2+} in root development (Byrt et al. 2018; Machado and Serralheiro 2017). According to Lutts et al. (1996a) and knowing that a significant proportion of all ions is entering via the bypass flow as suggested by Yeo and Flowers (1986), a decrease in transpiration rates of rice salt-resistant cultivars should have led to a decrease in the content of other ions, especially in Ca^{2+} and it was also noteworthy that such a decrease occurred mainly in salt-sensitive cultivars.

1.2.1.4 Photosynthetic pigments and photosynthesis

Photosynthesis is a crucial biochemical pathway by which plants convert solar energy into chemical energy and grow. The reduction in photosynthetic rates in plants under salt stress is mainly due to stomatal closure, desorganization of chloropalst ultrastructure, reduction in water potential, the high concentrations of salt ions accumulated in the chloroplasts leading to a decrease in the photosynthetic pigments concentration and in alteration of photosystem stability (Parihar et al. 2015).

The electron microscopy showed that the thylakoidal structure of chloroplast was notably disorganized, the number and size of plastoglobuli increased and the starch content decreased in NaCl-treated plants (Hernandez et al. 1999; Hernandez et al. 1995). Furthermore, electron microscope studies on the leaves of several potato cultivars after application of salt at various development stages showed that salt-induced changes were more marked in chloroplasts than in other organelles (Bruns and Hecht-Buchholz 1990). The authors showed that salt reduced the number and depth of the grana stacks and caused swelling of the thylakoids. Changes in the chloroplasts were always greater in the palisade than in the spongy parenchyma and large

starch grains in the chloroplasts indicated disturbed transport of assimilates.

Under salt stress, the decrease in leaf's content of photosynthetic pigments is a common observation, and in many studies, chlorophyll concentration has been used as a sensitive indicator of the cellular metabolic state (Chutipaijit et al. 2011). Indeed, there was a linear decrease in the levels of total chlorophyll, chlorophyll-a, chlorophyll-b, carotene and xanthophyll as well as the intensity of chlorophyll fluorescence in mungbean under increasing concentrations of NaCl (Saha et al. 2010). Compared to plant control, the photosynthetic pigment contents decreased on an average, by 31% for total chlorophyll, 22% for chlorophyll-a, 45% for chlorophyll-b, 14% for carotene and 19% for xanthophyll. According to Amirjani (2011), the reduction of chlorophyll a (33%) and b (41%) contents of rice leaves was detected after NaCl treatment (200 mM, 14 days). Further studies on rice plant have also showed the detrimental effect of salt stress on photosynthetic pigments content (Ali et al. 2004; Lutts et al. 1996b; Sultana et al. 1999).

In addition to the desorganization of chloroplast ultrastructure and the reduction of photosynthetic pigments content, salt stress causes either short or long-term effects on photosynthesis in relation to salt-induced decrease in gaz exchange. As an attempt to reduce water losses resulting from transpiration, salinity induces stomatal closure which has a direct impact on gaz exchange and results in a decreased level of CO_2 fixation (Wungrampha et al. 2018). It is commonly considered that the impact of stomatal closure is higher on transpiration than on CO_2 exchange, which commonly results in an increased water use efficiency, at least for moderate stress intensities. It is also believed that water stress is primarily responsible for stomatal closure although recent studies clearly showed that an impact of ion toxicities on anion transport systems at the guard cell level is an important component explaining stomatal closure on a long term basis (Saito and Uozumi 2019). An impact of salt on stomatal density in developing leaves may also directly influence net photosynthesis, although experimental approaches are contradictory regarding this impact (Isayenkov and Maathuis 2019).

The short term effects occurs after a few hours or within 1 or 2 days of the onset of exposure and this response is important, as there is

complete cessation of carbon assimilation within hours. The long-term effect occurs after several days of exposure to salt and reduction in carbon assimilation is due to the salt accumulation in developping leaves (Munns and Termaat 1986). In four cultivars of *Oryza sativa* L., a gradual decrease in the photosystems I and II activity as well as in chlorophyll (Chl) fluorescence transients and emission at 688 nm was observed with an increase in NaCl concentration. This decrease was more pronounced in salt-sensitive cultivars as compared to the tolerant ones while a drastic decrease in net photosynthetic rate was found in both cultivar types (Tiwari et al. 1998). Still on the rice plant, Amirjani (2011) observed the inhibition of the maximum quantum yield of PSII (F_v/F_m), photo yield of PSII (Φ_{PSII}), non-photochemical quenching (NPQ) and net photosynthetic rate in the seedlings exposed to NaCl (200 mM) for 14 days. According to Parida and Das (2005), decreases in photosynthetic rate are due to several factors such as (1) dehydration of cell membranes which reduce their permeability to CO_2 , (2) salt toxicity, (3) reduction of CO_2 supply because of hydroactive closure of stomata, (4) enhanced senescence induced by salinity, (5) changes of enzyme activity induced by changes in cytoplasmic structure, and (6) negative feedback by reduced sink activity.

1.2.1.5 Water relation

According to Soria and Cuartero (1993), reduction of plant water uptake with salinity could be related to reductions in morphological and/or physiological parameters like leaf area, stomatal density, and stomatal closure (stomatal conductance and transpiration). Relative water content, leaf water potential, water uptake, transpiration rate, water retention, stomatal conductance and water use efficiency decreased due to short-term salinity stress in jute (Chaudhuri and Choudhuri 1997). Given that the water potential is an important physiological parameter for determining the water status of the plants, Romero-Aranda et al. (2001) showed that an increase of salt in the root medium can lead to a decrease in leaf water potential and, hence, may affect many plant processes. At very low soil water potentials, this condition interferes with plant's ability to extract water from the soil and maintain turgor while at low or moderate salt concentration (higher soil water potential), plants adjust osmotically (accumulate solutes) and maintain a potential gradient for the influx of water (Parihar et al. 2015).

The osmotic component of salt stress predominates when the reduction in plant growth is mainly due to a high concentration of salts in the growing substrate that decreases the roots ability to absorb water. Eaton (1942) demonstrated that chloride salinity was twice as inhibitory as sulfate salinity per milliequivalent of salt in his sand-culture solutions and the osmotic potentials in the media were equal when the chloride solutions had half the concentration of his sulfate solutions on a milliequivalent/liter basis. Assessing the effects of salt stress on growth, mineral nutrition and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) cultivars differing in salinity resistance, Lutts et al. (1996a) found that the osmotic potentials of the roots and of the oldest and youngest leaves were lower in the salt-resistant than in the salt-sensitive genotypes, differences among genotypes increasing with stress intensity. According to these authors, the Na^+ and Cl^- accumulation in oldest leaves of salt-resistant cultivars did not result in a decrease in water content while such a decrease occurred in salt-sensitive cultivars. Flowers et al. (1991) already suggested that a decrease in water content of the leaf tissue could be due to an apoplastic accumulation of Na^+ in salt-sensitive cultivars, leading to a cellular dehydration and a loss of water resulting from a poor stomatal control in these genotypes.
Some analyses are more and more frequently devoted to the effect of salt stress on aquaporins and the ultimate consequences of these modifications on the plant water status: both chloride ions and salt-induced ABA may have an impact on this aspect (Isayenkov and Maathuis 2019).

1.2.1.6 Reactive Oxygen Species (ROS) and oxidative damage

About 2.2 billion years ago molecular oxygen was introduced in our environment by the O_2-evolving photosynthetic organisms and ROS have been the uninvited companions of aerobic life (Halliwell 2006). ROS are a group of free radicals, reactive molecules, and ions that are derived from O_2. It has been estimated that about 1% of O_2 consumed by plants is diverted to produce ROS in various subcellular loci such as chloroplasts, mitochondria and peroxisomes (Sharma et al. 2012). Depending on their concentration in plants, ROS are well recognized for playing a dual role as both deleterious and beneficial species. At high concentration ROS cause damage to biomolecules, whereas at low/moderate concentration it acts as second messenger in intracellular

signaling cascades that mediate several responses in plant cells (Yang and Guo 2018).

Salinity stress, commonly NaCl and Na_2SO_4, lead to the overproduction of reactive oxygen species in plants. The overproduced ROS are then highly reactive and toxic and cause damage to protein, lipids, carbohydrates and DNA (Fig.5) which ultimately results in oxidative stress (Gill and Tuteja 2010). The ROS comprises both free radical ($O_2^{\cdot -}$, superoxide radicals; $OH^{\cdot}$, hydroxyl radical; $HO_2^{\cdot}$, perhydroxy radical and $RO^{\cdot}$, alkoxy radicals) and non-radical (molecular) forms (H_2O_2, hydrogen peroxide and $^1O_2$, singlet oxygen).

According to Hernandez et al. (2000), salt stress can lead to stomatal closure, which reduces CO_2 availability in the leaves and inhibits carbon fixation which, in turn, causes exposure of chloroplasts to excessive excitation energy and overreduction of photosynthetic electron transport system leading to enhanced generation of ROS and induced oxidative stress. Low chloroplastic CO_2/O_2 ratio also favors photorespiration leading to increased production of ROS such as H_2O_2. Furthermore morever, high salt concentrations lead to overproduction of the ROS by impairment of the cellular electron transport within different subcellular compartments such as chloroplasts and mitochondria, as well as from induction of metabolic pathways such as photorespiration (Tanou et al. 2009).

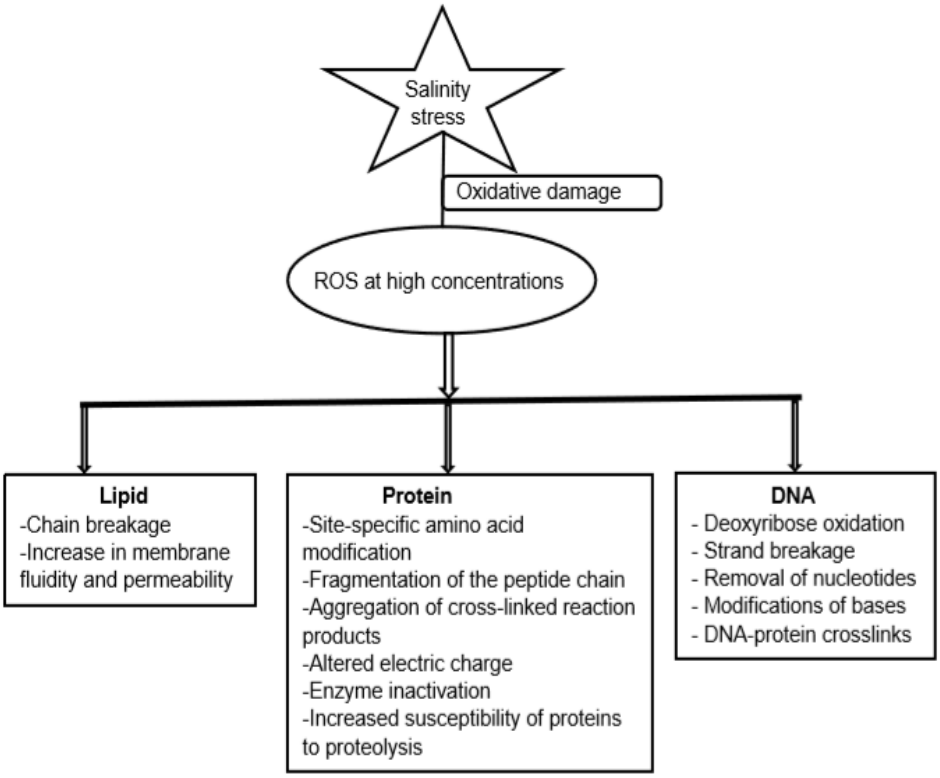

Figure.5. Reactive oxygen species (ROS) induced oxidative damage to lipids, proteins, and DNA (adapted from Sharma *et al.*, 2012).

According to Gill and Tuteja (2010), the antioxidant defense machinery protects plants against oxidative stress damages. Plants possess very efficient enzymatic (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione peroxidase, GPX; guaicol peroxidase, GOPX and glutathione-s-transferase, GST) and non-enzymatic (ascorbic acid, ASH; glutathione, GSH; phenolic compounds, alkaloids, non-protein amino acids and α-tocopherols) antioxidant defense systems which work in concert to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging of ROS. Almost all components of this complex network are involved in plant response to salinity (Ahanger et al. 2017; Lv et al. 2019; Sharif et al. 2019; Singh et al. 2017).

Dionisio-Sese and Tobita (1998) studied the possible involvement of activated oxygen species in the mechanism of damage by NaCl stress in leaves of four rice cultivars (*Oryza sativa* L.) exhibiting different sensitivities to NaCl. The salt-sensitive varieties, Hitomebore and IR28, exhibited a decrease in superoxide dismutase activity and an increase in peroxidase activity under high salinization while the salt-tolerant variety Pokkali, showed only slight increase and decrease in superoxide dismutase and peroxidase activity, respectively. On the other hand, the putative salt-tolerant Bankat variety, which showed a slight stimulation in growth rate similar to Pokkali at moderate salinity level, exhibited symptoms of oxidative damage during salt stress similar to the salt-sensitive varieties rather than the salt-tolerant one. The authors confirmed that free radical-mediated damage of membrane may play an important role in the cellular toxicity of NaCl in rice seedlings and that salt-tolerant varieties exhibit protection mechanism against increased radical production by maintaining the specific activity of antioxidant enzymes.

The comparative alterations of short term (12 days) NaCl stress and recovery on lipid peroxidation and antioxidants in roots of two rice cultivars differing in salt tolerance were studied by Khan and Panda (2008). Both peroxide content and lipid peroxidation measured in terms of MDA level increased and the ratio was higher in *Begunbitchi* compared to *Lunishree* cultivars. Although SOD, CAT and GR activities decreased in the stressed roots, CAT activity also increased in recovered roots of both the cultivars while the POX activity increased in stressed and recovered roots of both *Lunishree* and *Begunbitchi* cultivars. The authors concluded that improved tolerance to salt stress may be accomplished by increased capacity of antioxidative system. Cell bio-membranes, and especially lipid components, are specific targets for oxidative stress and any modification in plasma menbrane or endomembranes composition may lead to altered properties (Guo et al. 2019; Xiaoxiao et al. 2019).

1.2.1.7 Yield

Throughout civilized history, a high concentration of salt ions in arable soil has always been considered as one of the abiotic stresses very harmful to agricultural production and more specifically in most domesticated species which have been shown to be more sensitive to salt stress: among major crops, only coton and sugarbeet have direct

halophyte ancestors and may exhibit, to some extent, some constitutive salt-resistant mechanims (Lv et al. 2019; Sharif et al. 2019). Therefore, the above mentioned effects of salt stress on plants ultimately lead to reduction of crop yield which is the most countable effect of salt stress in agriculture.

Studying the effects of salinity stress on the yield of two canola cultivars (*Brassica napus* L.), Zadeh (2007) found that the number of flowers, flower primordia, siliquas, seeds per siliqua and 1000-seed weight all decreased and the most inhibitory effect was observed in the most stressed treatment with more effect on salt-sensitive cultivar than on salt-tolerant one. Salt sensitivity of rice was studied to determine salinity effects on yield components (Zeng and Shannon 2000b) and the results showed that the reductions in seedling survival, tiller number per plant and spikelet number per panicle were the major causes of yield loss in M-202 cultivar. Other studies have also proven the deleterious effect of salt stress on yield components in rice cultivars (Ali et al. 2004; Asch et al. 2000; Asch et al. 1999; Khatun and Flowers 1995; Shereen et al. 2005; Sultana et al. 1999; Zeng and Shannon 2000a; Zeng et al. 2001) and according to Abdullah et al. (2001), sterility and significant reductions in seed set in rice were not merely due to reduction or inhibition of different biochemical constituents and physiological functions, but were mainly due to limitation of soluble carbohydrate translocation in primary and secondary spikelets, accumulation of more sodium and less potassium in all the floral parts, and highly significant inhibition of specific activity of starch synthetase in developing rice grains, thus resulting in failure of seed set.

1.2.2 Mechanisms of salinity tolerance

1.2.2.1 Is salt resistance an objective or a subjective concept?

From a fundamental point of view, stress is a set of physiological responses exhibited by an organism in response to an external constraint (Levitt, 1980). This implies that "salinity" *stricto sensu* is the constraint and that the response is in fact the "stress". There is commonly confusion in this respect and in the mind of numerous people, including scientist, salt stress refer to the presence of an excess of soluble salt in the substrate (Ruan et al., 2010; Ketehouli et al., 2019). Hence, another option is to talk about an "external constraint" (salt, in our case) and an "internal constraint" (metabolism alteration induced by the osmotic and ionic component of salinity).

Similarly, there is very often a confusion between the notion of resistance and the notion of tolerance. There is no consensus in this regard among scientists and even physiologists do not agree to what is behind these terms (Levitt, 1980; Lutts and Lefèvre, 2015). It has been previously considered that "resistance" is a generic term which refers to the capacity of a plant to display its whole cycle and to set viable seeds despite the presence of an external constraint (Levitt, 1980; Ruan et al., 2010). Resistance may include two types of strategy: the first one is "avoidance" which implies that the plant "avoid" the internal constraint while the second one is "tolerance" which implies that the plant is able to maintain its metabolism despite the presence of this internal constraint. Discrimination between the two strategies is not easy to establish for physical stress, such as extreme temperature or high light intensity. In contrast, for "chemical constraint", things are easier and the fact that a given plant does not absorb Na^+ is frequently considered as an avoidance process (and allow scientist to define "excluders") while tolerance commonly implies that plants absorb ions (and behave as "includers").

To some extent, we believe that such discrimination is somewhat misleading and constitutes an oversimplification. First because it is based on the ionic component of salt stress while the osmotic component is precisely related to the external ions, those which are not absorbed by excluders, implying that "avoidance" as previously defined does not imply avoidance to osmotic stress but only to ionic stress. It may even be considered that ion absorption allow the plant to reduce its own osmotic potential meaning that plants which are behaving as salt-

tolerant includers in fact avoid the osmotic stress, at least on a short time basis, until the toxicity of ions is so high that it modifies plant metabolism (Zulfiqar et al., 2019). Secondly, discriminating between avoidance and tolerance tends to suggest that the two types of strategies are incompatible and that a plant behave as an excluder OR as an includer. Things, for sure, are not so simple, and the two types of strategy are not mutually exclusive. Indeed, a plant may, at the same time regulate toxic ion absorption and display metabolic adaptation to cope with those ions which are already absorbed. Both types of strategy imply a metabolic cost, and the most "salt-resistant" plant will probably be the plant which finds the best compromise between among a large set of complementary responses.

Even if we consider "salt-resistance", it is not always easy to identify the best criteria to consider that a plant is "salt-resistant" or "salt-sensitive". Situation is clear for halophyte plant species, which are precisely defined on the basis of their salt-resistance and are commonly found in environments characterized by a high level of salinity (Ruan et al., 2010; Lutts and Lefèvre, 2015; Ketehouli et al., 2019). For glycophyte species, things are by far more difficult. The previous definition (the capacity of a plant to display its whole cycle and to set viable seeds) should probably be considered as the best one and is established on a botanical perspective. For cultivated plant species (and all cultivated plant species except *Amaranthus cruentus* and *Chenopodium quinoa* are considered as true glycophyte (Flowers and Yeo, 1995)), yield is regarded as the best criteria. But the physiological strategies which are behind yield maintenance are completely different depending on the considered species and the harvested organs. For example, in an agronomical perspective, salt-resistance in wheat implies that plants remain able to set seed even if root development is impaired while salt resistance in sugar beets is precisely based on tap root growth and sugar accumulation even if salinity implies sterility in this biannual plant species. A huge amount of papers are published each year on salt resistance of cultivated plant species. For practical reasons, most of them imply a short-term exposure which do not consider yield itself, but plant growth. Is growth a reliable indicator? It could be for cultivated plant species, since yield maintenance imply activities of source organs. But reliability of growth-related parameters is not a guarantee for the whole plant kingdom. Once again, from an ecological point of view, the priority for the plant exposed to salinity in its natural environment is to survive and set seeds: from a metabolic point of view,

plant growth is expensive and it might be hypothesized that in some cases, the best strategy for the plant is to limit vegetative growth and affect a part of energy to protective mechanisms and save the remaining part for the reproductive phase of development.

Hormesis, which is a positive impact of very low doses of stressor on plant growth and metabolism, has often been considered in the past as an artefact but now appears more and more as a reality which rely on numerous biochemical cues (Poschenrieder et al., 2013). It illustrates that the link between "stress" and "plant growth inhibition" is weak in some cases. Another important aspect is the capacity of the plant to recover after the stress relief. The development of a given plant might be inhibited but the plant remains able to resume growth and development when the constraint disappears. At the opposite, a plant which remains able to still grow during the stress period could spend too much energy and this may compromise full efficient metabolism recovery when the stressing factor disappear. According to a "classical" view, the first one might be considered as "sensitive" while the second one will be classified as "resistant" when focusing only on the stress period, although this is not fully relevant from the ecological reality. Recovery from stress is frequently analyzed in the literature in relation to water stress or exposure to extreme temperatures since those parameters may rapidly fluctuate on a short-term basis (Rueh et al., 2019; Foresta et al., 2019; Loka et al., 2020). In contrast, ion toxicities are considered to remain more or less constant on a short-term basis but we still lack precise information regarding this specific point and it could be interesting to analyze fluctuations of salinity in field conditions during the plant cycle considering evapotranspiration leading to salt accumulation on the one hand and pluviometry leading to salt dilution on the other hand, and to integrate, if required, salinization related to irrigations schemes.

It is well known that within cultivated plants, response to salinity differ among cultivars. It is not realistic to consider two groups: salt-sensitive on the one hand and salt-resistant on the other hand. Salinity resistance is not a qualitative Mendelian character and there is a continuous variation between extremes (Flowers and Yeo, 1995). Hence, considering a given cultivar as "salt-sensitive" or "salt-resistant" often depends on the set of cultivars that are considered in a study. For rice, more than 100 000 cultivars and local landraces are available in the seed bank of IRRI. For glycophyte species, "salt resistance" is always a relative concept…meaning that one cultivar quoted as "salt resistant" is

in fact “less sensitive” than another one but this classification is highly dependent on the limited set of cultivars that are considered in the study. This is typically the case for IKP, which is considered here as a sensitive cultivar because we used (see the experimental parts) other cultivars which are by far less sensitive, although in some studies, IKP is sometimes reported to be moderately resistant (Lutts, personal communication).

1.2.2.2 Salt-resistant: an evolutionary perspective.

It is well established that life originates from the sea, and that the first living cells thus appear in a medium with a high salt concentration and a low water potential. Even if ocean at the origin contains less salt than today (26-29 gr.L^{-1} against 30-40 gr.L^{-1} in today’s ocean according to Railsback et al., 1990), it corresponds to a very low water potential and suggests that salt itself is not incompatible with life. Most plants, however, are clearly typical glycophyte species and halophyte only represent a very small proportion of the plant kingdom (2600 true halophytes for more than 200 000 angiosperms according to Menzel and Lieth (2003)).

This tends to suggest that the first terrestrial plants should have been salt-resistant and that his trait was then rapidly lost during subsequent evolution. Channing and Edwards (2009) indeed identified fossils in Scotland from the early Devonian (around 400 million year ago) showing that the corresponding plant was salt-tolerant. Nevertheless, the usual option that land plant originates from the sea is not so easily accepted by some members of the scientific community who consider that a pre-adaptation to a fresh water life was probably occurring allowing the plant to acquire nutrient from a low concentration of nutrient (Rodriguez-Navarro and Rubio, 2006). This is still a matter of passionate debate.

Salinity resistance is a very complex trait which require the simultaneous presence of a huge number of properties interacting with each-others in an adapted sequence (Yeo and Flowers, 1986). If one single property is absent, then the whole organism will be salt-sensitive, suggesting that salinity resistance is by far easier to be lost than to be acquired.

While animals or fungal cells possess Na^+-ATPases, plants do not appear to express, higher plants rely only on Na^+/H^+ transporter for

internal Na^+ compartementation (Benito and Rodriguez-Navarro, 2003) but some mosses, such as *Physcomiteilla patens* express two ATPase similar to fungal ENA-Na^+-ATPase (Lundle et al., 2007). This, however, is a specific case, and Aronson (1989) listed only few frequencies of salt tolerance in bryophyte and pteridophyte (with even, in this last class, one single fern genus exhibiting clear halophyte species).

Salt glands are present only in a few orders of flowering plants and their distribution suggest at least three independent origins occurring during the evolution of the plant kingdom (Flowers et al., 2010). Some others suggest a polyphyletic origin for salt resistance in plants, which then can be quite different from their common ancestor (if any) and salt-resistance seems to have evolved multiple time (Rozema and Flowers, 2008) and even related lineages may develop different strategies for dealing with environmental salt. Bennett et al. (2013) similarly found that in grasses, salt-tolerance has evolved over 70 times but that even if salt-resistance evolves surprisingly, its evolution does not often give rise to a large number of salt tolerant species, thus suggesting that salt-resistance is an evolutionary labile trait in grasses.

Another point of view is to consider that inducible genes contributing to salinity resistance are still present in numerous plants, including glycophytes, but that the most salt-resistant plant are those which are able to trigger the right response at the right place more rapidly than the sensitive one (Baillo et al., 2019). As a consequence, salinity resistance might be a consequence of the kinetics of the response and not only the nature of the response. The most salt-resistant plants should be the most vigilant in order to prevent symptoms appearance, while the most sensitive may trigger similar response a long time after symptom appearance, at a time when it is difficult to identify a specific physiological response as an attempt of adaptation or as a consequence of injuries. We should thus focus on stress sensing, signaling and efficiency of signal transduction pathways. This view could be valid for distinct cultivars belonging to the same species but the situation may be different when comparing species clearly separated and distant at the phylogenic level (Iseki et al., 2016).

Three distinct types of plant response or tolerance to salinity stress were proposed by Munns and Tester (2008):

1. *Tolerance to osmotic stress*. During the first time of salt stress, osmotic stress reduces cell expansion in root tips and young leaves, and causes stomatal closure. The less effective response to osmotic stress could lead to high leaf growth as well as to increased stomatic conductance; only the plant growing in soil with sufficient water will benefit from this leaf growth. The fact that cell wall properties exhibits a higher extensibility as a result of modification in rheological properties also help to maintain cell elongation despite a salt-induced decrease in cell turgor (Ali and Yun 2017; Meng et al. 2018; Mishra and Tanna 2017).

2. *Na+ exclusion from leaf blades*. Na^+ exclusion by roots ensures that Na^+ does not accumulate to toxic concentrations within leaves. However, in case of high salt stress, the Na^+ exclusion at the root level is exceeded and this leads to a strong accumulation of Na^+ at the leaf level; which disrupts the cell metabolic processes. The toxic effects are observed after a few days or weeks of plant exposure to salinity stress depending on the crop species, the nature of the salt ions and the stress level. The first observed symptom is the premature death of older leaves.

3. *Tissue tolerance*, i.e., tolerance of tissue to accumulated Na^+, or in some species, to Cl^- or SO_4^{2-}. Tolerance requires compartmentalization of salt ions at the cellular and intracellular level to avoid toxic concentrations within the cytoplasm, especially in leaf's mesophyll cells.

Plants have adopted the biochemical and molecular mechanisms to cope with salt stress. Biochemical pathways leading to products and processes that improve salt tolerance are likely to act additively and probably synergistically (Iyengar and Reddy 1996). Biochemical strategies include (i) selective accumulation or exclusion of ions, (ii) control of ion uptake by roots and transport into leaves, (iii) compartmentalization of ions at the cellular and whole-plant levels, (iv) synthesis of compatible solutes, (v) change in photosynthetic pathway, (vi) alteration in membrane structure, (vii) induction of antioxidative enzymes, and (viii) induction of plant hormones (Fig. 6).

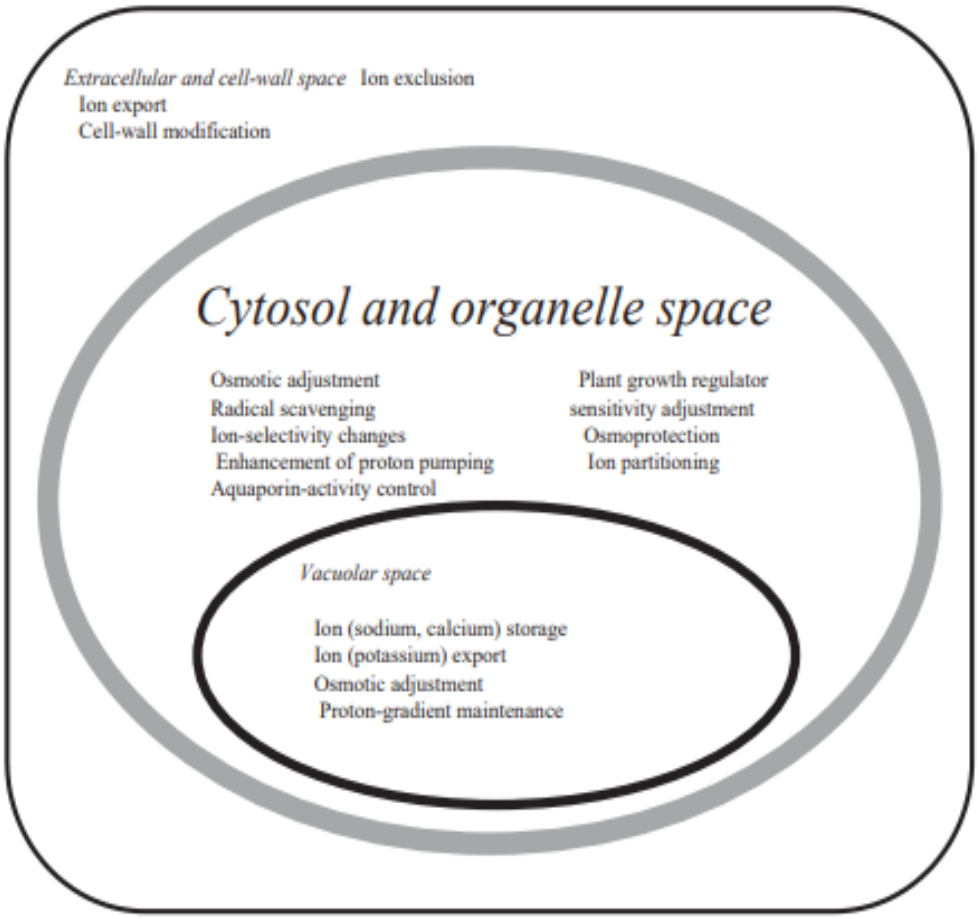

Figure.6. Biochemical functions associated with tolerance to plant salt stress. The schematic presentation of a plant cell includes three compartments that are defined by the plasma membrane and tonoplast (reproduced from Bohnert and Jensen, 2001).

There has been increasing interest in studying the omics tools to identify and understand salt tolerance components and mechanisms at molecular level (Isayenkov and Maathuis 2019; Jha et al. 2019; Singh et al. 2017; Yang and Guo 2018). Below, we will show the roles of genomics, transcriptomics and proteomics in salt stress tolerance.

1.2.2.3 Genomic level

Tolerant plants may possess some unique stress-responsive genes which are absent in susceptible plants. Profiling at the genome level, when combined with systematic genetic analysis, promises to reveal much of the signaling networks that control stress tolerance (Munns 2005). The author classifies the genes involved in salt stress tolerance into three functional groups: (1) those that control salt uptake and transport; (2) those that have an osmotic or protective function; and (3) those that could make a plant grow more quickly in saline soil.

1.2.2.3.1 Genes that control salt uptake and transport

Increasing evidence exists for the role of some members of the *HKT* gene family in retrieval of Na^+ from the xylem. In *Arabidopsis*, the importance of Na^+ retrieval from the xylem as a primary controller of shoot Na^+ concentration and plant salinity tolerance is suggested by forward genetic studies that have revealed *AtHKT1;1* (Fig.7) as a primary determinant of these parameters (Berthomieu et al. 2003; Gong et al. 2004). Furthermore, good evidence exists that a closely related gene, *TmHKT1;4-A2*, is the candidate gene for the Na^+ exclusion (*Nax1*) locus in durum wheat (Huang et al. 2006), which is associated with Na^+ exclusion and a high leaf K^+ /Na^+ ratio. The protein encoded by this gene retrieves Na^+ from the xylem, and has activity in the leaf sheaths as well as in the root (James et al. 2006).

Analyzing the *AtHKT1* expression in transgenic plants containing an *AtHKT1* promoter-GUS construct, some researchers showed the expression of this gene in vascular tissues and further in situ hybridizations showed expression in phloem tissues (Berthomieu et al. 2003). Taken together with the previous reports showing the Na^+ selectivity of *AtHKT1*, a model was proposed in which *AtHKT1* would mediate Na^+ loading into the phloem in leaves, and also Na^+ unloading from the phloem sap in roots. Several studies have shown that members of *HKT* gene family play important roles in long-distance Na^+ transport and plant salt tolerance.

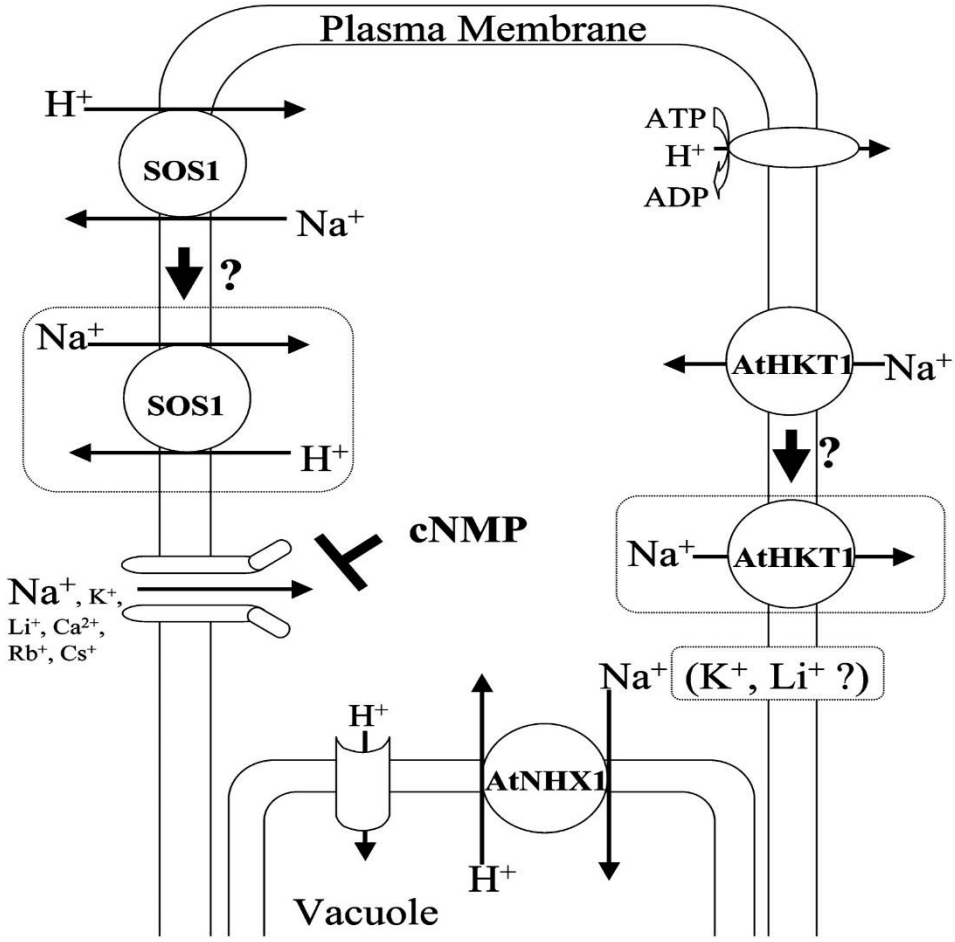

Figure.7. Schematic drawing of a simplified plant cell model representing Na^+ transport proteins (Horie and Schroeder 2004).

Na^+ extrusion at the root-soil interface, as well as some level of Na^+ efflux in every other cell type to maintain ion homeostasis, is presumed to be of critical importance for the salt tolerance of glycophytes (Tester and Davenport 2003). Indeed, efficient efflux of Na^+ to the soil solution must function in the roots of several species to minimize net uptake because unidirectional influx of Na^+ is rapid and greatly exceeds the rate of accumulation. The Na^+/H^+ antiporter salt overly sensitive (*SOS1*) (Fig.7) is the only Na^+ efflux protein at the plasma membrane of plants characterized so far. In *Arabidopsis*, ion homeostasis is mediated mainly by the *SOS* signal pathway, which consists of three main components. *SOS1* encodes a plasma membrane Na^+/H^+ antiporter that is involved in sodium extrusion and in controlling long-

distance Na^+ transport from the root to shoot (Shi et al. 2000; Shi et al. 2002). The authors hypothesized a long-distance Na^+ transport model, in which under moderate stress, *SOS1* functions to load Na^+ into the xylem in roots for Na^+ transfer and storage in leaf mesophyll vacuoles. Under severe Na^+ stress, *SOS1* is proposed to function in unloading Na^+ from the root xylem to reduce Na^+ damage of leaves that might be caused by exceeding the capacity of Na^+ sequestration in leaf cell vacuoles (Shi et al. 2002). *SOS3* encodes an EF-hand Ca^{2+}-binding protein that functions as a calcium sensor for salt tolerance (Liu and Zhu 1998) while *SOS2* interacts with and is activated by *SOS3* (Qiu et al. 2002). The *SOS2*/*SOS3* kinase complex phosphorylates and activates *SOS1*.

Studying the conservation of the Salt Overly Sensitive pathway in rice, Martínez-Atienza et al. (2007) reported that the rice transporter, denoted by *OsSOS1*, proved a capacity for Na^+/H^+ exchange in plasma membrane vesicles of yeast (*Saccharomyces cerevisiae*) cells and reduced their net cellular Na^+ content. Moreover, *OsSOS1* suppressed the salt sensitivity of a *sos1-1* mutant of Arabidopsis. Based on *SOS1* expression profile, Zhu (2003) suggested several roles for this gene in the plant. Indeed, the three main roles are: Na^+ efflux into the root medium; buying time for Na^+ storage in the vacuole by slowing down Na^+ accumulation in the cytoplasm; and controlling long-distance Na^+ transport between roots and leaves by loading Na^+ into and unloading Na^+ from the xylem and phloem.

At high Na^+ concentration in the cytoplasm, vacuolar sequestration of Na^+ not only lowers Na^+ concentration but also contributes to osmotic adjustment to maintain water uptake from saline solutions (Zhu 2003). According to the author, other organelles such as plastids and mitochondria, may also accumulate some Na^+ and thus contribute to the overall subcellular compartmentation of Na^+. Vacuolar sequestration of excess Na^+ is accomplished by tonoplast Na^+/H^+ antiporters such as those belonging to the Na^+/H^+ exchanger (*NHX*) family (Fig.7) in rice (Pardo et al. 2006). The authors showed that there is a passive leakage of Na^+ back to the cytosol from vacuoles (possibly via tonoplast nonselective cation channels), requiring constant resequestration of Na^+ into vacuoles. Constitutive overexpression of *NHX1* increases both Na^+ accumulation and Na^+ tolerance in Arabidopsis, suggesting that more efficient sequestration may improve tissue tolerance, perhaps by

reducing cytosolic Na^+ concentrations (Gaxiola et al. 2001). Analyzing the function and intracellular localization of the product of the Na^+/H^+ antiporter gene (*OsNHX1*) cloned from rice (*Oryza sativa*), Fukuda et al. (2004) found that the *OsNHX1* gene encodes a vacuolar (Na^+, K^+)/H^+ antiporter and the treatment with high concentrations of NaCl or KCl increased the transcript levels of *OsNHX1* in rice roots and shoots. The authors confirmed that the tonoplast-induced *OsNHX1* plays important roles in the compartmentation of excess Na^+ and K^+ into the vacuoles, and they concluded that the amount of antiporter is one of the most important factors determining salt tolerance in rice.

1.2.2.3.2 Genes with an osmotic or unknown protective function

The small organic compounds called osmolytes, osmoprotectants or compatible solutes provide a protective function to the plant cell under osmotic stress. To accommodate the ionic balance in the vacuoles, cytoplasm accumulates these low-molecular-mass compounds because they don't interfere with normal biochemical reactions (Parida and Das 2005). With accumulation proportional to the change of external osmolarity within species-specific limits, the osmolytes have an active role in osmotic regulation, in maintaining membrane integrity, stabilizing proteins, alleviating ionic toxicity, protecting cellular components, scavenging reactive oxygen species, and defending antioxidant compounds as well as balancing cellular redox under salinity stress (Hasanuzzaman et al. 2019). Other genes products include enzymes that "mop up" free radicals, and proteins that protect the formation or stability of other proteins. Munns (2005) reported the four main classes of solute that could have an osmotic or protective role: N-containing solutes such as proline and glycine betaine; sugars such as sucrose and raffinose (which are both non-reducing sugars); straight-chain polyhydric alcohols (polyols) such as mannitol and sorbitol; and cyclic polyhydric alcohols (cyclic polyols) such as D-ononitol and D-pinitol (Table 2). However, not all crop species are able to effectively synthesize osmoprotectants. The creation of transgenic plants via the transfer of genes responsible for the osmolytes synthesis could be an alternative for improving rice production.

Several researchers have conducted studies on protective effects of exogenous osmolytes in mitigating salt stress-induced damages in *Oryza sativa* and they observed a marked improvement in the tolerance

level of the cultivars (Nounjan et al. 2012; Sobahan et al. 2012; Sobahan et al. 2009; Theerakulpisut and Phongngarm 2013; Wutipraditkul et al. 2015). The fact that salt stress was applied for rather short time is however misleading: laboratory approaches do not provide realistic proof that salinity tolerance is improved at all development stages and will afford a higher yield in the specific cases of cultivated plants. This could be especially the case for some compounds such as glycinebetaine, which is not naturally produced by some important crops such as rice and tomato. Transgenic approaches aiming to transfer genes responsible for GB synthesis in these species often lead to negative results because these plants, even if they produce this compound are not able to use it efficiently because they lack the information necessary to allow interaction between this osmoprotectant and their cellular targets (Acosta-Motos et al. 2017; Fedotova 2019; Mansour and Ali 2017). Modulating proline synthesis is not an easy task, considering the complexity of the metabolic pathways involved in proline synthesis, and the interaction with the phytohormonal status of the stressed plants (Anwar et al. 2018; Llanes et al. 2018; Per et al. 2017).

Table 2. Major categories of solutes with possible function as osmolytes or protectants, and results of key overexpression transformation studies, summarized by Munns (2005).

		Overexpression studies with whole plants that resulted in increasing salt tolerance			
Solute type	Function	Solute	Gene	Species	Conc.
N-containing solutes such as proline, glycine betaine, trigo-nelline	Tugor maintenance. Possibly redox regulation, to buffer cellular redox potential as metabolic activities slow down during stress, and so to promote recovery from stress.	Proline	*P5CS mod*	Tobacco	60 mM
		Glycine betaine	*codA*	Rice	5 mM
Trehalose	Signalling, possibly through hexose sensing	Trehalose	*otsA*, *otsB*	Rice	300 µM
Straight-chain polyhydric such as mannitol and sorbitol	Tugor maintenance, particularly in the cytosol. Mannitol may be involved in hexose sensing or in mopping up hydroxyl radicals.	Mannitol	*mt1D*	Wheat	2 mM
		Mannitol	*mt1D*	Tobacco	6 mM
		Sorbitol	*S6PDH*	Persimmon	60 mM

Table 2. *Continued*

		Overexpression studies with whole plants that resulted in increasing salt tolerance			
Solute type	Function	Solute	Gene	Species	Conc.
Cyclic polyhydric alcohols such as myo-inositol, ononitol and pinitol	Tugor maintenance in certain halophytes. Might also mop up hydroxyl radicals	Ononitol	*imt1*	Tobacco	35 mM
Sugars such as fructose, sucrose, glucose and raffinose	Tugor maintenance, carbohydrate storage	-	-	-	-

All the summarized studies used 100–200 mM NaCl for 2–4 weeks in most cases, and increased the salt gradually at 25–50 mM d^{-1}. *P5CS* (Δ-1-pyrroline-5-carboxylate synthetase modified from *Vinca aconitifolia*); *codA* (choline oxidase from *Arthrobacter globiformis*); *otsA* and *otsB* (trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase from *Escherichia coli*); *mt1D* (mannitol-1-phosphate dehydrogenase from *E. coli*); *S6PDH* (sorbitol-6-phosphate dehydrogenase modified from apple); *imt1*(d-*myo*-inositol methyltransferase from *Mesembryanthemum crystallinum*).

In addition to the osmolytes production, aerobic organisms evolved a complex antioxidant system, which protect the cells against oxidative damage caused by the reactive oxygen species (ROS). Considering the plant cells, the mecanisms involved in ROS producing and scavenging are found in different organelles such as chloroplasts, mitochondria, and peroxisomes. In the section below, the protective molecules are taken as the gene product, rather than the gene.

The nonenzymatic components of the antioxidative defense system include the major cellular redox buffers ascorbate (AsA) and glutathione (γ-glutamyl-cysteinyl-glycine, GSH) as well as tocopherol, carotenoids, and phenolic compounds (Sharma et al. 2012). They interact with numerous cellular components and in addition to crucial roles in defense and as enzyme cofactors, these antioxidants influence plant growth and development by modulating processes from mitosis and cell elongation to senescence and cell death (de Pinto and De Gara 2004).
The enzymatic components of the antioxidative defense system comprise of several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), enzymes of ascorbate-glutathione (AsA-GSH), cycle ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Noctor and Foyer 1998). These enzymes operate in different subcellular compartments and respond in concert when cells are exposed to oxidative stress.

According to Teixeira et al. (2006), the rice APX gene family encodes functionally diverse isoforms localized in different subcellular compartments and the transcript accumulation analysis showed specific expression patterns for each member of the APx family according to

developmental stage and in response to salt stress, revealing the complexity of the antioxidant system in plants. The *OsAPX3* was localized in the peroxisomes while *OsAPX6* was found in mitochondria. These authors reported the functional diversity of the rice APx genes and suggest complementation and coordination of the antioxidant defenses in different cellular compartments during development and salt stress. Other studies also revealed the inductive response of the antioxidative defense system by salt stress in the rice (*Oryza sativa* L.) (Demiral and Türkan 2005; Dionisio-Sese and Tobita 1998; Khan and Panda 2008; Lee et al. 2001; Menezes-Benavente et al. 2004; Vighi et al. 2017).

1.2.2.3.3 Genes that control cell and tissue growth rates

A plant exposed to salt stress quickly enters a quiescent state followed by the growth resumption, often at a growth rate much lower than that of a control plant. The osmotic effect of salinity stress is thought to be the cause of the quiescent state initiation while no effect could be attributed to salt ions accumulation in the plant (Roy et al. 2014). Under saline conditions, the repression of the genes responsible for signal transduction via gibberellin and brassinosteroid synthesis leads to the reduction of cell cycle activity and meristem size (West et al. 2004). Wang et al. (1998) reported that a potentially important link between stress and cell division was revealed by induction of *ICK1* in Arabidopsis by abscisic acid. *ICK1*, a cyclin dependent-protein-kinase inhibitor, might hinder cell division by reducing the activities of cyclin-dependent protein kinases that help to drive the cell cycle. Salt and water stress might inhibit cell division by causing the accumulation of abscisic acid, which, in turn, induces *ICK*. Additionally, salt stress-induced cell wall stiffening limits cell elongation (Tenhaken 2015). The key players in this process are peroxidases present in the apoplast, which use ROS as a co-substrate. During the early stages of stress, high apoplastic ROS levels induce peroxidase activity and then, peroxidase cross-links phenolic compounds and glycoproteins of the cell wall, resulting in reduced extensibility of the cell wall and limited cell expansion (Tenhaken 2015).

According to Munns (2005), genes that could increase the growth rate of plants in saline soil would influence the rate of production of new leaves and roots by controlling the rate of cell division, the development of new primordia for shoot or root branching, the rate of cell wall expansion, or the dimensions of differentiated cells. The author found that these genes would also influence the rate of photosynthesis by regulating stomatal aperture or the dimensions of mesophyll cells, which influences not only the leaf morphology but also the transpiration efficiency. After the quiescent state, the growth resumption of the plant exposed to salinity stress relies on hormonal cues, sensitivity of early signaling, and the plant ability for acclimation (Munns and Tester 2008). Production of stress granules (SGs), a group of cytoplasmic foci that assemble in response to stress, has an important role in growth resumption. SGs act as centers of messenger (m) RNA regulation

through selective re-initiation or degradation of mRNA (Weber et al. 2008).

The SGs produced are involved in stabilization of the *GA20ox3* transcripts, a key enzyme for gibberellin biosynthesis (Yan et al. 2014). Geng et al. (2013) suggest a crucial role of ABA in growth resumption of the salt-treated plants. Zhu et al. (2015) observed that rice plants over-expressing *OsMYB91* showed reduced plant growth and accumulation of endogenous ABA under control conditions. Under salt stress, the over-expression plants showed enhanced tolerance with significant increases of proline levels and a highly enhanced capacity to scavenge active oxygen. Furthermore, the authors found that *SLR1*, the rice homolog of Arabidopsis *DELLA* genes that have been shown to integrate endogenous developmental signals with adverse environmental conditions, was highly induced by *OsMYB91* over-expression, while the salt-induction of *SLR1* expression was impaired in the RNAi plants. Based on these results, it is clear that *OsMYB91* is a rice gene involved in abiotic stress tolerance as well as in improvement of plant growth by regulating *SLR1* expression.

The previous studies have evidenced that the constitutive expression of some stress related genes such as *CBF1, DREB1A, ATHB7* and yeast trehalose synthase, caused the slow growth of transgenic plants (Holmström et al. 1996; Liu et al. 1998; Soderman et al. 2000; Söderman et al. 1996). *CBF1, DREB1A* and *ATHB7* are cold- or drought-inducible genes that are not expressed under normal growth conditions while trehalose might be a signalling molecule for growth control as well as for stress tolerance.

The extensibility of the plant cell wall increases with prolonged exposure to salt stress. During the later stages of salt stress, where the availability of phenolic compounds and glycoproteins is low, high ROS levels in the apoplast result in increased OH^- radicals, which cleave sugar bonds in polysaccharides in the cell wall (Tenhaken 2015). This together with activity of xyloglucan-modifying enzymes and expansins present in the apoplast result in cell wall loosening, allowing growth recovery.

According to the previous studies, it is clear that salinity tolerance in rice requires the combination of several different genes. Several researchers also proved that the mechanisms involved in salinity

tolerance are diverse; making it a complex and polygenic trait (Bohnert et al. 2001; Munns and Tester 2008; Tuteja 2007). Therefore, it is evident that effective plant improvement to salinity tolerance requires multiple genes involved in the principal mechanism of the processes such as signaling, ion homeostasis, osmotic adjustment, vacuolar compartmentalization of ions, restoration of enzymatic activity, oxygen free radical scavenging and photorespiration (Bohnert et al. 2001).

1.2.2.4 Transcriptomic level

A transcriptome is the complete set of transcripts representing all messenger RNA molecules in a given cell at a particular time point. Transcriptomics, also termed as expression profiling, generally involves a systematic and comprehensive study of all the RNA transcripts that captures gene expression (spatial and temporal expression) of a cell and tissue of an organism under a given biological context (Duque et al. 2013). According to Zhou et al. (2007), the identification of genes regulated by salinity stress has several meanings. First, it provides a more comprehensive understanding of the transcriptional responses to this stress. Second, it provides a starting point for further elucidating the role of individual genes in stress responses, which will be of great value in crop engineering. Third, it aids in the identification of stress responsive promoters and responsible cis-elements within them that are important for both basic study and crop engineering applications.

With the rapid improvement in the sequencing technologies, the sequencing of more and more crop plant genomes is being completed (Lister et al. 2009; Mardis 2008) . Being a model for monocots and having a small genome size among cereals, rice genome was the first cereal that was completely sequenced (Jiang and Ramachandran 2010). Over 95% of the Japonica genome (372.1 Mb) was covered by the map-based rice genome sequence assembly (Sasaki 2005) and about 56,278 genes (loci) were listed in the Institute for Genomic Research (TIGR) which is released as Rice Genome Annotation Database (release 5) in 2007 (Ouyang et al. 2006). Rice has the richest set resources available for plant genomic studies.

At the transcriptomic level, plants respond to salinity stress by activating a range of genes involved in stress signaling, transcription regulation, ion transport, and biosynthesis of specific metabolites of

complex signaling pathways (Cotsaftis et al. 2011; Kawasaki et al. 2001; Kumar et al. 2013; Walia et al. 2007). A study on genetic variability based on the evaluation of the expression profiles of genes involved in response to saline stress is very useful in rice crop. A comparison between two or more genotypes with contrasting salt responses is beneficial in the discovery of new tolerance mechanisms in rice (Formentin et al. 2018).

The transcriptome responses of two related rice genotypes, IR64 and PL177, with contrasting salt tolerance were characterized under salt stress and salt+abscisic acid (ABA) conditions (Wang et al. 2015c). In PL177, salt specifically upregulated genes involved in several pathways underlying salt tolerance, including ABA-mediated cellular lipid and fatty acid metabolic processes and cytoplasmic transport, sequestration by vacuoles, detoxification and cell wall remodeling in shoots, and oxidation–reduction reactions in roots. Exogenous ABA was able to alleviate the salt stress effect on the salt-sensitive line IR64, indicating that the ABA-mediated system for salt tolerance is at least partially impaired in IR64. Indeed, exogenous ABA greatly up-regulated the genes involved in the lipid and fatty acid metabolisms and cytoplasmic transport in shoots of both genotypes under salt stress, suggesting that enhanced lipid metabolism might also contribute to enhanced salt tolerance of rice.

The transcriptomic analysis conducted on tolerant rice-plants supported the setting up of an adaptive program consisting of sodium distribution preferentially limited to the roots and older leaves, and in the activation of regulatory mechanisms of photosynthesis in the new leaves (Formentin et al. 2018). In contrast, the authors confirmed that in sensitive variety, RNA-seq analysis revealed a misleading response, ending in senescence and cell death.

Shankar et al. (2016) reported that the comparison of transcriptomes of a drought-tolerant [Nagina 22 (N22)] and a salinity-tolerant (Pokkali) rice cultivars with IR64 (susceptible cultivar) revealed variable transcriptional responses under control and stress conditions. A total of 801 and 507 transcripts were exclusively differentially expressed in N22 and Pokkali rice cultivars, respectively, under stress conditions. A larger number of transcripts encoding for members of NAC and DBP transcription factor (TF) families in N22 and members of bHLH and C2H2 TF families in Pokkali exhibited differential regulation under

desiccation and salinity stresses, respectively. Transcripts encoding for thioredoxin and involved in phenylpropanoid metabolism were up-regulated in N22, whereas transcripts involved in wax and terpenoid metabolism were up-regulated in Pokkali.

Other studies revealed various salt stress-responsive TFs, like AP2/EREBP, NAC, bHLH, HB, MYB, MYB-related, WRKY, C2H2, and bZIP to be enriched in N22 and Pokkali cultivars under stress conditions (Garg et al. 2016; Lu et al. 2010; Walia et al. 2005). Transcription factors are promising targets for the improvement of plant resistance to abiotic stresses but their expression need to be regulated during the development and occur at the correct place which requires the selection of adapted promoters before using the corresponding gene for transgenic approaches (Baillo et al. 2019; Mishra and Tanna 2017).

In order to compare the rice plant responses to drought and high-salinity stress, a 70mer oligomer microarray covering 36,926 unique genes or gene models was used to profile genome expression changes in rice shoot, flag leaf and panicle under drought or high-salinity conditions (Zhou et al. 2007). While patterns of gene expression in response to drought or high-salinity stress within a particular organ type showed significant overlap, comparison of expression profiles among different organs showed largely organ-specific patterns of regulation. Moreover, both stresses appear to alter the expression patterns of a significant number of genes involved in transcription and cell signaling in a largely organ-specific manner.

Other studies showed that the transcriptome analyses in different rice cultivars provide new insights into adaptive and salinity stress responses (Chao and Lin 2018; Dai Yin et al. 2005; Rabbani et al. 2003; Sengupta et al. 2019; Ueda et al. 2006; Wang et al. 2018).

1.2.2.5 Proteomic level

Wilkins et al. (1996) revealed the term “proteome” to refer to the total set of proteins encoded by the genome of an organism. “Proteomics” is the global study of the proteins comprising the proteome, including the changes in structure and abundance in response to developmental and environmental cues. In recent years, the term proteomics has also been applied to all the proteins expressed in a particular organelle or tissue or in response to a particular stress. Functional gene expression patterns

is only achieved by proteomic analysis. Furthermore, proteins undergo significant levels of post-translational modification of their primary sequences followed by targeted proteolysis. Thus, quantitative analysis of gene expression at the protein level is essential for determining plant response to the stresses among others salt stress. Several researchers have applied proteomics to identify salt-responsive proteins in different rice organs like root, leaf lamina, leaf sheath, panicles, anthers and plasma membrane.

Proteomic comparisons of salt stress-tolerant and stress-sensitive genotypes revealed numerous constitutive and stress-induced differences in rice root proteins (Salekdeh et al. 2002). RS5, a 22 kDa protein, was up-regulated as much 3-fold in Pokkali (salt-tolerant genotype) but no significant change was observed in IR29 (salt-sensitive genotype). The protein showed sequence homology with ASR1 class of proteins whose main function would be the protection of DNA structure during osmotic stress (Riccardi et al. 1998). In the absence of stress, ascorbate peroxidase was 4.4 times more abundant in Pokkali roots than in IR29 roots and after salt stress, the abundance of the protein increased in both cultivars. Ascorbate peroxidase catalyzes the conversion of H_2O_2 to H_2O and O_2 and helps to protect cells from Reactive Oxygen Species (ROS). Caffeoyl-CoA *O*-methyltransferase (CCOMT) was also markedly up-regulated by salt-stress in Pokkali but little changed in IR29. CCOMT plays an essential role in the synthesis of guaiacyl lignin units as well as in the supply of substrates for the synthesis of syringyl lignin units. Increased lignification may help to reduce the bypass water flow that allows Na^+ ions to enter roots via an apoplastic route (Yeo et al. 1999).

Parker et al. (2006) performed the proteomic analysis of short- and long-term salt-stress-responsive proteins in the rice leaf lamina. The authors identified approximately 2500 protein species of which 32 were observed to be significantly regulated by salinity. Tandem mass spectrometry allowed to identify more precisely 11 of these 2500 proteins; their location, role and molecular function are described in the table 3.

Proteomic approach had been also applied in order to analyze salt-responsive proteins in rice leaf sheath (Abbasi and Komatsu 2004). Eight proteins showed 1-to-3-fold up-regulation in leaf sheath, in response to 50 mM NaCl for 24 h. Among these, three proteins were

unidentified (LSY081, LSY62, LSY363) while five proteins were identified as fructose bisphosphate aldolases, photosystem II (PSII) oxygen evolving complex protein, oxygen evolving enhancer protein 2 (OEE2) and superoxide dismutase (SOD). Gill et al. (2015) reported that SODs constitute the first line of defense against salt stress-induced ROS and its reaction products. Aldolases catalyze the cleavage of fructose-1-6-bisphosphate into D-glyceraldehyde-3-phoshate producing dihydroxyacetone phosphate and ATP. A possible reason for up-regulation of aldolases is that salinity causes oxidative stress leading to an anaerobic condition and so, acclimation to anoxia seems to be associated with greater ability to synthesize ATP in glycolysis and ethanolic fermentation (Andrews et al. 1994). Increased expression levels of OEE2 might be needed to repair protein damage caused by either NaCl or dissociation.

To further understand the mechanism of plant response to salinity at the reproductive stage, a proteomic approach had been employed to profile the protein changes of rice young panicle under salt stress (Dooki et al. 2006). The authors identified the proteins involved in several salt responsive mechanisms which may increase plant adaptation to salt stress including higher constitutive expression level and upregulation of antioxidants, up-regulation of proteins involved in translation, transcription, signal transduction, and ATP generation. Among the proteins identified, the up-regulation of stress-induced protein Sti1 may decrease the sterility of pollen during anther development (Kurek et al. 2002). The putative r40c1 protein was up-regulated up to four-fold in response to salt stress and it has been suggested that this protein is involved in water loss prevention and cell wall rigidity (Moons et al. 1997). The GR-RBP protein which increased more than twofold may stabilize mRNA and enhance synthesis of specific proteins during salt stress. The up-regulation of Guanine nucleotide-binding protein β-subunit-like protein (GPB-LR) might act on cell division and signaling during stress condition (Chen 2001; Smith et al. 1999). Other proteins up-regulated are involved in down-regulation of ROS accumulation (Nucleoside diphosphate kinase), in carbon metabolism and energy transduction (Triosephosphate isomerase, dihydrolipoamide dehydrogenase and cytosolic malate dehydrogenase) and in thylakoid formation or lipid deposition (enoyl-ACP reductase).

Table 3. Identified rice leaf lamina proteins altering in expression after short- and long-term salt stress (Parker et al. 2006)

Protein ID	Location	Role	Molecular function	Expression
Putative chloroplast phosphoglycerate kinase	Plastid	Calvin cycle	Phosphoglycerate kinase activity	Short
ATP synthase β subunit	Plastid	ATP biosynthesis	Nucleotide binding, ATP binding	Short and long
Rubisco activase large phosphoglycerate kinase	Plastid	Calvin cycle	Catalytic chaperone	Short and long
Ferritin	Plastid	Iron homeostasis	Iron sequestration	Short and long
S-adenosylmethionine synthetase 1	Unknown	One carbon metabolism	Methionine adenosyltransferase	Long
S-adenosylmethionine synthetase 2	Unknown	One carbon metabolism	Methionine adenosyltransferase	Long
Putative porphobilinogen deaminase	Plastid	Porphyrin biosynthesis	Hydroxymethylbilane synthase activity	Long
Putative superoxide dismutase [Cu-Zn], chloroplast	Plastid	Superoxide metabolism	Copper, zinc superoxide dismutase activity, metal ion binding	Long
Translation initiation factor 5A [*Oryza sativa*]	Nuclear, Cytosolic	Cell cycle	mRNA shuttling	Long

To determine the molecular mechanisms of salt tolerance at the reproductive stage, anther proteomic patterns for two contrasting rice genotypes IR64 (salt sensitive) and Cheriviruppu (salt tolerant) under NaCl salinity were compared by Sarhadi et al. (2012). Of 454 proteins spots detected reproducibly on two-dimensional electrophoresis gels, 38 showed significant changes in at least one genotype in response to stress and therefore, the authors identified 18 proteins that were involved in several processes that might increase plant adaptation to salt stress, such as carbohydrate/energy metabolism, anther wall remodelling and metabolism, and protein synthesis and assembly. Coming back to some of these proteins, three isoforms of fructokinase-2 were upregulated only in cherivirupu under salt stress and this upregulation might result in increased starch content in pollen, which would support pollen growth and development under salt stress. A dirigent-like protein was up-regulated in IR64 under salt stress, but did not show any significant change in Cherivirupu. Dirigent-like proteins are involved in anther dehiscence, lignin biosynthesis and can strengthen/repair sterility (Liu and Bennett 2011). α-L-Arabinofuranosidase/β-D-xylosidase isoenzyme ARA-I was downregulated in salt-sensitive cultivar but did not show any significant change in salt-tolerant one. As Fulton and Cobbett (2003) have categorised this protein as cell wall hydrolysing enzyme, Sarhadi et al. (2012) concluded that downregulation of α-L-Arabinofuranosidase in the anther cell wall of IR64 under salt stress might delay the degradation of cell wall tissues and obstruct anther dehiscence, leading to increased sterility. Two differentially expressed proteins were also identified in mitochondria (mitochondrial prohibiting complex protein 1 and putative mitochondrial processing peptidase) and are involved in protein synthesis and assembly.

The signal sensing, perception and transduction processes involved in cellular responses to salt stress are mainly located in the plasma membrane (PM). For better understanding the PM proteome response to salt stress, proteome was extracted from a root plasma-membrane-rich fraction of a rice salt tolerant variety, IR651, grown under saline and normal conditions (Nohzadeh Malakshah et al. 2007). Comparative two-dimensional electrophoresis revealed that 24 proteins were differentially expressed in response to salt stress and most of the proteins identified are known to be involved in several important mechanisms of plant adaptation to salt stress. These include regulation of PM pumps and channels (HIR protein), membrane structure (Plant-

specific PM/lipid-raft-associated proteins), oxidative stress defense (1,4-Benzoquinone reductase), signal transduction (GF14a and GF14b proteins), protein folding (PDI protein) and the methyl cycle (methionine synthase).

Overall, the previous results show that the protein expression profile in rice under salt stress is largely organ-specific and differs significantly depending on the tolerance level of the genotype considered.

1.3 Soil salinization process and description of the lower valley of the Rusizi plain

1.3.1 Soil salinization process

Soil salinization is a process of enrichment in soluble salts to a level that may have detrimental effects on agricultural production, environmental status and economic well-being (Rengasamy 2006). Salinized soils are characterized by high levels of soluble salts and/or Na^+ both in solution and on the surface phase (Qadir et al. 2007).

The progressive saturation of the exchange complex by the Na^+ ion is called sodization (Zaman et al. 2018). The authors confirm that under certain conditions, this last process is accompanied by the alkalization process which is the release of the Na^+ ions and the decrease of H^+ ions in the soil solution, causing the increase of pH and the dispersion of clays.

Soil salinization and sodization are gaining agricultural land daily, especially in arid and semi-arid regions.The world's surface area occupies about 13.2 billion ha, but no more than 7 billion ha are arable and 1.5 billion ha are cultivated (Tanji 2002). Of the cultivated lands, about 0.34 billion ha (23%) are saline (salt-affected) and another 0.56 billion ha (37%) are sodic (sodium-affected). Overall, the author show that saline and sodic soils cover about 10% of the total arable lands and exist in over 100 countries.

1.3.1.1 Origin of salt

The origin of soil salts may be natural or anthropogenic (human-induced). The former results in primary salinization and the latter, secondary salinization.

In natural conditions, the presence and concentration of salts at the terrain surface and in deeper soil profiles are controlled by geologic, geomorphic, climatic, and hydrologic factors (Metternicht and Zinck 2008). According to the authors, all salts originate from the weathering of the primary minerals contained in crystalline rocks and from this main source, salts are redistributed at the earth surface and become constituents of sedimentary rocks and unconsolidated sedimentary deposits. Weathering processes break down rocks and release soluble

salts of various types, mainly chlorides of sodium, calcium and magnesium, and to a lesser extent, sulphates and carbonates (Munns 2005). The principle salt sink for dissolved mineral salts is the world oceans while inland salt sinks include saline soils, saline ground water bodies, saline lakes, saline marshlands, saline playas, and salt domes.

According to Tanji (2002), other natural sources of soil salts may include atmospheric deposition of oceanic salts along coastal areas, seawater intrusion into estuaries and streams due to tidal events, seawater intrusion into ground water basins in coastal areas due to excessive ground water pumping, rising ground waters in low-lying topography from saline aquifers, salts released from volcanic activity and rainfall leaching of saline land forms.

Secondary salinity results from human activities that change the hydrologic balance in the soil between water applied (irrigation or rainfall) and water used by crops (transpiration). The most common causes are (i) land clearing and the replacement of perennial vegetation with annual crops, and (ii) irrigation schemes using salt-rich irrigation water or having insufficient drainage (Munns 2005). Tanji (2002) reported that anthropogenic sources of soil salts also include residual salts from soil and water amendments (gypsum, elemental sulfur, acids), animal wastes (manures and wash waters), chemical fertilizers, disposal of oil and gas field brines, applied sewage sludges and effluents.

1.3.1.2 Determination of soil salinity and sodicity properties

1.3.1.2.1 Soil salinity monitoring and measuring

Conventional ways of determining changes in soil salinity are based on field observation and laboratory analysis (Metternicht 2017). Some indicators of increasing salinity include: the reduced or no seed germination, patchy crop establishment, foliage damage (leaf burn) and the occurrence of naturally growing halophytes (indicators plants). The nonbiological indicators of salinity problems include the white crusts and efflorescences in saline soils as well as the presence of white spots and streaks. The affected area also worsens after a rainfall and waterlogging (Shahid et al. 2010). Visual assessment of salinity only provides a qualitative indication and the identified symptoms can easily

be confused with symptoms due to biotic or abiotic factors other than salinity. Visual assessment does not give a quantitative measure of the level of soil salinity. That is only possible through concentration measurements of mineral salts adsorbed on solid particles and present in soil solution. According to Tanji (2002), the dissolved mineral salts constitute a mixed electrolyte of cations and anions. The major cations in saline soil solutions consist of Na^+, Ca^{2+}, Mg^{2+}, and K^+ and the major anions, Cl^-, SO_4^{2-}, HCO_3^-, CO_3^{2-} and NO_3^-. The elements like B, Sr^{2+}, SiO_2, Mo, Ba^{2+} and Al^{3+} can also contribute to salinization process in hypersaline soils and waters.

In laboratory, the soil salinity level is often measured by estimating the concentration of total dissolved salts (TDS) in soil water; the measured parameter is electrical conductivity of soil solution. The higher the concentration of salts dissolved in soils water, the more ions to carry an electric current and the higher the EC (Rengasamy et al. 2010).

Soil salinity can be determined from measurements made 1) on saturated paste extract of soil samples, 2) on extracts of soil to water suspensions of diffirent rations, 3) on soil water samples collected in situ, usually with vacuum extractors, 4) in soil, using buried porous salinity sensors which imbibe and equilibrate with the soil water, 5) in soil, using four-electrode probes, or 6) remotely by electromagnetic induction techniques (Rhoades and Loveday 1990). Zaman et al. (2018) confirm that EC measurement at field capacity (fc) is the most relevant representing field soil salinity but the main constraint in such measurements is the difficulty to extract sufficient soil water. The compromise proposed by the author is an EC measurement from extract collected from saturated soil paste and the relationship between EC_{fc} to EC_{se} is generally $EC_{fc} = 2EC_{se}$ for most of the soils,except for the sand and loamy sand textures.
Although the use of saturation extracts as a method of measuring and referring salinity provides a direct relationship with the field moisture range for most soils, Sonmez et al. (2008) showed that the soil water suspensions of different ratios such us 1:1, 1:2, 1:5 and 1:10 can be more easily made and extracted than obtaining saturation extracts.
The unit of EC measurement has been customarily reported in micro-mhos per centimeter (µmho/cm), or in milli-mhos per centimeter (mmho/cm). In the international system of units (SI), EC is reported as siemens per meter (S/m), or as decisiemens per meter (dS/m). One dS/m being equivalent to one mmho/cm.

TDS is estimated by the following relationship (Bresler et al. 2012; Tanji 2002):

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

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

In addition to the visual assessment and measurements taken in the laboratory, soil salinity can also be monitored by remote sensing tools. This technique offer complementary data, and often constitute a less costly, more versatile and timely option to conventional monitoring, especially at regional or smaller scales (Metternicht and Zinck 2003).

1.3.1.2.2 Soil sodicity monitoring and measuring

When sodium is adsorbed by soil particles above a certain level, the soil becomes "sodic" and soil structure and hydraulic properties deteriorate. Therefore, sodicity refers to the proportion of Na^+ compared to other cations, mainly Ca^{2+}, Mg^{2+}, K^+ and Al^{3+} (Tanji 2002). Visual indicators of soil sodicity consist of: poor penetration of rain water (surface ponding), rain drop splash action (surface sealing and crusting), cloudy or turbid water in puddles, soil often black in color due to the formation of a Na-humic substances complex, difficulty to get soil saturation extracts in laboratory due to a filter blockage with dispersed clay (Zaman et al. 2018).

According to Rengasamy (2010b), these effects of sodicity (characterized by exchangeable sodium percentage, ESP, of soil or sodium adsorption ratio, SAR, of soil solution) are evident only when Ca^{2+}, Mg^{2+} and K^+ ions are leached below a 'threshold level" and the ionic strength of soil solution is low.

ESP is determined through measurement of exchangeable sodium (ES) and cation exchange capacity (CEC), as below:

$$ESP\ (\%) = 100\frac{ES}{CEC}$$

Where ES and CEC are represented as meq/100g soil. CEC refers to sum of exchangeable Na^+, K^+, Ca^{2+} and Mg^{2+}.

A more commonly measured sodicity parameter is the SAR, the ratio of Na^+ to Ca^{2+} plus Mg^{2+} in the soil solution:

$$SAR = C_{Na} / \sqrt{\frac{C_{Ca+C_{Mg}}}{2}}$$

Where C_{Na}, C_{ca} and C_{Mg} are mesured in meq/L or mmol/L.

1.3.1.3 Classification of soils affected by salinity

Based on pH_{se}, EC_{se} and ESP parameters, salinized soils have been classified into different groups as presented below.

Table 4. Soil classification by salinity and sodicity status (Richards 2012)

Soil class	EC_{se} (dS/m)	ESP (%)	pH_{se}
Normal	<4	<15	<8.5
Saline	>4	<15	<8.5
Sodic	<4	>15	>8.5
Saline-sodic	>4	>15	<8.5

The index "se" recalls the measurement made in the extract of the saturated paste

The USDA salinity laboratory staff (Richards 2012), classified the soils into three distinct categories (Table 4): saline, sodic, and saline-sodic soils. According to the author, since 50 per cent reduction in yield takes place at EC_{se} of 4 dS/m for most agricultural crops, this was proposed as the critical value to distinguish saline from non-saline soils. Similary, as the physical properties, especially the permeability of the soil, was significantly affected at ESP more than 15, that was taken as the critical value for differentiating sodic from normal soils.

1.3.1.3.1 Saline soils

A soil which contains soluble salts in amount in the root-zone which are sufficiently high enough to impair the growth of crop plants is defined as saline (Rengasamy 2010b). However, given that salt injury depends on species variety, plant growth stage, environmental factors, and the nature of the salts, it is very difficult to define a saline soil precisely. Despite these obstacles, the limits commonly considered to confirm that soil is salinized are EC_{se} more than 4 dS m^{-1}, pH_{se} less than 8.5 and ESP<15. Owing to the presence of excess salts and the absence of significant amounts of exchangeable sodium, saline soils generally are flocculated; and, as a consequence, the permeability is equal to or higher than that of similar non saline soils (Chhabra 2017).

The use of good quality water and good quality soils are the two strategies that can be adopted to limit the gradual salinization of arable land. However, good quality water and soil are in short supply in many areas, especially in dry areas; where the need for irrigation is high, precipitation is low and surpassed by evapotranspiration. Therefore, other suggestions and techniques would try to improve crop production in salinized soils as shown below.

Leaching. Generally, this technique consists of leaching the salts downward beyond the root zone by using excess water in order to drastically reduce the amount of total dissolved salts in soil solution (Dudley et al. 2008). However, Ben-Gal et al. (2008) proved that this method had negative impacts associated with water conservation and groundwater pollution.

Drainage. Drainage difficulties related to the low soil permeability or the presence of a high groundwater table, usually contributes to soil salinization (Rengasamy 2006). This obstacle can be circumvented via artificial subsurface drainage of salinized croplands; technique that allow to improve and reclaim salinized soils, as well as to solve associated problems of waterlogging (Feng et al. 2017; Singh 2015; Valipour 2014). To perform well an artificial drainage system, the horizontal and vertical subsurface drainage pipes, relief wells, pumping wells and digging of drain ditches may be combined in order to control groundwater levels and to impose efficient drainage of the root-zone

following rain and irrigation events (Benyamini et al. 2005; Feng et al. 2017).

Biological methods. Soil salinization process can also be controlled by using halophyte species for agriculture. This strategy consists of domesticating wild species that are highly resistant to salinity and able to produce valuable biomass (Rozema and Flowers 2008). Another avenue proposed by some researchers is genetic cross-breeding of plants in order to produce the more salt-tolerant cultivars (Chinnusamy et al. 2005). As an example, *Salicornia bigelovii* is a potential oil-seed crop which can be irrigated with a highly salinized water and produce a yield almost two times greater than sunflower (Rozema and Flowers 2008). Halophyte plants could also reclaim saline soil. According to Hasanuzzaman et al. (2014) and Ravindran et al. (2007), some plants may extract salt from the soil with the absorbed water and accumulate it in their tissues. Such desalination process could be a cheaper and highly environmentally sound way for remediation of salinized soils, but it must be properly developed (Hasanuzzaman et al. 2014). An example proving the effectiviness of phytoremediation technique is the case of plants *Suaeda maritima* and *Sesuvium portulacastrum* which have been shown to be able to remove approximately 500 kg of NaCl from a 0.01 km^2-salinized field over a period of 4 months (Ravindran et al. 2007).

Water desalination. In irrigated areas, the farmers have widely adopted the use of desalinated water over the past 15 years and this technique has led to a rapid increase in crop production (Cappelle et al. 2015). Rebhun (2004) and Welle et al. (2017) showed that water desalination technologies offer a potential remedy to soil salinization in irrigated crop lands by allowing farmers to use treated saline water from various sources, including saline groundwater, ocean water, or treated waste water.

1.3.1.3.2 Sodic soils

Sodic soils exhibit an ESP>15% and show an EC_{se} < 4 dS m^{-1}. The pH_{se} generally ranges between 8.5 and 10 and may be even as high as 11 (Bresler et al. 2012). The low EC_{se} and high ESP tend to de-flocculate soil aggregates and, hence, lower their permeability to water. Dispersed and dissolved organic matter present in the soil solution of highly sodic soils may be deposited on the soil surface by evaporation, thus causing darkening and giving rise to the term "black alkali" (Siyal et al. 2002).

Regarding the sodic soils, the clays dispersion is the consequence of the saturation of the exchange complex by the sodium ion on the one hand and the subsequent increase of the pH on the other hand. The large hydration radius of Na as well as its low ionic charge (+1) explain its low flocculation power compared with other cations such as Ca and Mg (Horneck et al. 2007). The authors proved that the low ionic charge of Na does not allow the cohesion of clay particles as is the case for high-valence cations (Ca, Mg, Al, etc.).

According to Richards (2012), the soil solution of sodic soils, although relatively low in soluble salts, has a composition that differs considerably from that of normal and saline soils. In addition to dominant anions such as chloride, sulfate and bicarbonates; small amounts of carbonates are also found in sodic soils. The high pH values combined with the presence of large amounts of carbonate ions generate precipitation of Ca and Mg cations; hence, the soil solutions of sodic soils usually contain only small amounts of these cations, sodium being the predominant.

Sodic soils can be improved by adding calcium/magnesium and/or organic matter and by reducing mechanical soil disturbance as this breaks apart clay aggregates and encourages dispersion (Rengasamy et al. 2010). The Na attached to the exchange complex is gradually replaced by the Ca ion having a higher flocculating power, which increases the soil structural stability. Indeed, the increased moisture retention and hydraulic conductivity of the soil lead to increased yields.

1.3.1.3.3 Saline-sodic soils

Saline-sodic soils contain high amount of soluble salts (EC_{se}> 4 dS m^{-1}) wich can be hurmful to the growth of most crop plants and sufficient ESP (>15%) that can degrade the soil structure. The pH_{se} may be less or more than 8.5 (Table 4).

If the excess soluble salts are leached downward, the properties of these soils may change markedly and become similar to those of sodic soils (Richards 2012). According to the author, as the concentration of the salts in the soil solution is lowered, some of the exchangeable sodium hydrolyzes to form sodium hydroxide and this may change to sodium carbonate upon reaction with carbon dioxide absorbed from the atmosphere. Under such conditions, soil leaching leads to soil alkalization (pH readings above 8.5), the particles disperse, and the soil becomes unfavorable for the entry and movement of water and for tillage. The management of saline-sodic soils continues to be a problem until the excess salts and exchangeable sodium are removed from the root zone and a favorable physical condition of the soil is reestablished (Richards 2012).

1.3.2 Description of the Rusizi plain

1.3.2.1 Geographical location

Often referred to as "The Heart of Africa" because of its shape and central location in Africa, Burundi is part of the African intertropical region. Burundi is a landlocked country located between 2°20' and 4°30' South latitude and 28°50' and 30°53' East longitude, at 1430 Km from the Indian Ocean, 1900 Km from the Atlantic Ocean, 3800 Km from the Mediterranean Sea and 3500 Km from the Cape. In Western Burundi is Lake Tanganyika, which is one of the lakes in the East African Rift Valley.

Burundi is bounded by Rwanda to the north, the Democratic Republic of Congo (DRC) to the west and Tanzania to the south and east (Fig 8). It covers an area of 27834 km^2, of which 2000 km^2 is occupied by the Burundian part of Lake Tanganyika.

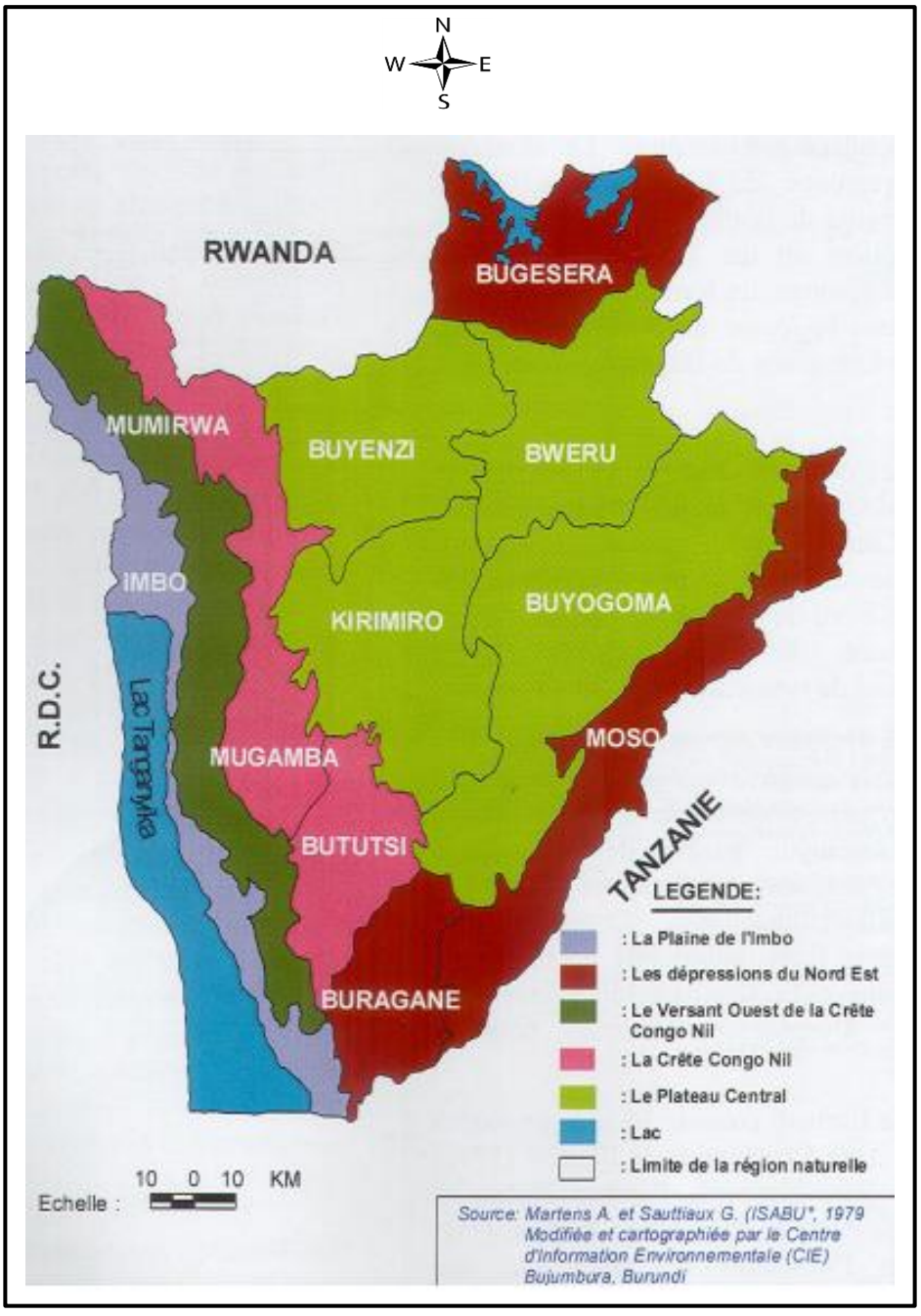

Figure 8. Map of the ecoclimatic regions of Burundi (ISABU, 1979)

The Rusizi plain or Imbo-Center, partly concerned by this study, is bounded to the east by the Bujumbura-Muzinda road, to the north by the Piedmont and the Nyamika river, to the west by the Rusizi river and to the south by Lake Tanganyika and Bujumbura city. It rises in successive steps towards the north and eastward in the Piedmont area. With a width greater than 20 km to the south, it shrinks considerably northward to form only a thin strip of width less than 5 km.

1.3.2.2 Geomorphology and geology

1.3.2.2.1 Geomorphology

The Rusizi plain descends gradually southward, from 860 m at its northern limit to 774 m at the lake level. The softly undulating plain is formed by a succession of individualized reflates by the hydrographic network, by breaks of slopes or by simple inflections of the slope. On this relief, a loose hydrographic network is printed in the central part, denser at the edge of the rocky foothills.

From the East to the West, the Rusizi lowlands are covered by the alluvial deposits of the Rusizi river, the lacustrine formations and the alluvial deposits of the Rusizi as well as Lake Tanganyika tributaries (Hakiza 2002)

The Rusizi forms a large alluvial plain in the old lacustrine formations. It has developed meanders, marshes and islands on a band ranging from 20 to 80 m wide. At the height of the northern limit of the lower Rusizi, cliffs overlook the plain from 30 to 40 m. As we approach the lake or the Rusizi delta, the differences in altitude between the old and fluvialed lacustrine formations decrease; they are only 2 to 3 m in the vicinity of the confluence Kajeke-Rusizi. From the Rusizi, going westward or eastward, it is possible to observe:

- the current alluvial deposits, temporarily exposed, which constitute discontinuous and narrow cords, with marshy formations dotted with small pools and ponds;
- the recent alluvial deposits overlooking the Rusizi, from 4 to 10 m during low water periods. Stratified alluvial deposits are, in places and periodically, influenced by the saline waters of the Rusizi river;

- the ancient alluvial deposits which cover the high terraces above 10 m from the current bed of the Rusizi. These terraces are distinctly individualized and very wide (200-400 m) or reduced to mere inflections in the slope.

The alluvial deposits of the Rusizi and its tributaries surround the lacustrine formations. They occupy the central part of the plain; by the lake. Their thickness would be in the range of 1500 to 2000 m.

The rivers that cross the lowlands have been superimposed on the lacustrine formations. At the entrance to the plain, the rivers gradually deposit kidding materials from the ridge and foothills. Thick alluvial deposits currently cover a large part of the Rusizi plain. By converging from the foothills to the Rusizi, we successively traverse the Piedmont deposition, the current alluvial deposits of the Rusizi as well as Lake Tanganyika tributaries, the alluvial lakes and alluvial deposits of the Rusizi. All these alluvial deposits are made up of fine or medium sands, gravel sands, clays, and sand clays, often accompanied by carbonate concretions.

1.3.2.2.2 Geology

The geological history of Burundi in general and the Rusizi plain more particularly is linked to the great tectonic movements that shaped the Eastern Africa relief, and especially to the great fracturing of the continent which, at the end of the tertiary has , among others, individualized the Tanganyika Gulf (Reekmans 1980).

The Rusizi lowland occupies the bottom of a ditch that, like the Lake Tanganyika ditch, would result from an earlier episode to be attached to the lower or earlier Pleistocene. This plain, up to 20 km wide, is a river-lacustrine plain backed up to the east at the foothills of the ridge and surrounded to the west by the Rusizi River. The Rusizi River and its tributaries, and even runoff, spoof alluvial materials of various origins: some from the dismantling of Precambrian rocks of the Congo-Nile ridge that are metamorphic (crystalline shale , gneiss, Amphibolite, Pyroxenites, quartzites feldspathic) and other volcanic zones in South Kivu, DRC. These sedimentary materials are deposited on the formations of the Rusizi lowland formerly submerged under Lake Tanganyika and whose exondation occurred at the Pleistocene-Holocene boundary (Ilunga 2007).

1.3.2.3 Climatology

1.3.2.3.1 Temperature

The thermal characteristics of the lower Rusizi plain can be synthesized as follows:

- the average annual temperature is in the order of 25.4 °C (Table 5); This value is close to that found for the waters of Lake Tanganyika;
- the amplitudes of annual variations are low; There are minima in the dry season and maxima at the beginning of the rainy season;
- the average daily amplitudes are stronger in the dry season than in the rainy season;
- extreme amplitudes are relatively large; absolute maxima are recorded during the rainy season and absolute minima during the dry season.

1.3.2.3.2 Precipitation

In the lower Rusizi plain, the annual average of precipitation is around 850 mm (IGEBU 2018). The altitude does not play practically any role in this regard, it is the situation in relation to the Lake and especially the proximity of the Piedmont that determine the rainfall regime. We find, indeed, that the regions furthest from the ridge and the Lake suffer from a chronic deficit of precipitation. It should also be noted that these precipitations, mostly from violent thunderstorms due to intense thermal convection of the air, are very unevenly distributed throughout the year and that it is not uncommon to raise in the middle of the rainy season periods of total drought lasting 15 to 20 days. For four months (June, July, August and September), monthly precipitation remains below 50 mm. It is important to mention that the average precipitation is lower than the average of potential evapotranspiration (PET). PET calculations based on area temperature data gave a PET average of 979 mm (Nijimbere, 2014), 15% higher than the average of annual precipitation. Conditions of high temperatures, low precipitations and high evapotranspiration lead to arid and semi-arid climates, which is the case of the Rusizi plain.

Table 5. Monthly average temperature at the Imbo-Centre station (IGEBU 2018)

	Month											
	J	F	M	A	M	J	J	A	S	O	N	D
Temperature (°C)	24.2	24.6	24.1	24.8	25	25.1	27.4	27.5	26.1	25.6	25.1	24.8

1.3.2.3.3 Relative humidity

According to IGEBU (2018), the average daily relative humidity of the air ranged from 56% to 93% with an average of 73% during the year 2018. The daily minima are in the order of 50% at 6:00 am while the maxima fluctuate between 70% and 80% at 6:00 pm. During the year, the humidity is higher during the rainy season (November to May) than the rest of the year. This is the result of the much larger cloud cover during this period.

1.3.2.3.4 Winds

From the deviated trade winds in the Tanganyika corridor, the winds blow mostly from the South sector. In addition to these prevailing winds, it is also important to note the presence of local winds, the most frequent of which are: the Lake breeze and the land breeze. The Lake breeze blows from the Lake to the land especially during the dry season, it rises at noon and disappears at sunset. The land breeze blows from land to the Lake for most of the night and fades away with the sunrise. Strong winds are rare and the average wind speed value for the year 2018 is 1.3 m/s (IGEBU 2018). High velocities are observed during the dry season (June, July, August and September).

1.3.2.3.5 Insolation

It can be estimated that the annual average insolation in the Rusizi plain is in the order of 55% of the theoretical maximum sunlight possible. It is thus one of the sunniest areas of the country. Maximum sunshine is reached during the dry months (June to August) while a minimum is reached during the rainy months. The average annual insolation raised at the perimeter of the Imbo-Centre during the year 2018 was 2200 hours.

1.3.2.4 Hydrology

The Rusizi river discharges into Lake Tanganyika (average level: 774 m) the excess waters of Lake Kivu (average level: 1.462 m) whose watershed covers approximately 7.300 km². It also drains 1.500 km² from the Lake Tanganyika Basin (Frankart and Herbillon 1971).
In Rusizi plain, the hydrographic network is sparse, at least in the central part of the plain. The rivers that run down the ridge approach

the lake formations in deep valleys and continue their course between high slopes, steeply inclined. In the center of the plain, however, certain rivers, such as Kajeke and Ninga, crisscross the ancient lacustrine alluviums between the barely drawn banks; downstream, however, their collection is accentuated when they join the Rusizi. Deep-casted depressions, without apparent connection with the current network, deeply incise the lake landscape on the edge of the alluvial plain of Rusizi. The regime of permanent rivers is characterized by very strong floods, often violent, causing floods.

The Rusizi waters have an electrical conductivity between 750 and 1.400 micromhos/cm (Reekmans 1980) with significant seasonal variations showing high values in the dry season. Carbonates and bicarbonates are the best represented anions, while magnesium and sodium dominate among the cations. This salinity decreases from upstream to downstream due to the influence of the salt-poor tributaries. However, local infiltration of poor quality groundwater would counterbalance the beneficial effect due to the tributaries flow.
According to the irrigation water classification realized by the United States Salinity Bureau (Richards 1969), Rusizi waters are highly saline (class C3) with a low alkalization hazard (class S1). The Rusizi waters, with a sodium absorption coefficient (SAR) of 2.66, are very dangerous for irrigation given their extremely high "Na_2CO_3 residue". Its value is close to 5.06 mmolc / L, and water with a residue greater than 2.50 mmol/L is considered unsuitable for irrigation (Frankart and Herbillon 1971).

1.3.2.5 The origin of salts and the damage caused by the salinization process in the Rusizi plain

According to Nijimbere (2014a), the primary source of the salinity in Rusizi lowland would be related to the volcanism of Kivu and the hydrothermalism of its watershed. This option was further justified by the fact that the analysis of the chemical composition of lava from the volcanic regions of South Kivu, Lake Kivu and the thermal springs of the Cibitoke region, all located in the northern part of the Rusizi watershed, revealed reconciliations between their salts and those of the Rusizi waters. Overall, the author found that appreciable of cations (Na^+, K^+, Mg^{2+}, and Ca^{2+}) would be derived from the volcanic rocks alteration. The atmosphere would be the primary source of the CO_3^{2-} and HCO_3^- anions while the anions like Cl^- and SO_4^{2-} would be of

hydrothermal and volcanic origin. During Burundi's civil war from 1993 until 2003, irrigation and drainage canals were not maintained and this led to a high accumulation and redistribution of salts in the arable area of the Rusizi plain. This secondary source of salinity has resulted in the salinization of a large area of the plain where 15% of the arable land has been seriously affected and 182 fields of at least 0.5 ha each have been completely abandoned (Kabwa 2010a). The gradual salinization of the Rusizi plain also leads to a drastic decrease in crop yields that we cannot quantify due to the lack of numerical and up-to-date data.

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EXPERIMENTAL PART

The results of this part are presented in the form of scientific articles published, submitted or to be submitted in a journal. The choice of this form of presentation will generate some redundancies in “introduction” as well as in “material and methods” sections.

Chapter I. Comparative effects of chloride and sulfate salinities on two contrasting rice cultivars (*Oryza sativa* L.) at the seedling stage

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Abstract

In field conditions, soil salinity may be due to an excess of different soluble salts. In order to compare the impact of chloride and sulfate salinities on rice, two contrasted cultivars (IKP: salt-sensitive and Pokkali: salt-resistant) were exposed to iso-strength Na^+ nutrient solutions (NaCl 50mM or Na_2SO_4 25mM; EC: 5.31 dS m^{-1}) for 2 weeks under controlled environmental conditions. It was found that NaCl was more toxic than Na_2SO_4, especially for the salt-sensitive IKP. Sodium and proline accumulation were higher while shoot osmotic potential was lower in NaCl-treated plants than in those exposed to Na_2SO_4. Chloride-treated plants exhibited a higher shoot malondialdehyde concentration, suggesting a higher level of lipid peroxidation while Na_2SO_4-treated plants presented a slightly higher total antioxidant activity. Pokkali was more tolerant than IKP to both types of toxicities although it accumulated similar concentration of toxic ions. Pokkali was able to reduce the root osmotic potential and to quickly recycle oxidized glutathione to reduced glutathione, which may help the plant to more efficiently control its oxidative status in stress conditions. It is concluded that different salts may have distinct impacts on the plant physiology and that differences may vary according to the considered cultivar.

Key words: chloride; ion-specific toxicity; *Oryza sativa*; osmotic adjustment; salinity; sulfate

Introduction

Salinity is one of the major abiotic stresses limiting agricultural production in many areas of the world with adverse effects on germination, plant vigor, and crop yield. More than 900 million hectares of land have been damaged by salt in the word (including 45 million hectares of irrigated lands) and 1.5 million hectares are taken out of production each year as a result of high salinity levels in the soil (Rengasamy 2006).

A given soil is considered as salty if it exhibits an apparent electrical conductivity higher than 4 dS m^{-1} (Daliakopoulos et al. 2016). The major cations issued from soluble salts in saline soils comprise sodium (Na^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}), whereas the most prevalent anions are chloride (Cl^-), sulfate (SO_4^{2-}) and carbonates (including bicarbonates). Among those, sodium chloride is predominant worldwide and much of the research studying the plant physiological responses to salinity rely on experiments focusing on NaCl stress only (Chinnusamy, Jagendorf, and Zhu 2005). Some areas, however, may also be affected by an excess of sulfate salts. This is especially the case in the San Joachim valley in California and in Dakota (Rhoades 1989, Thapa, Wick, and Chatterjee 2017), in large parts of India, Egypt, and Argentina (Bowman, Cramer, and Devitt 2006, Devinar et al. 2013), and in Burundi where the lower valley of the Rusizi plain is affected by sodium sulfate from volcanic origin (Nijimbere 2014).

Salinity induces several types of constraints on plants: the low osmotic potential of soil solution compromises water uptake and induces a physiological drought while accumulation of toxic ions results in a wide range of metabolic disorders. Salinity leads to the overproduction of reactive oxygen species (ROS) which are highly toxic and may cause damages to proteins, lipids, carbohydrates and DNA (Ahanger et al. 2017). In order to cope with those deleterious impacts of salinity, some plants are able to (i) perform osmotic adjustment through the accumulation of organic compatible osmoprotectants such as proline and carbohydrates allowing a decrease of plant water potential and the protection of enzymes and cellular structures (Bohnert and Jensen 1996) (ii) efficiently regulate ion absorption and translocation in order to limit toxic ions accumulation and salt-induced

deficiencies in essential elements (Hasegawa 2013, Maathuis, Ahmad, and Patishtan 2014) (iii) scavenge reactive oxygen species by triggering antioxidants synthesis (glutathione, ascorbate, tocopherol) and antioxidative enzymes activation (superoxide dismutase, catalase, peroxidases, etc.) (Bose, Rodrigo-Moreno, and Shabala 2014, Ozgur et al. 2013). Endogenous antioxidants are hydrophilic (ascorbate, glutathione, flavonoids, anthocyanins) or lipophilic (α-, δ-, and γ- tocopherol, γ-oryzanol, carotenoids) compounds which may be differently affected by contrasting environmental constraints but data regarding ion-specific impact of salinity on those two classes of compounds remain scanty.

Despite its economic importance which has been evidenced by several authors (Samson et al. 2016, Thapa, Wick, and Chatterjee 2017), plant responses to Na_2SO_4 salinity only received minor attention. The type of salinity may not only influence quantitative yield-related parameters but also the quality of harvested products (Navarro et al. 2002). In the cultivated halophyte Chenopodium quinoa, Wu et al. (2016) recently reported that Na_2SO_4 increased grain protein content and seed densities whereas NaCl did not exhibit significant effects. Sodium sulfate was shown to be more toxic than NaCl in potato (Bilski, Nelson, and Conlon 1988), wheat (Datta et al. 1995), tall fescue (Gao, Li, and Chen 2012), and Brassica rapa (Reich et al. 2017) while an opposite trend was reported for pea (Hasson-Porath, Kahana, and Poljakoff-Mayber 1972, Mor and Manchanda 1992), soybean (Gupta and Gupta 1984), barley (Curtin, Steppuhn, and Selles 1993), and pepper (Navarro et al. 2002). Such discrepancies may be at least partly explained by the relative proportions of the other mineral components present in the media. Indeed, tolerance to chloride salts was reported to be directly influenced by phosphorous nutrition to a higher extent than tolerance to sulfate salinity (Manchanda, Sharma, and Bhandari 1982, Mor and Manchanda 1992). In contrast, sulfate salinity has a more obvious deleterious impact on Ca^{2+} absorption and translocation than NaCl stress (Bilski, Nelson, and Conlon 1988, Curtin, Steppuhn, and Selles 1993, Han, Gao, and Li 2014). The fact that different authors used different nutrient solution may partly explain contradictory data. Genetic differences between tested species may also be involved in the different behavior observed by different authors. Even

cultivars from a given plant species are expected to differ in their relative sensitivities to NaCl and Na_2SO_4 (Rogers, Grieve, and Shannon 1998).

Rice (*Oryza sativa* L.) is considered as a salt-sensitive species but data related to the comparative behavior of plants facing NaCl and Na_2SO_4 toxicities are surprisingly rare. Kimura, Okumura, and Yamasaki (2004) demonstrated that both chloride and sulfate ions provided root growth stimulation when applied in small amounts as ammonium salts. At higher concentrations such as those prevailing in saline conditions, chloride was reported to be more toxic than sulfate in wild rice (*Ziziana palustris*) (Fort et al. 2014). According to Khare, Kumar, and Kishor (2015), Na^+ and Cl^- assume distinct negative impacts on rice and have additive effects when added as NaCl salts. Khare, Kumar, and Kishor (2015) also considered that salt treatment caused an imbalance in non-enzymatic antioxidants, which was greater under NaCl than Na^+ and Cl^- separately. According to Lefèvre, Gratia, and Lutts (2001), the ionic component of salt stress may trigger plant response even on a short-term basis and these authors noticed that KCl was more toxic than NaCl which could be related to a higher accumulation of Cl^- in the former case than in the latter. A similar trend was observed in mature embryo-derived calli where Na_2SO_4 was clearly less toxic than NaCl (Lutts, Kinet, and Bouharmont 1996) but no data are available for the whole plant behavior.

The paucity of data regarding rice response to both types of salinity prompted us to compare the behavior of plants from two contrasted rice cultivars (I Kong Pao: salt-sensitive and Pokkali: salt-resistant) exposed to iso-strength Na^+ nutrient solutions at the young seedling stage, which is commonly considered as one of the most salt-sensitive developmental stage in *Oryza sativa* (Lutts, Kinet, and Bouharmont 1995).

Material and methods

Plant material and growing conditions

Seeds of the rice cultivars I Kong Pao (IKP; salt-sensitive) and Pokkali (salt-resistant) were obtained from IRRI (International Rice Research Institute; Philippines). Seeds were germinated on two layers of filter paper (Whatman No. 2) moistened with 10ml of sterile deionized water in a growth chamber at 25°C under a 12hr daylight period (120 µmol m^{2-} s^{-1}). Ten-day-old seedlings of the two cultivars were transferred into a phytotron and fixed on polystyrene plates floating on Yoshida nutritive solution (Yoshida et al. 1976). Illumination was provided by Sylvania fluorescent tubes (F96T12/CW/VHO) for 12hr day^{-1} at a photon flux density of 300 mmoles m^{2-} s^{-1}. Daytime humidity was between 60% and 80%, and the temperature was maintained at 29°C during the day and 26°C during the night. For each treatment, seedlings were distributed among three tanks (20 seedlings per tank) containing 50L of Yoshida nutritive solution (Yoshida et al. 1976). The solutions were renewed weekly and tanks were randomly rearranged in the phytotron. After 2 weeks of acclimatization in control conditions, seedlings were exposed for two weeks to 25mM Na_2SO_4 or 50mM NaCl (electrical conductivity of c.a. 5.31 dS m^{-1} for both solutions). The pH values recorded in the culture solutions salinized with 25 mM Na_2SO_4 and 50 mM NaCl were 5.81 and 5.82 respectively. In addition, a chemical speciation of the culture solutions salinized with NaCl and Na_2SO_4 was performed using Visual Minteq software. The identified species are presented in appendix 2 of this document. The concentration and activity rates of Na^+, Cl^- and SO_4^{2-} are presented in results section.

At the end of the experiment, 10 plants per treatment were harvested for mineral analysis. Roots were rinsed for 30s in deionized water to remove ions from the free spaces. Roots and shoots were separated and weighed. Samples were then dried during 72h at 70°C in an oven and dry weight was estimated. The remaining plants were quickly frozen in liquid nitrogen and stored at -80°C until subsequent biochemical analysis.

Estimation of ion content and osmotic potential

For shoots and roots, 20mg dry weight (DW) was digested with nitric acid (68%) at 80°C. After complete evaporation, residues were dissolved with HNO3 (68%) + HClcc (1:3, v/v). Solution was filtered using a layer of Whatman (85mm, Grade 1). The filtrate was used to determine the cations concentration (Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+},K^{+}, and Na^{+}) by flame emission using Atomic Absorption Spectrometer (Thermo scientific S series model AAS4, Thermo Fisher Scientific Waltham, MA, USA). Chloride was specifically extracted according to Hamrouni et al. (2011). Anions (PO_4^{3-}, S, and Cl^{-}) were quantified by liquid chromatography (HPLC-Dionex ICS2000, Dionex Corporation, Sunnyvale, California, U.S.A.).

For the determination of osmotic potential (Ψ_S), the last fully expanded leaf was removed from the plant and cut in small segments. The sample was then introduced in an Eppendorff tube perforated with four small holes, frozen in liquid nitrogen for 30s and warmed again at room temperature (three cycles of freeze/thaw) in order to break the cellular membrane (Lutts, Bouharmont, and Kinet 1999). Tubes were then encased in a second intact Eppendorff tube and centrifuged at 8000g during 15min at 4°C. The osmolarity of the collected tissular sap was assessed with a Wescor vapor pressure osmometer and converted to Ψ_S according to Van't Hoff equation.

Quantification of proline and total soluble sugars

For proline analysis (Bates, Waldren, and Teare 1973), 200mg fresh weight (FW) of shoots was frozen in liquid nitrogen, ground in a pre-chilled mortar, homogenized in 3ml of sulfosalicylic acid and then centrifuged at 5000g for 15min at ambient temperature. An aliquot of 1ml of ninhydrin solution (2.5g of ninhydrin dissolved in a mixture of 60ml glacial acetic acid and 40ml 6M phosphoric acid) and 1ml of concentrated acetic acid were added to the supernatant and the mixture was heated one hour at 90°C before stopping the reaction in the ice. The reaction mixture was extracted with 2ml of toluene, mixed vigorously with a test tubes stirrer for 15s. The chromophore-containing toluene was warmed to room temperature and absorbance was read at 520nm using toluene as a blank. The proline concentration was determined from standard curve.

Total soluble sugars were quantified according to Yemm and Willis (1954): 500mg FW of shoots was ground in a pre-chilled mortar with liquid nitrogen. An aliquot of 4ml of ethanol (70%) was added to the homogeneous powder and the mixture incubated on ice for 5min. After three successive centrifugations at 8000g, the pooled supernatants were mixed with 1ml of the anthrone (0.5g anthrone dissolved in 12.5 ml H_2O and 250 ml H_2SO_4), heated at 100°C for 10min and then incubated on ice. Absorbance was measured at 625nm and the calibration curve was performed via glucose.

Oxidative damages and total antioxidant activity and glutathione content

Lipid peroxidation was estimated as the amount of the thiobarbituric acid-reactive substances [mainly malondialdehyde (MDA)] determined by the thiobarbituric acid reaction (Heath and Packer 1968): 250mg FW was frozen with liquid nitrogen, crushed in a pre-chilled mortar and homogenized in 5ml of trichloroacetic acid (5% w/v). The homogenate was centrifuged at 12,000g for 10 min and filtered on Whathman filter Paper No. 1. An aliquot of 2ml of thiobarbituric acid 0.67% (w/v) was added to 2ml of supernatants and samples heated during 30 min at 100°C, cooled on ice and then centrifuged for one min at 2000g to eliminate turbidity. The absorbance was read at 532nm and the non-specific absorption at 600nm was subtracted. The concentration of MDA was calculated using a molar extinction coefficient of $155 mM^{-1}\ cm^{-1}$.

Total antioxidant was estimated on the basis of α,α-diphenyl-2-picrylhydrazyl (DPPH) method (Al-Temimi and Choudhary 2013). One-gram FW of shoots was frozen in liquid nitrogen, ground in a pre-chilled mortar and 10ml methanol were added to the homogenous powder and then centrifuged at 10,000g for 20min at 4°C. The recovered supernatant corresponded to the antioxidant activity measured in methanol extract (AOAM) fraction. The pellet was re-dissolved in 10ml of dichloromethane, centrifuged at 10,000g for 12min at 4°C and 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 activities (Arnao, Cano, and Acosta 2001): 2.85ml of DPPH solution (24mg DPPH in 100ml

methanol) was added to 150ml sample, the mixture incubated in the dark for 24hr at room temperature and the absorbance measured at 515nm.

For reduced (GSH) and total (GSHt) glutathione quantification, 200mg of frozen samples were extracted and derivatized by orthophthalaldehyde (OPA) according to Cereser et al. (2001). GSHt was quantified after a reduction step of oxidized glutathione (GSSG) by dithiotreitol (DTT) 25mM. Extracts were filtered through 0.45mm microfilters (Chromafil PES-45/15, Macherey-Nagel, Düren, Germany) prior to injection and OPA derivatives were separated on a reversed-phase HPLC column with an acetonitrile-sodium acetate gradient system and detected fluorimetrically. Five microliters of sample was injected into a Shimadzu HPLC system (Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with a Nucleodur C18 Pyramid column (125x4.6mm internal diameter; 5µm particle size; Macherey-Nagel). Derivatives were eluted in acetonitril gradient in a 50mM sodium acetate buffer pH 6.2 at 30°C at a flow rate of 0.7ml min^{-1}. Fluorimetric detection was performed with a spectra system Shimadzu RF-20A fluorescence detector at 420nm after excitation at 340nm. GSH was quantified using 9-point calibration curves with custom-made external standard solutions ranging from 0.0625 to 50mM and every 10 injections, a check standard solution was used to confirm calibration of the system. The recovery was determined using GSH as an internal standard and was always found to be higher than 98%.

Contribution of proline, K^+ and salt ions to osmotique adjustment

We also calculated the contribution rate of proline, K^+ and salt ions (Na^+, Cl^- and S^{6+}) to osmotic adjustment in shoots by applying Van't Hoff's law ($\pi = RT\frac{C}{M}$) where Π is the osmotic pressure in MPa, R: the universal gas constant (8.31 $J.mol^{-1}.K^{-1}$), T: the absolute temperature (K), C and M: the weight concentration and molar mass of the solute respectively. This calculation consider that proline accumulated only in the cytosol, which represent around 10% of the cell volume and that salt ions accumulated only in the vacuole, which represent around 90% of the cell volume.

Statistical treatment

Statistical analyses were performed using JMP Pro 13 software. Data were treated by variance analysis and means were compared using Tukey's HSD all-pairwise comparisons at the $p = 0.5$ level as a post-hoc test. The graphs were plotted using SigmaPlot 10.0 software.

Results

The pH values recorded in this experiment were within the acceptable range for good plant growth (5.5-6.5).

Considering that the identified species did not vary depending on the stress intensity, the data presented in appendix 2 still valid for all studies conducted in hydroponic culture. Regarding NaCl stress, the concentration and activity rates of Na^+ in culture solution were 97.9% and 79.29% respectively. The concentration and activity rates of Cl^- were 98.09% and 77.7% respectively. In Na_2SO_4-treated solutions, the concentration and activity rates of Na^+ were 94.98% and 75.5% respectively while the concentration and activity rates of SO_4^{2-} were 86.49% and 34.6% respectively.

The tall indica landrace Pokkali always exhibited a higher shoot biomass than IKP, whatever the treatment (Figure 1) while the root biomass was the same for the two cultivars. Chloride and sulfate salinities had no detrimental impact on the shoot DW of Pokkali. In contrast, chloride salinity reduced shoot DW in IKP comparatively to control, while plants exposed to sulfate salinity exhibited an intermediate behavior. Salinities had no impact on the root DW.

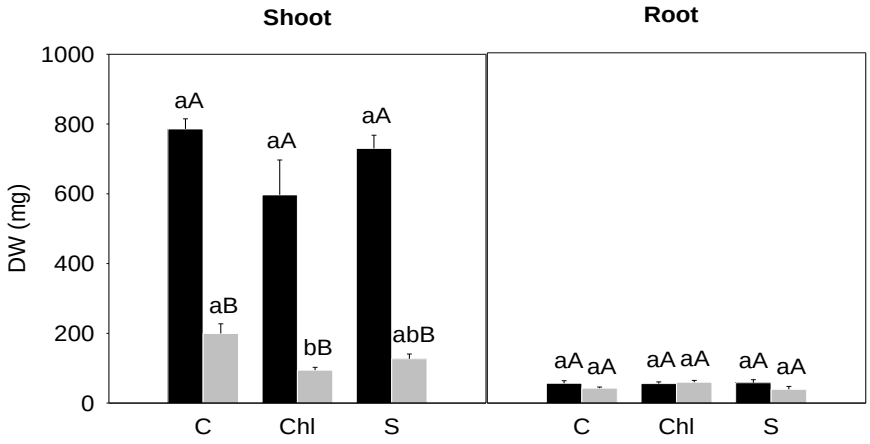

Figure 1. Dry weight of shoot and roots of rice seedlings from cv. Pokkali (black bars) and IKP (gray bars) cultivated during 2 weeks in control (C) conditions or in the presence of 50mM NaCl [chloride salinity (Chl)] or 25mM Na_2SO_4 [sulfate salinity (S)]. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments followed by the same lowercase letter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a particular treatment do not differ statistically.

For both cultivars, water content was higher in the roots than in the shoots (Table 1). The two types of salinity had no impact on Pokkali shoot and root water content. Chloride salinity drastically decreased water content in IKP while sulfate salinity had no impact on this parameter (Table 1).

The two types of salinity induced a strong increase in shoot and root Na^+ content (Figure 2). As far as shoots are concerned, Na^+ accumulation was obviously higher in plants exposed to NaCl salinity than in those exposed to Na_2SO_4 while an opposite trend was observed for roots. The two considered cultivars accumulated similar amounts of Na^+ under our experimental conditions. As expected, chloride accumulation was observed mainly in NaCl-treated plants. Pokkali accumulated more Cl^- than IKP in the shoot while the two cultivars did not differ for root Cl^- content. Sulfur accumulation was observed for Na_2SO_4-treated plants. Shoot S concentration was higher in Pokkali than in IKP and once again, no

difference between cultivars was recorded for root S concentration. The two types of salinity induced a decrease in K^+ concentration. At the shoot level of Pokkali, the recorded decrease was higher for chloride than for sulfate salinity while the two treatments induced a similar decrease in K^+ content in cv. IKP.

Table 1. Water content (%) in shoots and roots of rice seedlings from cv. Pokkali and IKP cultivated during two weeks in control (C) conditions or in the presence of 50 mM NaCl (chloride salinity (Chl)) or 25 mM Na_2SO_4 (sulfate salinity (S)). Each value is the mean of three replicates ± standard errors of the mean.

	Pokkali		**IKP**	
Treatment	Shoot	Root	Shoot	Root
C	84.67 ± 2^{aA}	91.9 ± 0.9^{aA}	83.45 ± 0.4^{aA}	87.6 ± 2^{aA}
Chl	83.25 ± 0.7^{aA}	92.3 ± 2^{aA}	70.7 ± 3.4^{bB}	72.9 ± 1.6^{bB}
S	83.38 ± 1^{aA}	89.76 ± 0.6^{aA}	83 ± 2.7^{aA}	90.6 ± 3.9^{aA}

Notes: Treatments followed by the same lower case letter for a particular cultivar do not differ statistically. Cultivars followed by the same upper case letter in a particular treatment do not differ statistically.

Sulfate salinity induced a significant decrease in the shoot Ca^{2+} and Mg^{2+} concentration in IKP but not in Pokkali (Table 2). Conversely, sulfate salinity induced a significant decrease in the shoot P content of Pokkali while such a decrease was recorded in response to chloride salinity in IKP. In the two studied cultivars, both types of salinity induced an increase in the shoot Mn^{++} content and in the root Fe and P concentration.

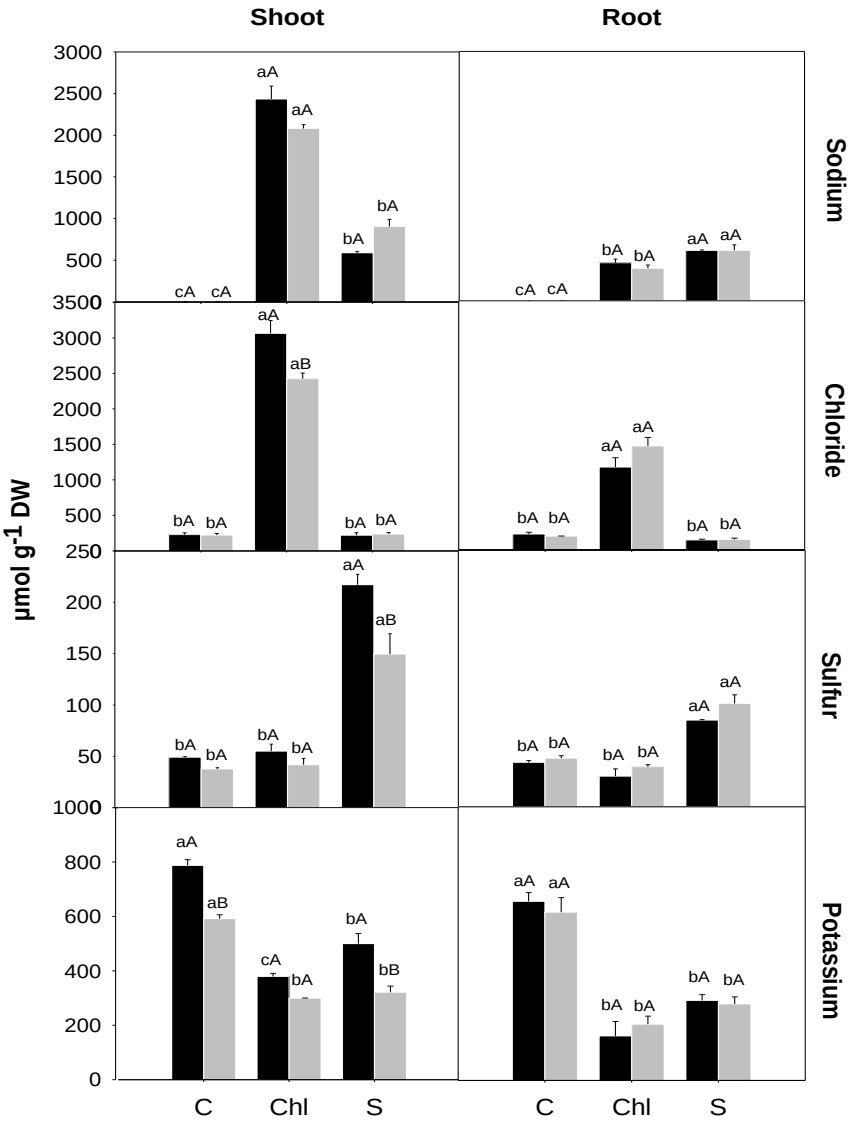

Figure 2. Mineral nutrient concentration in shoot and roots of rice seedlings from cvs. Pokkali (black bars) and IKP (gray bars) cultivated during 2 weeks in control (C) conditions or in the presence of 50mM NaCl [chloride salinity (Chl)] or 25mM Na_2SO_4 [sulfate salinity (S)]. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments followed by the same lowercase letter for a given cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a treatment do not differ statistically.

Table 2. Mineral content (Ca, Fe, P, Mg and Mn) in shoots and roots of rice seedlings from cv. Pokkali and IKP cultivated during two weeks in control (C) conditions or in the presence of 50 mM NaCl (chloride salinity (Chl)) or 25 mM Na_2SO_4 (sulfate salinity (S)). Each value is the mean of three replicates ± standard errors of the mean.

		(µmol g^{-1} DW)									
		Ca		**Fe**		**P**		**Mg**		**Mn**	
Cultivar	Treatment	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Pokkali	C	137.7 ± 4.2^{aA}	11.4 ± 0.87^{aA}	2.3 ± 0.08^{bA}	37 ± 6.8^{bA}	379.2 ± 28.5^{aA}	145 ± 8.3^{bA}	337.9 ± 45^{aA}	50.6 ± 4.8^{aA}	29.4 ± 4.3^{bA}	1.8 ± 0.5^{aA}
	Chl	$151 \pm 7.^{aA}$	26 ± 13.6^{aA}	5 ± 0.6^{aA}	274.2 ± 47.4^{aA}	307.5 ± 18.6^{abA}	362.3 ± 65.4^{aA}	306.5 ± 17^{aA}	54.6 ± 12^{aA}	40 ± 0.7^{abB}	0.81 ± 0.3^{aA}
	S	192 ± 36^{aA}	19.8 ± 3^{aA}	2.2 ± 0.28^{bA}	150.3 ± 17.1^{abA}	278.4 ± 12.4^{bA}	252.8 ± 16.5^{abA}	332.7 ± 29.8^{aA}	44.7 ± 1.3^{aA}	78.2 ± 18.7^{aA}	1.6 ± 0.05^{aA}
IKP	C	156.1 ± 2.5^{aA}	12.3 ± 3.3^{aA}	1.9 ± 0.2^{aA}	36.2 ± 4^{cA}	387.5 ± 12.6^{aA}	190.5 ± 13.2^{bA}	306.7 ± 12^{aA}	50.3 ± 3.4^{aA}	32.2 ± 1.7^{bA}	2.5 ± 0.5^{aA}
	Chl	122.6 ± 17.4^{abA}	20.35 ± 4.4^{aA}	4.3 ± 0.8^{aA}	307.3 ± 18.4^{aA}	256.6 ± 35.7^{bA}	460.4 ± 33.9^{aA}	245.3 ± 44.6^{abA}	47.6 ± 5.7^{aA}	62.9 ± 7.4^{aA}	1.9 ± 0.2^{aA}
	S	85.2 ± 3^{bB}	11 ± 1^{aA}	3.6 ± 1^{aA}	142.5 ± 27.5^{bA}	276.4 ± 26.4^{abA}	285.9 ± 11.8^{bA}	172 ± 6^{bB}	38 ± 2.3^{aA}	47.7 ± 0.8^{abA}	3 ± 0.4^{aA}

Notes: Treatments followed by the same lower case letter for a particular cultivar do not differ statistically. Cultivars followed by the same upper case letter in a particular treatment do not differ statistically.

The shoot Ψ_S (Figure 3[A]) decreased in response to chloride salinity in the two considered cultivars. Shoot Ψ_S significantly decreased in response to sulfate salinity in cv. Pokkali while cv. IKP showed an intermediate decrease. Whatever the treatment, shoot Ψ_S remained however similar in the two considered cultivars. In contrast, the root Ψ_S of salt-treated plants decreased in cv. Pokkali only and was always lower than in IKP (Figure 3[B]). The shoot proline concentration drastically increased in response to chloride salinity (Figure 3[C]) and was slightly higher in Pokkali than in IKP. Proline concentration also significantly increased in shoot of sulfate-treated plants but remained lower than values recorded for Cl-treated ones. In sulfate-treated plants, proline concentration was higher in IKP than in Pokkali. The total soluble sugar concentrations (Figure 3[D]) also increased in salt-treated plants but no difference was recorded neither between cultivars nor between the two types of salinity.

As evidenced by the results in table 3, the salt ions as well as K^+ more contributed to osmotic adjustment than proline and a great contribution was recorded specifically for Na^+ and Cl^- ions. Regarding rice cultivars, contribution rates of both proline and ions were higher for IKP than for Pokkali. The contribution rates of proline and salt ions were higher in NaCl- than in Na_2SO_4-treated shoots whatever considered cultivar while a reverse trend was observed for K^+.

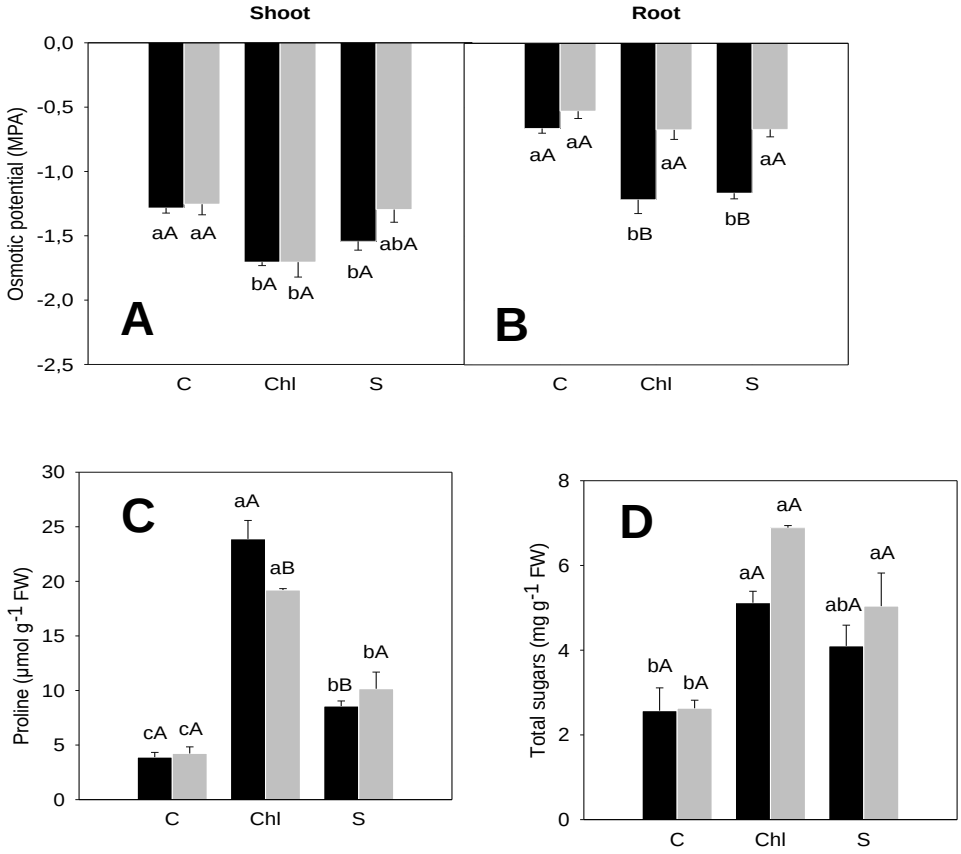

Figure 3. Shoot and root osmotic potential (Ψ_S), shoot proline and shoot total soluble sugar concentration in rice seedlings from cvs. Pokkali (black bars) and IKP (gray bars) cultivated during 2 weeks in control conditions (C) or in the presence of 50mM NaCl [chloride salinity (Chl)] or 25mM Na_2SO_4 [sulfate salinity (S)]. Each value is the mean of three replicates per treatment and vertical bars are standard error of the mean. Treatments followed by the same lowercase letter for a given cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a treatment do not differ statistically.

Table 3. Contribution rates of proline, K^+ and salt ions to osmotic adjustment in shoots.

Rice cultivar	Osmolyte, K^+ and salt ions	Applied salt stress	
		50 mM NaCl	25 mM Na_2SO_4
Pokkali	Proline	0.97%	0.55%
	Na^+	48%	15%
	Cl^-	58.6%	
	S^{6+}		6.1%
	K^+	8.63	17.2
IKP	Proline	1.5%	1.1%
	Na^+	80.1%	53.4%
	Cl^-	97%	
	S^{6+}		8.8%
	K^+	11.7	19.02

The shoot MDA concentration obviously increased in plants exposed to chloride salinity and was higher in IKP than in Pokkali (Figure 4[A]). Malondialdehyde concentration was only slightly higher in sulfate-treated plants than in controls, but the difference between treatments was not significant. As far as total antioxidant activity is concerned, both AOAM (Figure 4[B]) and AOAD (Figure 4[C]) fractions increased in salt-treated plants. Salt-induced increase was more marked for AOAM than for AOAD fraction and for sulfate than for chloride salinity. Pokkali tended to have slightly higher AOAM activities than IKP but the difference between cultivars was not significant.

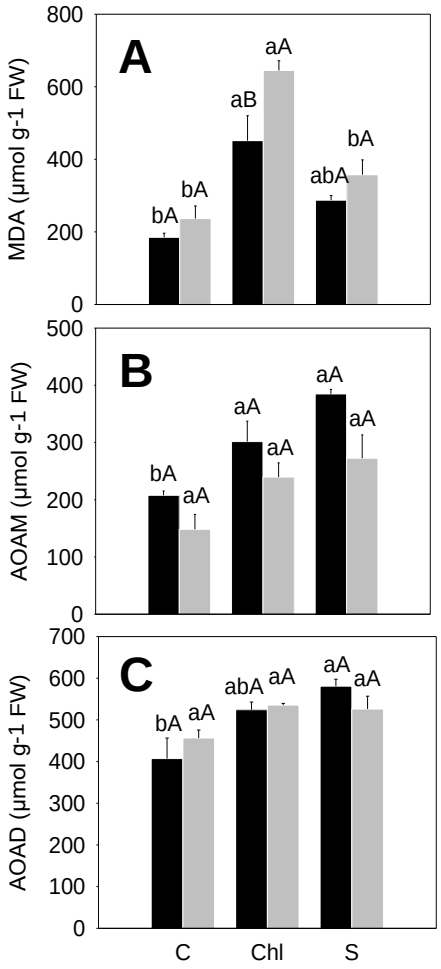

Figure 4. Malondialdehyde (MDA), hydrophilic (AOAM) and lipophilic (AOAD) antioxidants concentration in the shoots of rice seedlings from cv. Pokkali (black bars) and IKP (gray bars) cultivated during two weeks in control (C) conditions or in the presence of 50mM NaCl [chloride salinity (Chl)] or 25mM Na_2SO_4 [sulfate salinity (S)]. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments followed by the same lowercase letter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a particular treatment do not differ statistically.

Table 4 indicates that chloride salinity reduced the GSH concentration in Pokkali but the recorded decrease was not significant in IKP. In plants exposed to sulfate salinity, shoot GSH concentration was higher in Pokkali than in IKP. No difference was recorded between treatments for shoot GSSG concentration which also remained similar in the two considered cultivars. The GSH/GSSG ratio was always slightly lower in IKP than in Pokkali, whatever the considered treatment but difference was not significant. Chloride salinity reduced GSH/GSSG ratio in Pokkali but not in IKP.

Table 4. Oxidized (GSSG) and reduced (GSH) glutathione content in shoots of rice seedlings from cv. Pokkali and IKP cultivated during two weeks in control (C) conditions or in the presence of 50 mM NaCl (chloride salinity (Chl)) or 25 mM Na_2SO_4 (sulfate salinity (S)). Each value is the mean of three replicates ± standard errors of the mean.

		µmol g^{-1} FW		
Cultivar	Treatment	GSH	GSSG	GSH/GSSG
Pokkali	C	0.148 ± 0.008^{aA}	0.206 ± 0.007^{aA}	0.719 ± 0.03^{aA}
	Chl	0.089 ± 0.01^{bA}	0.211 ± 0.01^{aA}	0.429 ± 0.07^{bA}
	S	0.139 ± 0.01^{aA}	0.223 ± 0.02^{aA}	0.627 ± 0.02^{abA}
IKP	C	0.107 ± 0.01^{aA}	0.215 ± 0.02^{aA}	0.515 ± 0.04^{aA}
	Chl	0.080 ± 0.01^{aA}	0.226 ± 0.01^{aA}	0.349 ± 0.03^{aA}
	S	0.087 ± 0.004^{aB}	0.224 ± 0.02^{aA}	0.39 ± 0.01^{aA}

Notes: Treatments followed by the same lower case letter for a particular cultivar do not differ statistically. Cultivars followed by the same upper case letter in a particular treatment do not differ statistical.

Discussion

A non-negligible proportion of salinization in irrigated lands may be due to Na_2SO_4 and because rice is extremely important for human nutrition and rather salt-sensitive, there is an evident interest to compare the rice response to NaCl and Na_2SO_4 salinities. The present study revealed a higher toxicity of NaCl over Na_2SO_4, as found previously in studies dealing with other species (Curtin, Steppuhn, and Selles 1993, Gupta and Gupta 1984, Hasson-Porath, Kahana, and Poljakoff-Mayber 1972, Mor and Manchanda 1992, Navarro et al. 2002).

The results on the chemical speciation of the culture solutions revealed that the concentration and activity rates of the salt ions differed widely. Despite the equimolar concentration of Na^+ in the initial saline solution, the Na^+-concentration and activity rates were higher when Na^+ was accompanied by the chloride anion than by the sulfate anion. We also observed that the concentration and activity rates of Na^+ were almost the same as those of Cl^- in the case of NaCl salinity. Surprisingly, the concentration and activity rates of the SO_4^{2-} anion were significantly low and that could partly explain a less intense toxicity recorded for Na_2SO_4-treated plants as observed on several studied parameters.

The sodium ion accumulation in shoots was almost five times higher in the case of chloride stress compared to sulfate stress (Figure 2). This observation suggests that Na^+ uptake is influenced by the nature of anion excess present in nutrient solution. Distinct transporter proteins located in the plasma membrane are involved in Na^+ absorption but the majority of Na^+ influx into the plant cell is thought to occur via nonselective cation channels while Na^+ efflux appears to be mediated by Na^+/H^+ exchange (Hasegawa 2013, Maathuis, Ahmad, and Patishtan 2014). In the present study, salt-treated plants were exposed to similar Na^+ concentration and accumulated anions thus appear to influence Na^+ translocation in the plant. This view is supported by the fact that accumulated Cl^- in NaCl-treated plants was by far higher than sulfate accumulation in Na_2SO_4-treated ones. Since the initial concentration of Cl^- anion was two times higher than that of SO_4^{2-} anion in the saline solution, this ratio should normally be maintained with regard to their accumulation in plant. However, our results

showed that the chloride concentration in plant was sixteen times higher than that of sulfur. This could be explained by the combination of several factors such as the activity rate of each ion in the culture solution, the ion mobility, the duration of stress exposure as well as the stress intensity.
Since small amounts of Cl^- are required for normal metabolism, plants possess complex molecular mechanisms for chloride transport across root cell membrane and long distance transport of this element from the root to photosynthetic tissues (White and Broadley 2001). Regulation of these mechanisms probably plays an important role in salinity resistance but has been neglected up to now and this question needs to be addressed in the future.

In the present study, root growth estimated on a dry weight basis was not affected by salinity but the morphology of the root system was not studied. Zhou et al. (2011) clearly demonstrated that net Na^+ fluxes change significantly at anatomically distinct root zones of rice seedlings, mainly in relation to the development of exodermis and endodermis conditioning the importance of apoplastic by-pass flow. Local disruption of exodermis during secondary root development from the pericycle could also facilitate Na^+ transport. A differential impact of NaCl and Na_2SO_4 on anatomical features of rice root could not be ruled out and also requires further investigations.

Shoot Ca^{2+} content was affected by salinities in IKP while K^+ decreased in both cultivars. According to Choi et al. (2014) roots may elicit a calcium wave signal that propagates throughout the plant and may be responsible for the adaptative response of salt-treated plants. A decrease in Ca^{2+} concentration of Na_2SO_4-treated plants in IKP may reflect the inability of this cultivar to trigger a long-distance Ca^{2+}-signaling process. Calcium has a strong impact on the regulation of plasma membrane H^+-ATPase activity involved in Na^+ compartmentation preventing excessive accumulation of Na^+ in the cytosol and a decrease in Ca^{2+} may thus have a deleterious impact on cell metabolism (Morgan et al. 2014). Calcium also helps to maintain cell growth through its positive impact on cell wall composition (An et al. 2014). Frouin et al. (2018) recently confirmed that several quantitative trait loci for salt tolerance in rice are closed to those of genes

involved in calcium signaling and metabolism and that Pokkali is able to maintain an efficient Na^+-Ca^{2+} selectivity.

Manganese is also thought to assume positive functions in salt-treated plants. Rahman et al. (2016) provided evidences that Mn^{2+} improves ionic and osmotic homeostasis through decreasing Na^+ influx and increasing water status, and increasing ROS detoxification by increasing flavonoids, phenolics and ascorbate content. Aktas et al. (2005) even hypothesized that manganese itself exerts antioxidative effects in plant tissues by being oxidized from Mn^{2+} to Mn^{3+}. Both rice cultivars were able to increase shoot Mn content in response to NaCl and to Na_2SO_4 which should be regarded as an adaptative strategy to cope with salt stress and also suggest that roots did not retain absorbed Mn. In contrast, to Mn, shoot P content was reduced in response to NaCl in IKP and in response to Na_2SO_4 in Pokkali. A myriad of physiological properties rely on an adequate presence of P. It is unlikely that $H_2PO_4^-$ and Cl^- ions are competitive in terms of plant uptake. In the present work using hydroponic culture and which does not take into account the poor P mobility in the soil, it seems that P translocation from the root to the shoot was adversely affected since a decrease in the shoot P content occurred concomitantly with an increase in the root P content. The two types of salinity may thus act on P loading at the xylem level, but in contrast to Mn, the effect was cultivar-dependent.

Beside ion accumulation, plants exposed to salt stress are also exposed to a decrease in the external water potential (Chinnusamy, Jagendorf, and Zhu 2005, Lefèvre, Gratia, and Lutts, 2001, Lutts, Bouharmont, and Kinet 1999). Plant responses classically involve osmotic adjustment allowing the organism to maintain the water potential gradient required for water absorption. Although this osmotic constraint has been regarded as a shoot-ion independent process (Pires et al. 2015), it is noteworthy that in our study, shoot Ψ_S was lower for NaCl-treated plants than for Na_2SO_4-stressed ones. This observation might be related to higher accumulation of proline and soluble sugars, which are well known compatible organic compounds putatively involved in cytosolic osmotic adjustment (Tuteja 2007). The fact that these compounds increased to a higher extent in response to NaCl than to iso-osmotic Na_2SO_4 suggests that the accumulated ions and not only the external osmotic potential may somewhat influence over synthesis of these

organic compounds. However, as revealed by our results (Table 3) on contribution rate of proline, K^+ and salt ions to osmotic adjustment, the contribution rates of ions was far higher than that of proline in all cultivars and for both types of salt stress. Beside acting as an excellent osmolyte, Joseph et al. (2015) proved that proline plays major roles as antioxidative defense molecule and signaling molecule during stress. In our case and on the basis of its low contribution in the osmotic adjustment, the proline level may be more of a stress intensity signal than an indicator of stress tolerance. In line with our hypothesis, Ghars et al. (2008) showed that the accumulation of proline itself does not confer a higher tolerance to osmotic stress in *Thellungiella halophile*. It could be considered that proline is part of a signaling pathway and that higher proline content just reflects that the plant experiences higher stress intensity (Kaur and Asthir 2015; Kavi Kishor and Sreenivasulu 2014; Zhang and Becker 2015). Osmotic potential provides an information regarding the amounts of soluble compounds accumulated inside the cell plant but not on the nature of these compounds. In the specific case of salinity and considering our results on Na^+, Cl^- and S^{6+} contribution rates in osmotic adjustment, accumulation of salt ions may contribute to lowering osmotic potential but has major detrimental effects on the plant metabolism when they are not compartmentalized inside the vacuole cell. It is well-known that K^+ is clearly involved in osmotic potential leading to turgor maintenance in unstressed plants but in our study, the recorded salt-induced decrease in K^+ could affect negatively this function in the presence of NaCl or Na_2SO_4. We deduced that the higher the accumulation of proline or a given ion in the shoots, the higher its contribution rate to osmotic adjustment.

Oxidative stress due to synthesis of ROS is an important component of saline toxicity (Ahanger et al. 2017, Bose, Rodrigo-Moreno, and Shabala 2014). A higher MDA concentration in response to NaCl than Na_2SO_4 also confirms a higher toxicity of the former salt comparatively to the latter. This observation could be related to the fact that Na_2SO_4-treated plants more efficiently trigger antioxidant response than plants exposed to chloride toxicity, as evidenced by the recorded response for AOAM fraction. Unfortunately, we have not analyzed the nature and amount of ROS produced by the salt-treated plants; a study evaluating both antioxidant activity and ROS production would provide additional

information. Glutathione is a multifunctional water-soluble tripeptide containing a sulfhydryl group and assumes important role as a major antioxidant in all aerobic organisms, especially in stressed plants where ROS generation is accelerated (Gill et al. 2013). This important compound should be recovered in the AOAM fraction. However, the total glutathione (GSH + 2 x GSSG) content only slightly varied in Pokkali and remained remarkably constant in IKP (0.534mmol g^{-1} FW). Sulfur uptake and assimilation are essential for GSH synthesis. Despite an increase in sulfur content in sulfate-treated plants, no concomitant increase was recorded for glutathione content although this peptide contains a central cysteine with a thiol group. Conversely, GSH is also involved in sulfur assimilation pathway since it acts as a co-factor for the adenosine-5-phosphosulfate reductase, a key enzyme in sulfite synthesis (Khan et al. 2012). Since chloride salinity did not decrease sulfur content, and since sulfate salinity did not increase GSH synthesis, one may assume that under our experimental conditions glutathione metabolism remain constant. Hence, other antioxidant such as ascorbate may account for the recorded increase in the total antioxidant activities of the AOAM fraction in stressed tissues. Similarly, lipohilic antioxidant of the AOAD fraction are membrane-bound hydrophobic scavengers where they interrupt chain reaction of lipid peroxidation leading the MDA synthesis and compounds such as α-tocopherol might accumulate in sulfate-treated plants and thus account for their lower MDA content comparatively to chloride-treated ones.

The tall indica landrace Pokkali is frequently used as a salt-tolerant reference in studies devoted to rice response to NaCl (Frouin et al. 2018, Lefèvre, Gratia, and Lutts, 2001, Lutts, Kinet, and Bouharmont 1995). Although this cultivar displayed a quite higher shoot biomass than the semi dwarf salt-sensitive IKP, our study suggested that resistance of Pokkali was not due to a dilution effect of absorbed Na^+ since shoot Na^+ concentration estimated on a dry weight basis was similar in the two cultivars, even if shoot growth was affected in IKP and not in Pokkali. The NaCl-induced decrease in shoot K^+ was higher in Pokkali than in IKP, which confirms the recent analysis of Pires et al. (2015) assuming that K^+ content is not a sufficient trait to assess rice salinity resistance. Similarly, the two cultivars exhibited similar shoot Ψ_S in NaCl-treated plants but WC was drastically decreased in IKP, confirming that osmotic adjustment is necessary but not

sufficient to ensure the maintenance of an optimal plant water status. A poor stomatal regulation in salt-sensitive cultivars (Lutts, Bouharmont, and Kinet 1999) might explain the low WC recorded in IKP. Since WC was not affected in IKP in response to Na_2SO_4, one may assume that this salt was less toxic than NaCl in terms of plant water status alteration. Roots from Pokkali displayed osmotic adjustment properties that were not observed in IKP since no root Ψ_S decrease has been observed in this cultivar. This confirms that the roots of the salt-resistant cultivar have a distinct metabolic status comparatively to those of the salt-sensitive one. Our data also suggest that IKP less efficiently cope with salt-induced antioxidative stress comparatively to Pokkali: this is especially the case in NaCl-exposed plants where MDA was higher in IKP than in Pokkali. The total glutathione content remained statistically similar in the two cultivars but the GSH/GSSG ratio slightly differed. It appeared to be lower in IKP than in Pokkali, suggesting that GSSG recycling through glutathione reductase may be less efficient in the former than in the latter.

Conclusion

In conclusion, NaCl appeared more toxic than Na_2SO_4 and lead to a higher Na^+ accumulation in the shoot of rice. The moderate toxicity recorded for Na_2SO_4 could be due to its low concentration and activity rates in culture solution as well as to the low mobility of the SO_4^{2-} anion inside the plant. Calcium nutrition was impaired by Na_2SO_4 in the salt-sensitive cultivar while phosphorus translocation was affected by NaCl. Increase in the shoot Mn may be regarded as an attempt of adaptation to both types of salinity. Salt ions as well as K^+ ion more contributed to osmotic adjustment than proline. In the present study, proline may play a key role in salt stress sensing, perception and transduction. The salt-resistant cultivar Pokkali displayed a higher capacity of osmotic adjustment and maintenance of antioxidant status compared to the salt-sensitive IKP in response to both types of salinity.

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Interchapter I-II

What do we know?

The first chapter provided a positive answer to the first question listed in our thesis objectives and demonstrated that the anionic component of salt stress has a specific impact on the rice behavior for a same external Na^+ concentration. It also demonstrated that, for a short term exposure at the seedling stage, oxidative stress was higher in chloride-treated plants than in sulfate-exposed ones, and that the antioxidant status remained more efficient in the salt-resistant cultivar Pokkali than in the salt-sensitive cultivar IKP. Differences between NaCl and Na_2SO_4 impact on rice should not necessarily be regarded as a consequence of differential toxicities of anions, but might also be explained by the fact that NaCl-treated plants accumulated more internal Na^+ than Na_2SO_4-treated plants.

What is our question?

It is well known that in the plant kingdom the impact of abiotic stresses, including salinity, differ according to the plant development and that the underlying physiological processes involved in the response to salinity are not the same at all phenological stages. Rice is considered to be especially sensitive at the seedling and at the flowering stages. The next question is then to assess if the differential impact of NaCl and Na_2SO_4 on rice previously recorded at the vegetative stage is also observed at the reproductive phase of development and what could be the consequence on yield-related parameters.

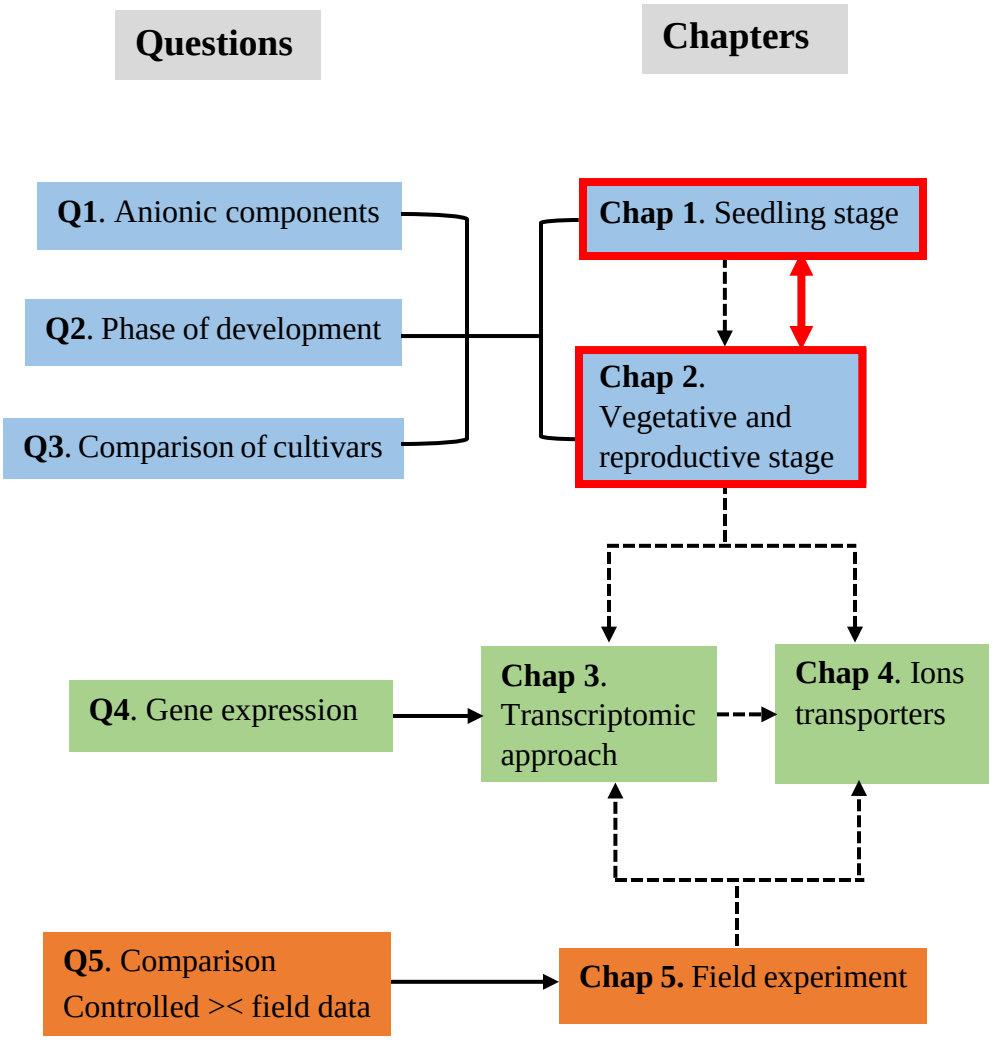
What is our strategy?

In the Chapter 1, we used two rice cultivars (IKP: salt-sensitive and Pokkali: salt-resistant). Plants were cultivared in semi-controlled environment in nutrient solutions, and the same system will be used in Chapter II. However, we decided to focus on one single cultivar (IKP; salt-sensitive) for the two following reasons: i) As a tall indica landrace, the cycle of Pokkali is quite longer than the cycle of IKP: this implies that the plant will not flower simultaneously, and that Pokkali will be therefore exposed to NaCl for a longer period than IKP and will not have the same chronological age ii) Pokkali is a photoperiodic sensitive cultivar and our experimental conditions do not offer full guarantee that flowering of

control plants in Pokkali will occur under optima conditions. In contrast, IKP is not photosensitive allowing us to more precisely analyze the impact of salt stress independently of light interference.

In Chapter II, salt stress will also be applied at the vegetative stage but will be maintained until harvest. This implies that the plant will be exposed to salt stress for a quite longer period than in the Chapter I and explains why we decided to slightly reduce the salt dose. The plant will be exposed to salinity during the whole cycle (except germination), implying that the salt impacts on flowering and yield-related parameters are not only the reflect of salt impact occurring at this specific stage, but may also occur as a delayed impact and ultimate consequence of deleterious effects occurring during the previous stage and this is why we also consider tillering in the next Chapter.

Photosynthesis is an important physiological function providing sugar and thus energy for flowering process and subsequent grain filling. As a consequence, a specific attention will be paid to photosynthetsis in the next Chapter.



Synthetic overview of the five research questions addressed during our thesis project and the specific source of their answers in the different chapters of the thesis.

Chapter II. NaCl and Na_2SO_4 salinities have different impact on photosynthesis and yield-related parameters in rice (*Oryza sativa* L.)

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Abstract

To elucidate the comparative effect of chloride and sulfate salinities on photosynthesis and yield components in rice, plants of *Oryza sativa* (cv. I Kong Pâo (salt-sensitive)) were exposed in nutrient solutions to 20 mM Na_2SO_4 or 40 mM NaCl (electrical conductivity of *c.a.* 4.30 dS m^{-1} for both solutions) from seedlings to maturity stage. Both types of salt induced a strong decrease in net photosynthesis (A_N) at the seedling and tillering stages while the intracellular CO_2 concentrations (C_i) remained unaffected. Instantaneous transpiration (E) and stomatal conductance (g_s) decreased at tillering and seedling stages respectively only in plants exposed to NaCl. Chloride salinity also strongly decreased photosynthetic pigments while no impact was detected in response to Na_2SO_4. All yield-related parameters were affected by salinities but NaCl was significantly more deleterious than Na_2SO_4 for the mean number of tillers produced per plant, spikelets sterility and non-viable pollen percentage. In contrast, both types of salinity similarly impacted the percentage of fertile tillers and 1000-grain weight. At the grain level, more than 90% of toxic ions (Na^+, excess of Cl^- and S^{6+}) accumulated in the hulls, thus preserving the internal part of the caryopses from ion injuries.

***Key words*:** chloride, sulfate, salinity, *oryza sativa*, photosynthesis, yield

Introduction

Soil salinity is one of the major environmental constraints on agriculture, affecting a large part of arable land throughout the world and costing billions of euros every year. High contents of soluble salts are reported in numerous areas of the world. Soil salinity is aggravated by secondary salinization due to unadapted irrigation and, on a long term basis, by global warming rising sea level with numerous consequences in coastal area (Bui, 2013; Wassman et al., 2004). Rice (*Oryza sativa* L.) is extremely sensitive to salinity and current guidelines indicate that rice yield decrease by more than 12% for every unit (dS/m) increase in electrical conductivity of root-zone saturated soil extract above 3 dS/m (Grattan et al., 2002).

Salt sensitivity depends on the developmental stage. As far as rice is concerned, early seedling growth and reproductive stages are the most salt-sensitive (Lutts et al., 1995; Moradi & Ismail, 2007; Zeng & Shannon, 2000; Zhu et al., 2001; 2004). Numerous authors reported that salinity delays flowering in rice and has a negative impact on several yield components including number of panicles, fertile tillers and spikelets per plant, floret fertility and individual grain size (Abdullah et al., 2001; Rao et al., 2008; Sultana et al., 1999; Zeng et al., 2003; Zeng et al., 2001; Zhu et al., 2004). Differential sensitivity during developmental stage is a major issue in the management of saline water for irrigations and Zeng et al. (2001) demonstrated that salinity has different impact if applied at booting stage and panicle initiation stage. In numerous rice production systems, however, salinity persists during most of the plant developmental stages. Beside salt-sensitivity at the flowering phase itself, ultimate consequence on rice yield also appear to be influenced by stand establishment and photosynthesis during the vegetative stage leading to preanthesis assimilates that will be retranslocated during grain filling (Kaddah, 1963; Sultana et al., 1999; Flowers & Yeo, 1981).

Salinity affects plants in two distinct phases. The first phase is the osmotic effect, which reduces external water potential and makes water uptake from roots thermodynamically hampered (Pardo, 2010; Roy et al., 2014). The second phase of salinity is ionic specific and is due to the accumulation to toxic concentrations of salt ions inducing tissue necrosis and early leaf

senescence (Lutts et al., 1996). Studying the effect of NaCl salinity on photosynthesis and dry matter accumulation in developing rice grains, Sultana et al. (1999) concluded that reduction in photosynthesis in the salinized rice plant depended not only on a reduction of available CO_2 by stomatal closure, but also on the cumulative effects of biochemical alterations, photosynthetic pigments degradation and structural modifications. Castillo et al. (2007) compared the effect of salt and iso-osmotic concentration of polyethylene glycol in two contrasted rice cultivars and concluded that plant response was mainly attributed to the osmotic component of salt stress. In contrast, Khatun & Flowers (1995) concluded that accumulation of Na^+ and Cl^- excess are responsible for salt-induced decrease in pollen viability and stigma receptivity.

Most studies dealing with salt stress focus on NaCl. In nature, however, the soil solution is a complex mixture of various ions, including not only Na^+ and Cl^-, but also Ca^{2+} and SO_4^{2-} (Bui, 2013; Samson et al., 2016). Some studies are dealing with realistic conditions and consider a mixture of various salts (Grattan et al., 2002; Khatun & Flowers, 1995; Rao et al., 2008) but only few works discriminated the impacts of the various salts (Aghajanzadeh et al., 2017; Gao et al., 2012). Recently, Irakoze *et al*. (2019) found that NaCl in seedlings of rice was more toxic than Na_2SO_4 and that sodium and proline accumulation were higher while shoot osmotic potential was lower in NaCl-treated plants than in those exposed to Na_2SO_4. No data, however, are available regarding the differential impacts of the two salts on yield-related parameters. Since Zhu et al. (2004) demonstrated that leaf K^+/Na^+ ratio quantified under stress conditions at the vegetative stage may be used as a predictive criterion for yield-potential in several rice cultivars, we hypothesize that sodium iso-molar solutions of NaCl would be more detrimental to yield than Na_2SO_4.

Material and methods

Plant material and growing conditions

Seeds of the rice cultivar I Kong Pao (IKP; salt-sensitive) were obtained from IRRI (International Rice Research Institute; Philippines). Seeds were germinated on two layers of filter paper (Whatman No. 2) moistened with 10 ml of sterile deionized water in a growth chamber at 25°C under a 12 h daylight period (120 µmol m^{-2} s^{-1}). Ten-day-old seedlings were transferred into a greenhouse and were fixed on polystyrene plates floating on Yoshida nutritive solution (Yoshida et al., 1976). Illumination was provided by LED LumiGrow Lights (650 W, red-blue) for 12 h d^{-1} at a photon flux density (PAR) of 150 µmoles m^{-2} s^{-1}. Daytime humidity was between 70% and 80%, and the temperature was maintained at 28 °C during the day and 26 °C during the night. For each treatment, seedlings were distributed among three tanks (twenty seedlings per tank) containing 50 l of solution. The solutions were renewed weekly and tanks were randomly rearranged in the greenhouse. After two weeks of acclimatization in control conditions, seedlings were exposed to 20 mM Na_2SO_4 or 40 mM NaCl (electrical conductivity of *c.a.* 4.30 dS m^{-1} for both solutions; Ψs = -0.088 MPa for control, -0.30 MPa for 40 mM NaCl and -0.22 MPa for 20 mM Na_2SO_4) until maturity stage.

The pH values recorded in the culture solutions salinized with 20 mM Na_2SO_4 and 40 mM NaCl were 5.74 and 5.64 respectively. In addition, a chemical speciation of the culture solutions salinized with NaCl and Na_2SO_4 was performed using Visual Minteq software. The identified species are presented in appendix 2 of this document. The concentration and activity rates of Na^+, Cl^- and SO_4^{2-} are presented in results section.

At the end of the experiment, three plants were tagged in each of the three replicate treatments. The number of tillers per plant and spikelets per panicle, the percentage of floret sterility and fertile tillers per plant as well as the 1000-grains weight were recorded from these samples. We referred to the flowering tillers as fertile tillers. The number of spikelets per panicle, the percentage of floret sterility and the 1000-grains weight were recorded on main stem.

Estimation of pollen viability and grain ion content

Pollen viability was estimated using the staining procedure of Alexander (1969). Pollen was collected immediately after anthesis and treated in a drop of freshly staining solution. The staining of 200-300 pollen grains from three individual panicles from each of three plants per treatment was observed under a microscope.

Measurements of ion content were performed on mature grains (considering separately husks (lemmas and paleas) and dehusked grains): 20 mg DW were crushed in powder 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^+ and Na^+) by flame emission using Atomic Absorption Spectrometer (Thermo scientific S series model AAS4). Chloride was specifically extracted according to Hamrouni et al. (2011). Anions (S^{6+} and Cl^-) were quantified by liquid chromatography (HPLC-Dionex ICS2000).

Photosynthesis-related parameters

Photosynthesis-related parameters were estimated at the seedling stage (14 days after stress imposition) and at the tillering stage (30 days after stress imposition). The leaf n°3 (blade fully unfolded) from the top of the plant (basipedal numbering) was selected for measurements. At the tillering stage, only the main stem was considered.

Gas exchange was recorded with an infrared gas analyzer (LCA4; ADC Bioscientific, Hoddesdon, Hertfordshire, UK) using a PLC Parkinson leaf cuvette on intact leaves for 1 min (20 records/min) and an air flow of 300 mL/min. Air taken from the greenhouse was sent to a chamber into which a leaf portion of 6.25 cm^2 was introduced. The net photosynthetic rate (A_N), the instantaneous transpiration rate (E) and the intracellular CO_2 concentration (C_i) were estimated on the third leaf. Six plants were measured for each treatment, and all measurements were performed

between 9:00 and 11:00 am. Stomatal conductance (g_s) was measured at the same leaves, using a porometer (AP4, Delta-T Devices, Cambridge, UK).

Chlorophyll a (*Chl* a), chlorophyll b (*Chl* b) and carotenoid. The pigments were extracted from shoot in 80% acetone. The *Chl* a, *Chl* b and carotenoid concentrations were determined by spectrophotometry according to the procedure described by Lichtenthaler (1987) and using the following equations:

Chl a (mg/l) = (12.25*A663.2)-(2.79*A646.8)
Chl b (mg/l) = (21.50*A646.8)-(5.10*D663.2)
Carotenoid (mg/l) = [(1000*A470)-(1.82*Chl a)-(85.02*Chl b)]/198

Statistical treatment of the data

Statistical analyses were performed using JMP Pro 13 software. Mean values and standard error (SE) were obtained from 6 replicates for photosynthesis-related parameters except the pigments content parameter where 3 replicates were considered as for yield components. Mean values and SE were analyzed using Tukey's HSD all-pairwise comparisons as a post-hoc test and a P value < 0.05 was considered to be statistically significant. A one-way analysis of variance (ANOVA) was performed for the yield components and pigments content parameters whereas for the photosynthesis-related parameters, a two-way ANOVA was performed to detect growth stage, treatment and their interaction effects. The graphs were plotted using Sigma Plot 10.0 software.

Results

The pH values recorded in this experiment were within the acceptable range for good plant growth (5.5-6.5). These pH values were approximately close to the pH values recorded in Chapter I. For both chapters, the pH values were relatively higher in Na_2SO_4- than in NaCl-treated solutions.

Considering that the identified species did not vary depending on the stress intensity, the data presented in appendix 2 still valid for all studies conducted in hydroponic culture. Regarding NaCl stress, the concentration and activity rates of Na^+ in culture solution were 98.14% and 79.4% respectively. The concentration and activity rates of Cl^- were 98.34% and 79.5% respectively. In Na_2SO_4-treated solutions, the concentration and activity rates of Na^+ were 95.6% and 76.9% respectively while the concentration and activity rates of SO_4^{2-} were 87.1% and 36% respectively. Compared to the results in Chapter I, the concentration and activity rates increased slightly for all treatments.

All plants remained alive until the end of the treatment. Net photosynthesis (Fig. 1) was lower at the tillering than at the seedling stage. At the two developmental stages, NaCl and Na_2SO_4 induced a similar decrease in A_N. In contrast, Ci values were not affected by salinities and remained similar at the two developmental stages. Instantaneous transpiration decreased at the tillering stage for plants exposed to NaCl salinity but not for control or plants exposed to Na_2SO_4. Both types of salinities induced a similar decrease in E values. At the seedling stage, stomatal conductance was reduced in response to NaCl only, while both types of salinity reduced g_s at the tillering stage. Intrinsic water use efficiency was calculated at the leaf level on the basis of instantaneous measurements of leaf photosynthesis and transpiration: [$WUE=A_N/E$)]. We found that WUE remained unaffected by salinities but decreased at the tillering comparatively to seedling stage (Table 1).

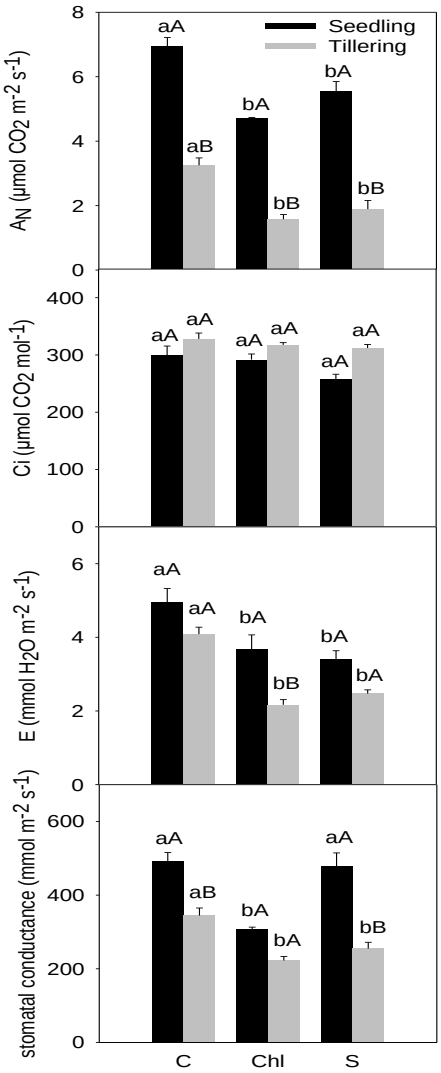

Figure.1. Effects of salinity stress application on mean net photosynthetic rate (A_N), intercellular CO_2 concentration (C_i), instantaneous transpiration rate (E) and stomatal conductance of IKP rice cultivar grown in Yoshida nutrient solution without salt stress (C), with 40 mM NaCl (Chl) or 20 mM Na_2SO_4 (S). Each value is the mean of six replicates per treatment and vertical bar is standard error of mean. Treatments followed by the same lower case letter for a particular vegetative stage do not differ statistically. Vegetative stage followed by the same upper case letter in a particular treatment do not differ statistically.

Table.1. Water use efficiency (WUE) measured at seedling and tillering stages of IKP rice cultivar grown in Yoshida nutrient solution without salt stress (C), with 40 mM NaCl (Chl) or 20 mM Na_2SO_4 (S).

	Seedling			Tillering		
	C	Chl	S	C	Chl	S
WUE	1.4 ± 0.09^{aA}	1.3 ± 0.1^{aA}	1.6 ± 0.12^{aA}	0.8 ± 0.08^{aB}	0.7 ± 0.09^{aB}	0.8 ± 0.08^{aB}

Notes: Treatments followed by the same lower case letter for a particular vegetative stage do not differ statistically. Vegetative stage followed by the same upper case letter in a particular treatment do not differ statistically.

At the seedling stage, chloride salinity induced a strong decrease in *Chl* a, *Chl* b and carotenoids content (Fig. 2) while Na_2SO_4 had no impact on the photosynthetic pigment concentration. At the tillering stage, chloride salinity reduced *Chl* a and carotenoids while Na_2SO_4, once again, had no significant impact on pigment content. The *Chl* a/*Chl* b ratio increased in rice seedlings exposed to NaCl.

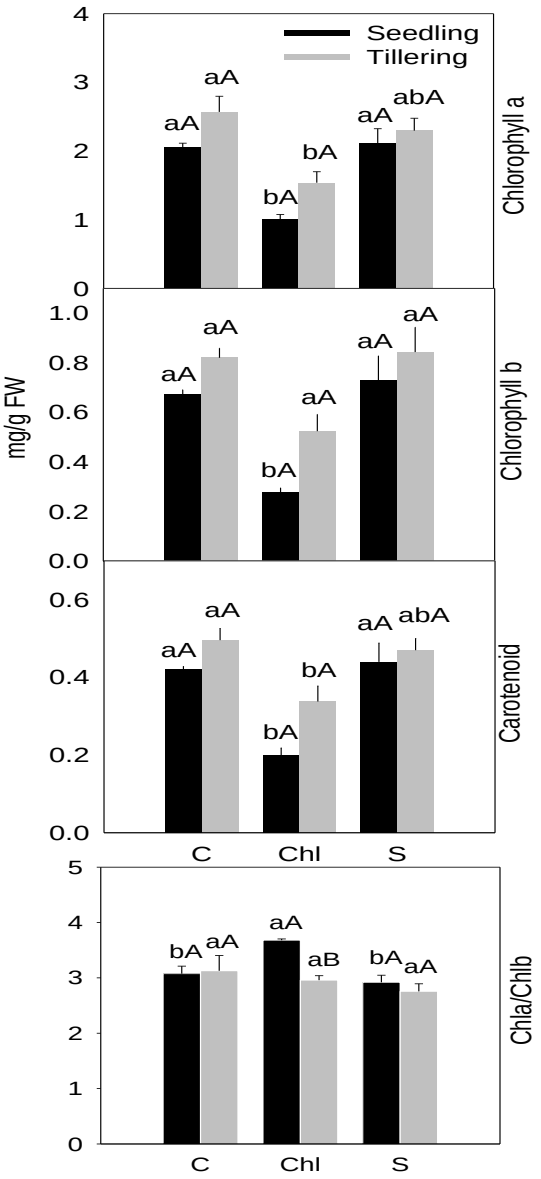

Figure.2. Effects of salinity stress application on chlorophyll and carotenoids content of IKP rice cultivar grown in Yoshida nutrient solution without salt stress (C), with 40 mM NaCl (Chl) or 20 mM Na_2SO_4 (S). Each value is the mean of three replicates per treatment and vertical bar is standard error of mean. Treatments followed by the same lower case letter for a particular vegetative stage do not differ statistically. Vegetative stage followed by the same upper case letter in a particular treatment do not differ statistically.

Net photosynthesis of the flag leaf was strongly reduced by both NaCl and Na_2SO_4 (Fig. 3), the minimal value being reported in response to NaCl, although the difference between the types of salt was not significant. All plants flowered and no delay in flowering was recorded for stressed plants (detailed data not shown). All yield-related parameters were affected by salinities (Fig. 4). The deleterious impact of NaCl was higher than the impact of Na_2SO_4 for the mean number of tillers produced per plant, spikelets sterility and non-viable pollen percentage, while both types of salinity had a similar impact on tiller fertility, 1000-grain weight and spikelet number per panicle.

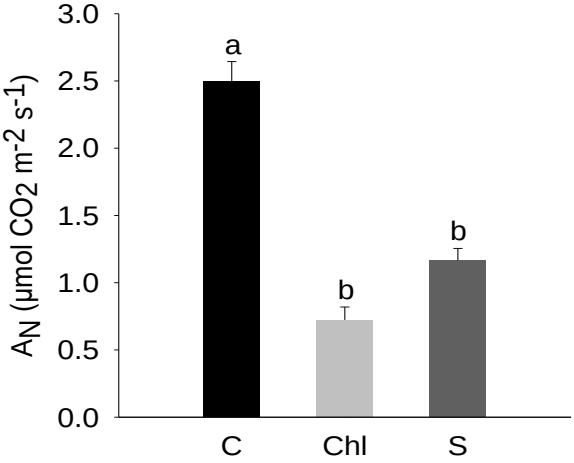

Figure.3. Effects of salinity stress application on mean net photosynthetic rate (A_N) measured on the flag leaf of IKP rice cultivar grown in Yoshida nutrient solution without salt stress (C), with 40 mM NaCl (Chl) or 20 mM Na_2SO_4 (S). Means with similar letters are not different at $p<0.05$ according to Tukey's multiple range test at 95%.

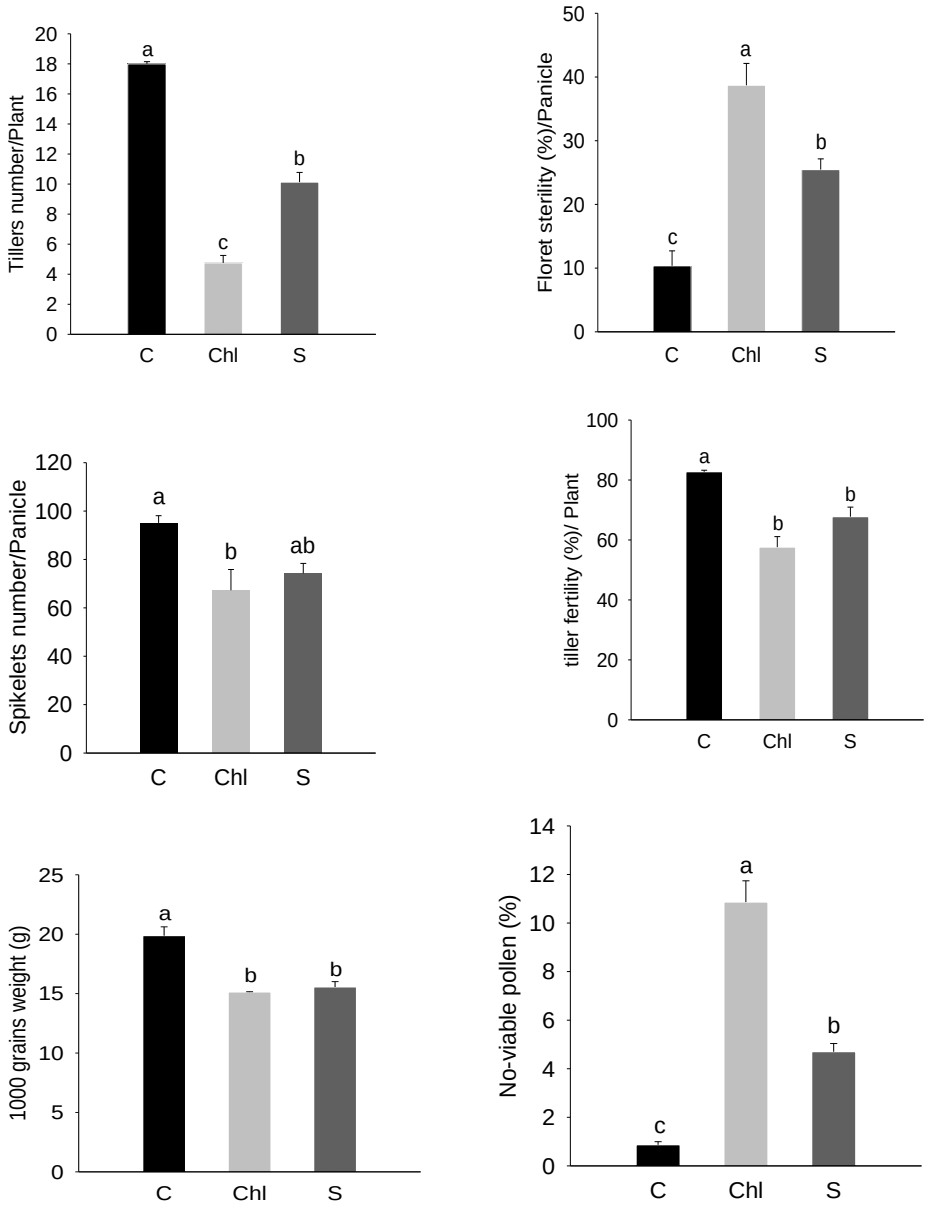

Figure.4. Effects of salinity stress application on vegetative and reproductive growth stages of IKP rice cultivar grown in Yoshida nutrient solution without salt stress (C), with 40 mM NaCl (Chl) or 20 mM Na_2SO_4 (S). Means with similar letters are not different at *p<0.05* according to Tukey's multiple range test at 95%.

For all analyzed elements, the mineral content was higher in the hulls than in the grain itself (Table 2). In both organs, Na^+ accumulation was lower in Na_2SO_4- than in NaCl-treated plants. Treatments had no significant impact on the relative Na^+ distribution and a mean percentage of 91.4% of total Na^+ was accumulated in the hulls. Both types of salinity reduced K^+ content: there was no significant difference between NaCl and Na_2SO_4 in this respect and salinities had no impact on the relative distribution between hulls and grains. Almost all recorded chloride (more than 95%) was found in the hulls. Chloride concentration increased by 730% in the hulls and by 960% in the grains of NaCl-treated plants. Sulfate salinity had no significant impact on Cl^- content but increased sulfur content to a higher extent in the hull (583%) than in the grain (152%).

Table.2. Mineral nutrient concentration measured in the grain and grain hull (lemma and palea) of IKP rice cultivar grown in Yoshida nutrient solution without salt stress (C), with 40 mM NaCl (Chl) or 20 mM Na_2SO_4 (S).

	µmol g^{-1} DW					
	Grain hull			Grain		
Ion	C	Chl	S	C	Chl	S
Na^+	42.8 ± 3.5^c	503.4 ± 3.2^a	314 ± 36.5^b	5.4 ± 0.06^c	51.7 ± 2.3^a	22.9 ± 3.5^b
K^+	920 ± 30^a	534.9 ± 15^b	550 ± 24^b	226 ± 1.5^a	141 ± 3.6^b	146 ± 7^b
Cl^-	199.3 ± 13^b	1455.6 ± 27^a	188 ± 14^b	6.7 ± 0.08^b	64.6 ± 3^a	7 ± 0.7^b
S^{6+}	48.7 ± 5.6^b	57.2 ± 4.3^b	284 ± 17^a	16.4 ± 1^b	15 ± 0.1^b	25 ± 2^a

Notes: Means with similar letters are not different at $p<0.05$ according to Tukey's multiple range test at 95%.

Discussion

The results on the chemical speciation of the culture solutions revealed that the concentration and activity rates of the salt ions differed widely. Despite the equimolar concentration of Na^+ in the initial saline solution, the Na^+-concentration and activity rates were higher when Na^+ was accompanied by the chloride anion than by the sulfate anion. We also observed that the concentration and activity rates of Na^+ were almost the same as those of Cl^- in the case of NaCl salinity. Surprisingly, the concentration and activity rates of the SO_4^{2-} anion were significantly low and that could partly explain a less intense toxicity recorded for Na_2SO_4-treated plants as observed on several studied parameters.

The present study confirms that flowering-related parameters are especially sensitive to salinity in rice but it also underlines that the type of salinity (chloride versus sulfate salinities) influences the plant response and that chloride salt was, on the whole, more toxic than sulfate salt. Some studies previously compared salinity and alkalinity impact on the reproductive structure in rice (Rao et al., 2008; Krishnamurthy et al., 2016) but, to the best of our knowledge, data regarding comparison between NaCl and Na_2SO_4 remain devoted to the vegetative phase of development (Rogers et al.,1998; Devinar et al., 2013; Han et al., 2014; Reich et al., 2017; Aghajanzadeh et al., 2018; Irakoze et al., 2019).

In order to unravel the putative influence of the counteranion, we used similar final Na^+ doses in the nutrient solution (Biliski et al., 1988) and the choosen doses induced a low salinity level (4.30 $dS.m^{-1}$). This salinity level was however sufficient to inhibit photosynthesis at vegetative stage and to induce floret sterility in relation to a decrease in pollen viability leading to a reduced number of filled grain per panicle.

Unlike the first chapter where salt stress reduced the plant water content, we found that stress did not affect WUE parameter in chapter 2. Therefore, the effective water management under salt stress conditions should be a tolerance strategy adopted by the plants.

Beside the stress intensity, the stage of stress imposition and duration of the stress also have an influence on flowering parameters. Some studies aiming to specifically analyze salt effect at the flowering stage apply stress at late-booting or at heading stage and for this purpose use high salt concentration (Chunthaburee et al., 2015; Lekklar et al., 2019; Thitisaksakul et al., 2015). Although they provide precious information on the genetic basis of salt-tolerance at the flowering stage, these studies are not considering the earlier step of inflorescence initiation, although the number of spikelets per panicle and panicle length are important parameters influencing the final yield in salt-treated rice (Zeng et al., 2001; Zhu et al., 2004). For the experiments performed in chapters 1 and 2, the plants of IKP cultivar were exposed to almost similar stress intensities (EC of *c.a.* 5.31 and 4.30 dS m^{-1} for both culture solutions respectively) but the duration of stress exposure was higher in chapter 2 than in chapter 1. In both cases, NaCl was always more toxic than Na_2SO_4. From our point of view, some of the studied parameters in the first chapter would have negatively affected the photosynthetic process and the yield parameters. Here we are referring to lipid peroxidation, osmotic stress leading to a decrease in plant water content as well as a great accumulation of salt ions in the plant impeding the dysfunction of the cell metabolic process. Very early at the vegetative stage, salt stress would hinder the panicle initiation leading to reduction of spikelet's number on the main panicle.

Long-term exposure to field-relevant levels of salinity also frequently induces a delay in flowering (Grattan et al. 2002). Such a delay was not observed in the present study since both control and stressed-plants simultaneously flowered. Flowering time under salt stress is genetically controlled in cereal plants (Ma et al., 2015) and it could not be excluded that IKP remains poorly affected in this respect. Park et al. (2013) reported that GIGANTEA protein (GI) encoded by a single gene and involved in the elicitation of photoperiod-dependent flowering may be degraded in the presence of salt, thus retarding the progression towards flowering; since IKP is a non-photoperiodic cultivar, it may be hypothesized that GI is not necessarily assuming a crucial role in the control of flowering in this cultivar.

Salt-induced decrease in pollen viability is an important component of stress impact on flowering. According to Khatun & Flowers (1995), NaCl reduced pollen staining and viability estimated by tetrazolium salt. According to these authors, Na and Cl per se are responsible for poor pollen viability, which also appear during pollen germination *in vitro*. Some authors, however, observed that in salt-treated plants, the anther tapetum retained most of the toxic ions and that the poor viability of the pollen should be ascribed to an impact on pollen maturation as a result of tapetum altered metabolism rather than to a direct accumulation of toxic ions (Ghanem et al. 2009). It is noteworthy, however, that in the present case, NaCl was by far more detrimental to pollen viability than Na_2SO_4. Since the ion content was not estimated on pollen, it is not possible to ascribe pollen viability decrease to ion accumulation. At the whole grain level, Na^+ accumulation was higher in NaCl- than in Na_2SO_4-treated pollen, but these observations valid for grain at the end of the maturation period is not a direct proof that Na^+ previously accumulated to a higher extent in pollen grain. Some authors however mentioned that Cl^- efflux is a crucial component for pollen tube growth (Zonia et al.2002). It could therefore be hypothesized that a modification in Cl^- occurring as a result of NaCl exposure but not in response to Na_2SO_4 may be deleterious for pollen viability. It has also to be mentioned that difference between the two salt treatments for pollen viability was estimated around 8% while difference for global florets fertility was 15%, thus suggesting that other components may be responsible for flower sterility. Khatun & Flowers (1995) reported that salinity may also induce alteration of stigma receptivity and additional localization of ions in the various flower organs should allow us to gain additional information on the underlying causes of salt-induced flower sterility. Beside the ionic component, the osmotic component of salt stress may also have a deleterious impact on flowering and yield-related parameters (Castillo et al., 2007), although this could hardly explain recorded differences between NaCl and Na_2SO_4 which are used in concentrations inducing comparable osmotic potentials in the nutrient solution.

Salinity induced a strong decrease in photosynthesis at both the vegetative and the flowering stage. The recorded decrease in A_N appeared similar for NaCl- and Na_2SO_4-treated plants and a salt-induced inhibition of

photosynthesis is commonly attributed to stomatal and to non-stomatal causes (Moradi & Ismail, 2007). At the seedling stage, NaCl and Na_2SO_4 had different impact on stomatal conductance suggesting that the recorded decrease in A_N recorded at this stage may be due to non-stomatal causes. According to Sultana et al. (1999), reduction of photosynthesis in salt-treated rice depend not only on a reduction of available CO_2 by stomatal closure, but also on the cumulative effect of biochemical constituents such as photosynthetic pigments, soluble carbohydrate and protein. This results in a low concentration of assimilates in the leaves, and poor translocation of assimilates from the source reduces grain growth which is especially sensitive at the milking stage. Regarding the Na_2SO_4-treated plants at seedling stage, we observed that the decrease in instantaneous transpiration rate (E) was not accompanied by the decrease in stomatal conductance (g_s). The reduction in E values should be linked to osmotic stress which decreases the amount of water entering the plant, and in this case, the stomatal conductance should not necessarily be affected since stomatal aperture also facilitates gaseous exchanges in addition to water-transpiration regulation. This could be further supported by the fact that the intercellular CO_2 concentration was not affected by the salt stress. At the vegetative stage, all photosynthetic pigments were more sensitive to NaCl than to Na_2SO_4 and this could be related to the fact than in IKP, Na^+ accumulation was shown to be higher in the former than in the latter case (Irakoze et al. 2019).

Imaizumi et al. (1990) demonstrated that the leaves (mainly flag leaves) are not the only contributor to grain filling. According to these authors, rice panicle itself (rachis-branches and spikelets) exhibit a photosynthetic activity contributing to grain filling and especially spikelets have a high photosynthetic capability which is similar to that of the flag leaf. In the present work, we demonstrated that majority of the translocated Na^+, Cl^- and S^{6+} present in the spikelets accumulated in the hulls. The comparison of chapters 1 and 2 showed that the salt ions accumulation in plant organs was as high at the vegetative as at reproductive stages. The accumulation of Na^+ ion was significantly higher in the grain hull and grain of NaCl- than Na_2SO_4-treated plants. Moreover, the accumulation of Cl^- anion was five times and two times higher than that of sulfur in grain hull and in grain respectively. If spikelets photosynthesis contributes to grain filling, an

important part of assimilates should be produced in the hulls. Ion accumulation at this level may be explained by the fact that these floral organs exhibit a xylem connection while phloem is involved in ion translocation to the endosperm. The hulls also present a high density of stomata, which contributes to transpiration and allows CO_2 fixation. Our data provided clear evidence that in NaCl-treated plants, more than 90% of the translocated Na^+ was accumulated in the hulls and the proportion was even higher for Cl^-. A similar observation is valid for Na^+ and S^{6+} in Na_2SO_4-treated plants. Such a high concentration of ion may impact spikelets photosynthesis and at least contribute to the decrease in grain weight. Hull may serve as a sink protecting young embryo and maturing endosperm from accumulation of toxic ions, as previously demonstrated for arsenic (Vromman et al., 2013). However, when the ratio of concentration hull/grain is calculated for Na^+ and Cl^-, no significant differences was observed for Na^+ between control and stressed plants (ratio from 8.0 to 14.9) as well as for Cl- (ratio of 29.8 in control and 28 in stressed plants), suggesting that processes operating in sodium and chloride concentration in the hulls are already operating in unstressed plants.

Negative impacts of salinity on flag leaf photosynthesis, number of spikelets per panicle and mean 1000-grain weight was similar for NaCl and Na_2SO_4. Although the number of spikelets per panicle decreased in salt-treated plants, salt impact on flag leaf photosynthesis was by far higher, and the decrease in sink organs could thus not compensate for photosynthesis inhibition. According to Abdullah et al. (2001) starch synthase activity (α1-4-glucan glucosyl transferase) in developing grain is inhibited by salinity and this could partly explain the salt-induced decrease in the mean 1000-grain weight. Moderate to high salinities may reduce amylose content and induce numerous functional changes in relation to pasting properties and gel consistency. Thitisaksakul et al. (2015) interestingly reported that salt-induced modification of enzyme activities during the grain filling is a function of timing and that the recorded effects are not the same for plants exposed to salinity since the seedling stage or since anthesis. According to this study, rice exposed for long period to low level of salt may even show adaptative responses to prolonged salt treatment and compensate a decrease in seed fertility by an increase in protein content.

Conclusion

In conclusion, NaCl appeared more toxic than Na_2SO_4 for numerous recorded photosynthetic parameters and yield components. The moderate toxicity recorded for Na_2SO_4 could be due to its low concentration and activity rates in culture solution as well as to the low mobility of the SO_4^{2-} anion inside the plant. Besides the salinity stress type, the plant stage and the stress duration also have an influence on photosynthetic and flowering parameters. Salt-induced yield decrease in rice could be attributed to both an inhibition of photosynthesis in leaves leading to a reduced translocation of assimilates toward the grain, and to a deleterious impact of salinity on the number and the efficiency of reproductive structures.

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Interchapter II – III

What do we know?

Chapter 2 confirmed the highest toxicity of chloride salinity comparatively to sulfate toxicity at the reproductive stage as previously reported for the vegetative seedling stage, but also affords a supplemental set of data in relation to the plant photosynthetic behavior. Moreover, Chapter 2 also demonstrated that although it is commonly considered as the least salt-sensitive stage in rice, tillering was also affected by the used doses of salt. This is especially the case for net photosynthesis and for stomatal conductance. Flag leaf itself is also deeply affected in salt-treated plants and this is a matter of concern since panicular leaf produces a high proportion of sugar and energy for subsequent flowering process and grain filling. Some deleterious effects of salt on yield-related parameters may also be explained by earlier events occurring before the reproductive stage: this is especially the case for the mean number of tillers per plant and for the number of spikelets on the main panicle since this last parameter is determined at the panicle initiation stage occurring a long time before booting and heading stage.

What is our question?

Yield-related parameters represent a long-term effect of salt stress and could be regarded as the ultimate consequence of a sequence of events occurring during the whole cycle of the plant for species, such as rice, which encounter a monocarpic senescence process.

Plant response to salt stress, however, occurs quite earlier: salinity sensing and signaling occur very quickly and they rapidly lead to modification in gene expression. Such modification may be regarded as a very important step since it will strongly influence the following sequence of biochemical events determining the subsequent morphological, physiological and agronomical response of salt-treated plants.

Hence, in the following chapter, our main question will be to compare NaCl and Na_2SO_4 impacts on gene expression in order to identify and to compare the "up-" and "down"-regulated genes in roots and shoots of rice plant.

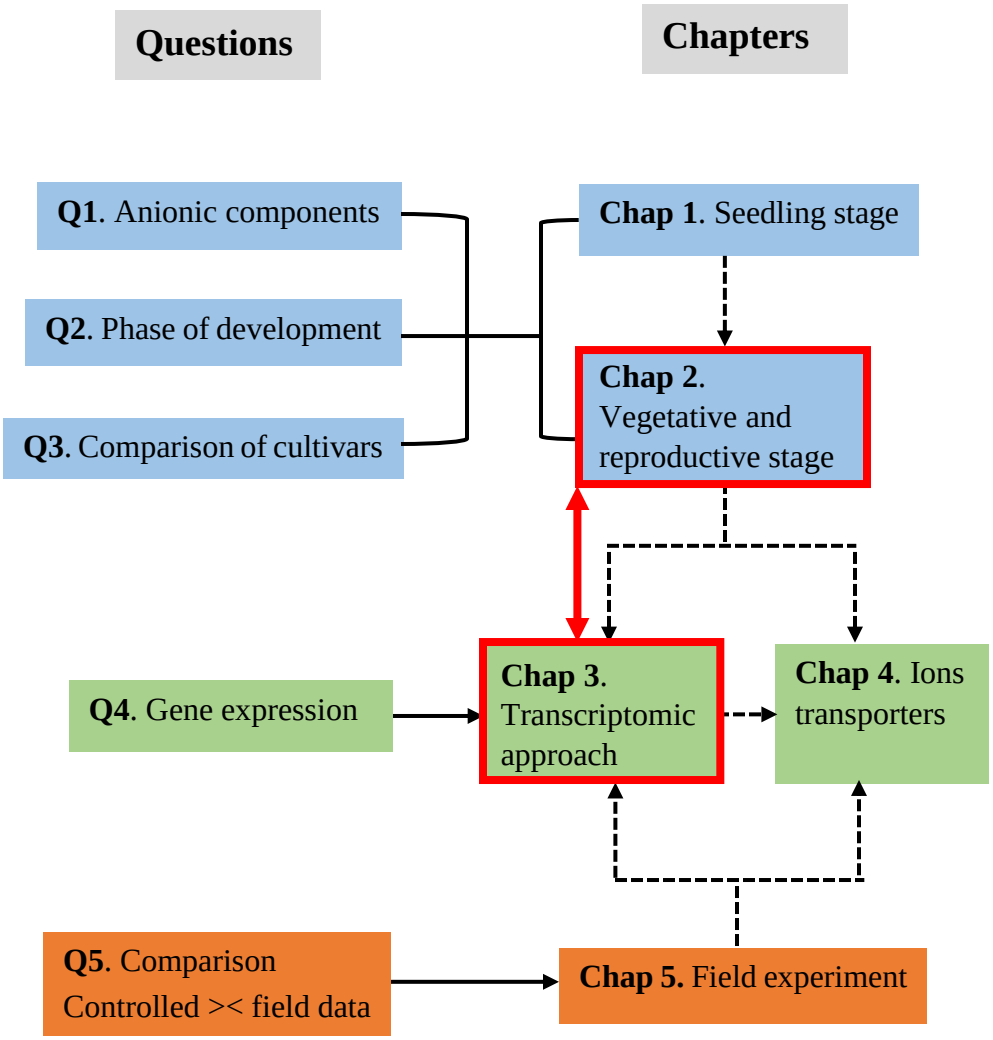
What is our strategy?

Although we already identified in Chapters I and II precise targets differently influenced by distinct types of salt, we do not have any exclusive at this stage regarding the genes to consider. Indeed, some of the analyzed properties are controlled by a huge number of genes while other genes of interest may impact some properties that we did not analyze in the previous chapters. We thus decided to perform a holistic microarray approach taking advantage of tools available for rice. This analyze will be performed at the transcriptomic platform of LIBST Institute in UCLouvain. This type of analyze is however time-consuming and very expensive. Considering our available financial support, it was impossible to conduct such analyze on more than one cultivar and to consider different developmental stages. We thus decided to focus on IKP (which was already considered in Chapter I and II) and to consider the salt-sensitive seedling stage.

In the literature, transcriptomic approaches commonly use short-term exposure to high stress intensities. It may indeed be argued that short-term exposure do not allow to clearly identify the "initial" reaction of stressed plants. The development of plant modify in a complex way the hormonal compounds which may strongly impact the expression of the genes of interest independently of the considered stress. On the other hand, even minor modifications in the environmental conditions (such as small variation of temperature or light intensities which may occur in our semi-controlled conditions) may interfere with the expression of genes putatively involved in stress response.

For all these reasons, we decided to limit the duration of stress exposure to 48h (2 days rather than 2 weeks in Chapter I). This choice however brings an additional problem in the specific case of salinity. In salt-treated plants, genes may be triggered by toxic ions accumulated inside the tissues. A short term of exposure of a few hours does not necessarily allow these ions

to reach toxic levels within the plant's organ. We thus decided to increase the external ion concentration (200 mM NaCl and 100 mM Na_2SO_4) to increase ion concentrations in the tissues, even after a short term exposure. However, by increasing external ion concentration, we also increased the osmotic component of the applied salt stress. Beside gene expression, it is thus necessary to monitor internal accumulation of ions and osmotic potential in relation to accumulation of osmotic compatible solutes putatively involved in osmotic adjustment (proline and soluble sugars in the case of rice).



Synthetic overview of the five research questions addressed during our thesis project and the specific source of their answers in the different chapters of the thesis.

Chapter III. Differential effects of sulfate and chloride salinities on rice (*Oryza sativa* L.) gene expression patterns: a comparative transcriptomic approach

Submitted in Plant Science

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Abstract

Salinity is among the most serious challenges to rice production in the word today. To elucidate genome-level responses to chloride or sulfate salinity stress in rice, I Kong Pao seedlings have been exposed to salt stress (EC of *c.a* 20 dS m^{-1} NaCl or Na_2SO_4) for 48 hours. A combined transcriptomic and physiological study has been performed after exposure to saline solutions in order to clear the main metabolic pathways involved in rice response to salt stress. Our results showed that contrasting genes were expressed under sulfate than chloride salinity, the difference being the most remarkable in root. Most of the genes involved in response to salt stress were up-regulated in Na_2SO_4-treated plants whatever the considered organs while more genes were down-regulated in NaCl-treated plants. The comparison of expression profiles among different salinity stresses showed largely stress-specific patterns of regulation. Moreover, both stresses appeared to alter the expression patterns of a significant number of genes involved in response to abiotic stimulus, signal transduction and ion transport in a largely organ-specific manner. Modification in some gene expression could be followed by modification in corresponding metabolic products and physiological properties. Our study offered the first comprehensive picture of genome expression modulation in response to sulfate salinity stress in two distinct rice organs and could be very useful in improving of rice plant tolerance to Na_2SO_4 stress.

Keys words: chloride, sulfate, stress, rice, transcriptomic and physiological studies.

Introduction

Saline soils are among the most serious challenges to crop production in the word today [1]. This is particularly the case in developing countries, where this environmental constraint severely limits plant growth and reduces crop production. Based on current population growth rate in developing countries, there is a great urgency to address this problem of salinity. Soil salinization is related either to geological reasons but also to secondary salinization resulting from human activities in relation to inadequate irrigation practices coupled with poor drainage and the practice of soil fertilization [2]. Each year, a large part of arable lands is destroyed by salt accumulation and is accentuated by climate change: it is estimated that by 2050, half of the word's arable lands will suffer from salinity [3.4]. All soils exhibiting an electrical conductivity higher than 4 $dS.m^{-1}$ should be regarded as saline but this definition does not impose restriction regarding the nature of soluble salts present in the soil. Beside NaCl, which is predominant in most areas, salinity may also result from the accumulation of Na_2SO_4 in numerous areas, leading to dispersion of soil particle and damaging soil structure by an increase in the compactness of soils [3,5]. In contrast to NaCl, the physiological impact of Na_2SO_4 salinity on plants remains poorly documented.

Salt stress reduce plant productivity by inhibiting a whole lot of metabolic activities [6]. A high accumulation of salt ions in soil solution hinders plant water uptake, inducing an osmotic component of salt stress while the progressive build-up of toxic ions is responsible of an ionic component of salt stress. Secondary stresses, such as oxidative stress, are induced as a consequence of both components [7,8].

Salinity-tolerant plants adopt effective mechanisms to reduce stress damage. Salt tolerant plants may exclude sodium, or compartmentalize it within vacuoles, apoplasm or trichomes [9]. Synthesis and accumulation of organic solutes known as osmolytes is also a good strategy to maintain metabolic activities at cellular level. The osmoprotectants like proline, quaternary ammonium compounds or sugars protect proteins and membranes against damage resulting from high concentration of inorganic ions and partial desiccation [10]. Some osmoprotectants also protect the

metabolic machinery against oxidative damage. Omics studies proved that salt tolerance is controlled by interactions between several independently regulated genes coordinated by temporally and spatially processes [11-13].

To gain access to new knowledge related to gene function and the regulatory mechanisms of biological processes that are associated with salt-stress responses, several techniques were used to identify plant genes involved in salt-stress tolerance. The previous studies showed that the molecular control mechanisms of salinity tolerance was based on the expression of salt-related-genes: (i) Genes involved in signaling cascade and in transcriptional control such as MyC, MAP kinases and SOS Kinases [14-16], phospholipases [17,18] and transcriptional factors such as HSF, CBF/DREB, ABF/ABAE families, MYB, ABRE and bZIP domain [19]; (ii) Genes that function directly in the protection of membranes and proteins such as heat-shock proteins (HSPs) and chaperones, late embryogenesis abundant (LEA) proteins [20] and osmoprotectants [21]; (iii) Genes that are involved in water and ions uptake and transport such as aquaporins and ions transporters [19].

The rice crop is very sensitive to salinity, more particularly at the young seedling and reproductive stages [22,23]; this complicates the identification of rice genes involved in salt-stress resistance. Improvement of salinity resistance in *Oryza sativa* thus appears as a priority, since improvement of irrigation management practices is difficult to implement on a large scale and often uneconomical, especially for developing countries. The salt-resistance is often genotype-dependent and even if the genus *Oryza* does not comprises halophyte, wild species have also been recently used to identify key genes contributing to an improved regulation of ion homeostasis [24].

Applied research on gene expression patterns of the plants growing in hostile environment has evolved rapidly after full genomic sequencing of Arabidopsis and rice (*Oryza sativa*), representing model systems for dicot and monocot plants, respectively. The cDNA microarrays offer a high-throughput approach to obtain comprehensive gene expression profiles [25-27]. In rice, the salinity stress response has been analyzed with microarray containing 1,728 cDNA clones from a root cDNA library of

salt-tolerant rice Pokkali [11]. In another study, Dai Yin et al. [28] have used cDNA microarrays containing ~9,000 unigenes to identify 486 salt responsive expressed sequence tags (ESTs) in shoots of the highly salt-tolerant rice variety, Nona Bokra. For the same purpose, an Affymetrix rice genome array containing 55,515 probe sets was used to profile the transcriptome of rice strains with salt-tolerant and salt-sensitive genotypes, revealing genome-wide differential transcription under salinity treatments during vegetative growth [29].

Some studies however focused on one single type of organ, considering either roots [30, 31] or leaves [29,32]. While patterns of gene expression in response to drought or high-salinity stress within a particular organ type showed significant overlap, comparison of expression profiles among different organs showed largely organ-specific patterns of regulation and both stresses appear to alter the expression of genes involved in transcription and cell signaling in a partly organ-specific manner [33]. Nevertheless, plants are complex organisms: root and shoot do not behave as independent organs and it has been widely demonstrated that root-to-shoot signaling implying hormonal compounds assume important functions in a coordinated response to salt stress [34]. This complexity imposes to consider both types of organ in the framework of the same experiment. Moreover, to the best of our knowledge, all transcriptomic approaches performed until now on salt-treated rice considered NaCl and no data are available for plants exposed to Na_2SO_4 treatments. Although recent studies provided useful information regarding the comparison between desiccation and salt stress [35], information is crucially lacking regarding the impact of the ionic component of salinity on gene expression.

Sodium sulfate was shown to be more toxic than NaCl in potato [36], wheat [37], tall fescue [38] and *Brassica rapa* [39]. In rice, however, a recent study demonstrated that NaCl was more toxic than Na_2SO_4 at the seedling stage, leading to a higher accumulation of Na^+ and proline in the salt-sensitive cultivar IKP while Na_2SO_4 induced a higher antioxidant activity in the salt-resistant genotype Pokkali which was able to more efficiently recycle oxidized glutathione (GSSG) to reduced form (GSH) [40]. An additional work confirms that yield-related parameters such as tiller

number, spikelet fertility and pollen viability were more affected by NaCl than by Na_2SO_4 in IKP rice cultivar (Irakoze, unpublished).

In this study, we compared, for the first time to the best of our knowledge, transcriptional profiling of I Kong Pao (IKP) genotype under NaCl and Na_2SO_4 stresses. The plants have been exposed to iso-strength Na^+ nutrient solutions at the young seedling stage, which is commonly considered as one of the most salt-sensitive developmental stage in *Oryza sativa* [22]. We intended to highlight the regulatory mechanisms adopted by the rice plant exposed to sulfate salt stress. The modification of the metabolic process and physiological properties of the rice plant according to the expression profile of the corresponding genes was also analyzed.

Materials and methods

Plant culture and growing conditions

Seeds of the rice cultivar I Kong Pao (IKP) were obtained from IRRI (International Rice Research Institute; Philippines). Seeds were germinated on two layers of filter paper (Whatman no. 2) moistened with 10 ml of sterile deionized water in a growth chamber at 25°C under a 12 h daylight period (120 µmol m^{-2} s^{-1}). Ten-day-old seedlings were transferred into a phytotron and fixed on polystyrene plates floating on Yoshida nutritive solution [41]. Illumination was provided by Sylvania fluorescent tubes (F96T12/CW/VHO) for 12 h d^{-1} at a photon flux density (PAR) of 300 µmol m^{-2} s^{-1}. Daytime humidity was between 60% and 80%, and the temperature was maintained at 29°C during the day and 26°C during the night. For each treatment, seedlings were distributed among five pots (six seedlings per pot) containing 1.3 l of solution. The solutions were renewed weekly and pots were randomly rearranged in the phytotron. After two weeks of acclimatization in control conditions, seedlings were exposed for 48 hours to 200 mM NaCl or 100 mM Na_2SO_4 (electrical conductivity of *c.a.* 20 dS m^{-1} for both solutions). The control treatment solution has not been salinized.

The pH values recorded in the culture solutions salinized with 100 mM Na_2SO_4 and 200 mM NaCl were 6.46 and 6.02 respectively. In addition, a chemical speciation of the culture solutions salinized with NaCl and Na_2SO_4 was performed using Visual Minteq software. The identified species are presented in appendix 2 of this document. The concentration and activity rates of Na^+, Cl^- and SO_4^{2-} are presented in results section.

Estimation of ion and water content, dry weight and osmotic potential

At the end of the experiment, 10 plants per treatment were harvested. Roots were rinsed for 30 s in deionized water to remove ions from the free spaces. Roots and shoots were separated and weighed. After drying the samples at 70°C during 72 hours in an oven, dry weight (DW) was estimated. The water content (WC) was calculated as: WC= [(Fesh Weight-Dry Weight)/Fresh Weight] x100.

For shoots and roots, 20 mg DW were 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 Whatman paper (85 mm, Grade 1). The filtrate was used to determine the cations concentration (K^+ and Na^+) by flame emission using Atomic Absorption Spectrometer (Thermo scientific S series model AAS4) and anions (sulfur) were quantified by liquid chromatography (HPLC-Dionex ICS2000). 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 x *g* for 10 min at 25°C was subsequently filtered and then quantified via liquid chromatography (HPLC-Dionex ICS2000).

For the determination of osmotic potential (Ψ_S), the last fully expanded leaf and the roots were removed from the plant and cut in small segments. The samples were then separately introduced in Eppendorf tubes perforated with four small holes, frozen in liquid nitrogen for 30 s and warmed again at room temperature (three cycles of gel/thaw) in order to break the cellular membrane [42]. Tubes were then encased in the second intact Eppendorf tubes and centrifuged at 8.000 x *g* during 15 min at 4°C. The osmolarity of collected tissular sap was assessed with the Wescor vapor pressure osmometer and converted to Ψ_S according to Van't Hoff equation.

Quantification of proline and soluble sugars

For proline analysis [43], 200 mg FW of shoots were frozen in liquid nitrogen, ground in pre-chilled mortar, homogenized in 3 ml of sulfosalicylic acid and then centrifuged at 5.000 x *g* for 15 min at ambient temperature. An aliquot of 1 ml of ninhydrin solution (2.5g of ninhydrin dissolved in a mixture of 60 ml glacial acetic acid and 40 ml 6 M phosphoric acid) and 1 ml of concentrated acetic acid were added to the supernatant and the mixture was heated one hour at 90°C before stopping the reaction in the ice. 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 with a spectrophotometer (Beckmann DU640) at 520 nm using toluene as a blank. The proline concentration was determined from standard curve.

For sugar detection and quantification, 500 mg FW of shoots were frozen in liquid nitrogen and ground in a pre-chilled mortar. An aliquot of 4 ml of ethanol (70%) was added to the homogeneous powder and the mixture deposited on ice for 5 min. After three successive centrifugations at 8.000x g, the supernatants were collected, put together and dried in a thermomixer using nitrogen (45 min at 50°C). Sugars were derivatized during 30 minutes at 65°C using 200 µl of an oximation solution (hydroxylamine chlorhydrate dissolved in a pyridine solution). 100 µl of hexamethyldisilazane (HMDS) and 10 µl of trifluoroacetic acid (TFA) were added to the samples. Monosaccharide and disaccharide concentrations were specifically determined by gas chromatography (GC-MS Thermo Scientific Trace 1310-iSQ QD, RXi®-5 SilMS-Restek-30 m x 0.25 mm i.d x 0.25 µm; USA) using helium as a carrier gas at a flow rate of $1ml.m^{-1}$. The injector and FID detector temperatures were 270° and 310° respectively. Oven temperature was held at 120°C for 3 min and then programmed to 230°C at $3°C.min^{-1}$. It was kept at this temperature for 12 min, then re-programmed to 270°C at $20°C.min^{-1}$ and held for 15 min.

RNA Isolation, Microarray and data analysis

Shoot and root of IKP were separated and immediately frozen in liquid nitrogen. Total RNA was isolated using the RNeasy Plant Mini Kit (Quiagen, Germany). The RNA quality and concentration were assessed using NanoDrop ND-1000 (Isogen Life Science, De Meern, The Netherlands) and the Bioanalyzer Agilent 2100 (Agilent Technologies).

Microarray analysis was performed according to Agilent Oligo DNA Microarray Hybridization protocols using the Agilent 44 K Rice Oligo DNA Microarray RAP-DB (Agilent Technologies, USA; G2519F#15241) (Project 2008) with two biological replicates. 10 µg of total RNA were used for the synthesis of fluorescently labeled cDNA via the Superscript Plus Indirect cDNA Labeling System (Invitrogen, Paisley,UK) [44].

Regarding the two-color hybridization, 0.7 µg of Alexa-555-labeled cDNA and 0.7 µg of Alexa-647-labeled cDNA were both used, and the hybridization mix was prepared with HI-RPM hybridization solution (Agilent Technologies). For each organ, three comparisons were made: gene expression level under NaCl stress compared to gene expression level in control plant taken as a reference (Chl), gene expression level under Na_2SO_4 stress compared to gene expression level in control plant taken as a reference (S) and gene expression level under Na_2SO_4 stress compared to gene expression level in plant stressed by chloride taken as a reference (S/Chl). Each comparison was repeated twice. Hybridization for 16h at 65°C in a rotating hybridization oven, and washings in GE solutions wash1 and pre-warmed wash2 were performed according to the manufacturer's protocols. The hybridized slides were scanned using an Agilent G2565B microarray scanner system (Agilent Technologies) and signal intensities were extracted by Feature Extraction software (Agilent Technologies). Gene expression analysis was performed using Gene spring GXv11.0 software (Agilent Technologies). Statistically significant differentially expressed genes were identified via a combined criterion of a fold change of 2.5 or greater and a P value <0.05 in the t-tests.

The gene function were categorized based on RiceNetDB (http://bis.zju.edu.cn/ricenetdb/) and Rice Annotation Project Data Base (RAP-DB: http://rapdb.dna.affrc.go.jp/).

Quantitative real-time PCR (qPCR)

Total RNA was isolated from 100 mg of shoots and roots using the RNeasy Plant Mini Kit (Quiagen, Germany). The potential contaminating genomic DNA was treated with DNase (Promega, Madison, USA). Reverse transcription was performed with 1 µg of total RNA using RevertAid Minus First Strand cDNA Synthesis Kit (Thermo Scientif, USA) following the manufacturer's recommendation. Each fragment was then amplified by PCR in triplicates in an ABI Prism 7000 SDS (Applied Biosystems, Carlsbad, CA, USA) using GoTaq® qPCR Master Mix (Promega, Madison, USA), and gene-specific primers are listed in table S1. The relative gene expression was evaluated using the comparative cycle threshold method [45] and actin was used as reference gene.

Statistical analysis

Each experiment was repeated three times and led to statistical similar results. Normality tests were performed and no further transformation of the raw data was required. An analysis of variance (ANOVA) was performed on all data set using JMP Pro 14 software, variance analysis with one factor (stress type) or with two crossed fixed factors (stress type x organ type).

Results

The pH values recorded in this experiment were within the acceptable range for good plant growth (5.5-6.5). These pH values were higher than the pH values recorded in the first two chapters. As in the first two chapters, the pH values were relatively higher in Na_2SO_4- than in NaCl-treated solutions.

Considering that the identified species did not vary depending on the stress intensity, the data presented in appendix 2 still valid for all studies conducted in hydroponic culture. Regarding NaCl stress, the concentration and activity rates of Na^+ in culture solution were 94.86% and 70.77% respectively. The concentration and activity rates of Cl^- were 95% and 70.85% respectively. In Na_2SO_4-treated solutions, the concentration and activity rates of Na^+ were 88.7% and 65.35% respectively while the concentration and activity rates of SO_4^{2-} were 76.33% and 22.48% respectively. Compared to the results in Chapter I and II, the concentration and activity rates significantly decreased in all treatments and more particularly in Na_2SO_4-treated solutions.

Physiological parameters

As shown on Fig. 1A, the DW was significantly reduced in shoots and roots (± 50%) of IKP seedlings exposed to chloride or sulfate stress comparatively to controls and no significant difference was recorded between the two types of salinity. NaCl stress reduced the shoot and root water content comparatively to controls, while seedlings exposed to Na_2SO_4 stress exhibited an intermediate behavior (Fig. 1B). As recorded in controls, the water content was higher in the root than in the shoot in salt treatments.

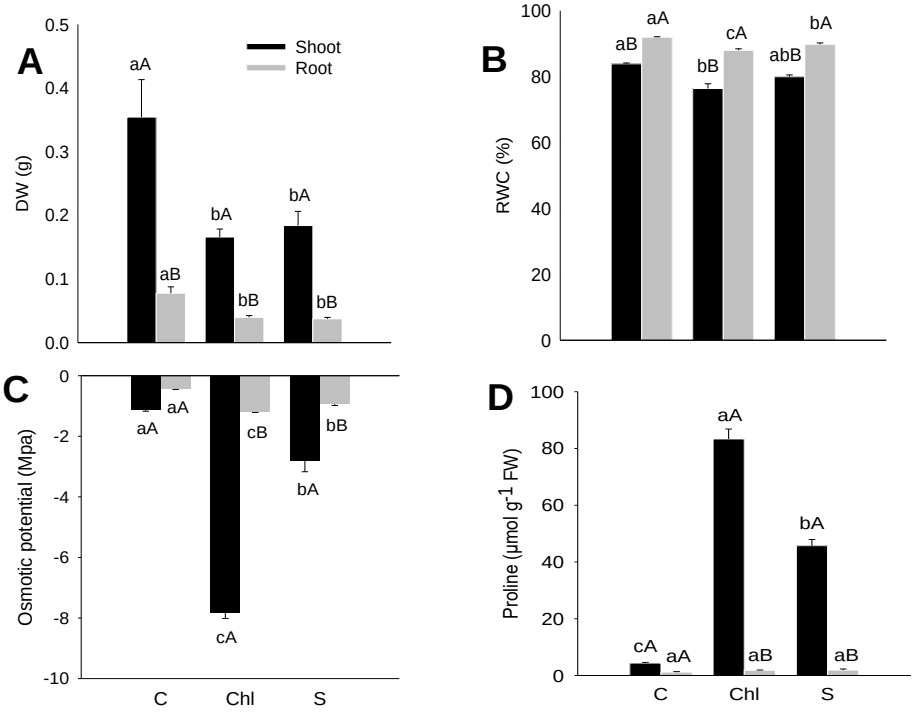

Figure 1. Dry weight (A), water content (B), osmotic potential (C) and proline concentration (D) as a result of 48h exposure to two different salt stress: control (C), NaCl 200 mM (Chl) and Na_2SO_4 100 mM (S). Each value is the mean of three replicates per treatment and vertical bar is standard error of mean. Treatments followed by the same lower-case letter for a particular plant organ do not differ statistically. Plant organs followed by the same upper-case letter in a particular treatment do not differ statistically.

The Fig. 1C shows that chloride salinity strongly decreased the shoot and the root Ψs comparatively to controls while Na_2SO_4-induced decrease in Ψs was by far more limited. The Ψs was significantly higher in root than in shoot for salt treatments. Comparatively to control, the accumulation of proline in the shoot drastically increased in response to NaCl salinity while Na_2SO_4 salinity still had an intermediate effect (Fig 1. D). The two types of salinity had no impact on root proline accumulation.

To assess the comparative effect of the two types of salt stress on sugar accumulation, we analyzed the concentration of sucrose, glucose, fructose, arabinose, xylose, mannitol, galactose, myo-inositol, stachyose, trehalose and raffinose in the shoot and root. As evidenced by Table. 1, the sucrose concentration significantly increased in shoot under sulfate stress comparatively to controls while a more limited impact was recorded for NaCl stress. In contrast, root sucrose concentration significantly increased under NaCl stress while no effect was detected under Na_2SO_4 stress. Sucrose concentration was higher in shoot than in root in all treatments. Free glucose was hardly detected in the shoot of control plants. The glucose concentration was higher under chloride than sulfate stress and glucose was not detected in roots whatever the treatments. The chloride stress significantly increased the fructose concentration in shoot comparatively to controls but the recorded increase was lower under sulfate stress. Fructose was not detected in root under salt stress. Arabinose, xylose, mannitol, galactose, myo-inositol, stachyose, trehalose and raffinose remained below the quantification limit, whatever the considered treatment.

As expected, chloride and sulfur significantly accumulated in NaCl- and Na_2SO_4-treated plants, respectively (Fig. 2). Chloride accumulation was higher in root than in shoot while no difference was observed between the two organs for sulfur accumulation. The two types of salinity induced a strong increase in shoot and root Na^+ content. However, Na^+ accumulation was higher in root under NaCl than Na_2SO_4 stress while no significant difference was recorded for shoot Na^+. For both stresses, Na^+ accumulation was higher in root than in shoot. Salt stress had no effect on K^+ accumulation in shoot comparatively to controls. In contrast, NaCl and Na_2SO_4 stresses significantly reduced K^+ accumulation in root and no significant difference was observed between the two salt stresses.

Table 1. Impact of 48h exposure to NaCl 200 mM (Chl) or Na_2SO_4 100 mM (S) salinities on sugar concentration in rice (*Oryza sativa*; cv; IKP). C, control. ND, not detected.

	µg g^{-1} FW					
	Sucrose		**Glucose**		**Fructose**	
Treatment	Shoot	Root	Shoot	Root	Shoot	Root
C	3561.05±77^{cA}	1051.9±114^{bB}	ND	ND	85.13±11^{c}	807±130
Chl	4376.5±52^{bA}	1651.8±248^{aB}	1557.2±95^{a}	ND	921.9±110^{a}	ND
S	4855.7±166^{aA}	965.8±30.45^{bB}	454.9±16^{b}	ND	636±24^{b}	ND

Each value is the mean of three replicates ±S.E. Treatments followed by the same lower-case letter for a particular plant organ do not differ statistically. Plant organs followed by the same upper-case letter in a particular treatment do not differ statistically.

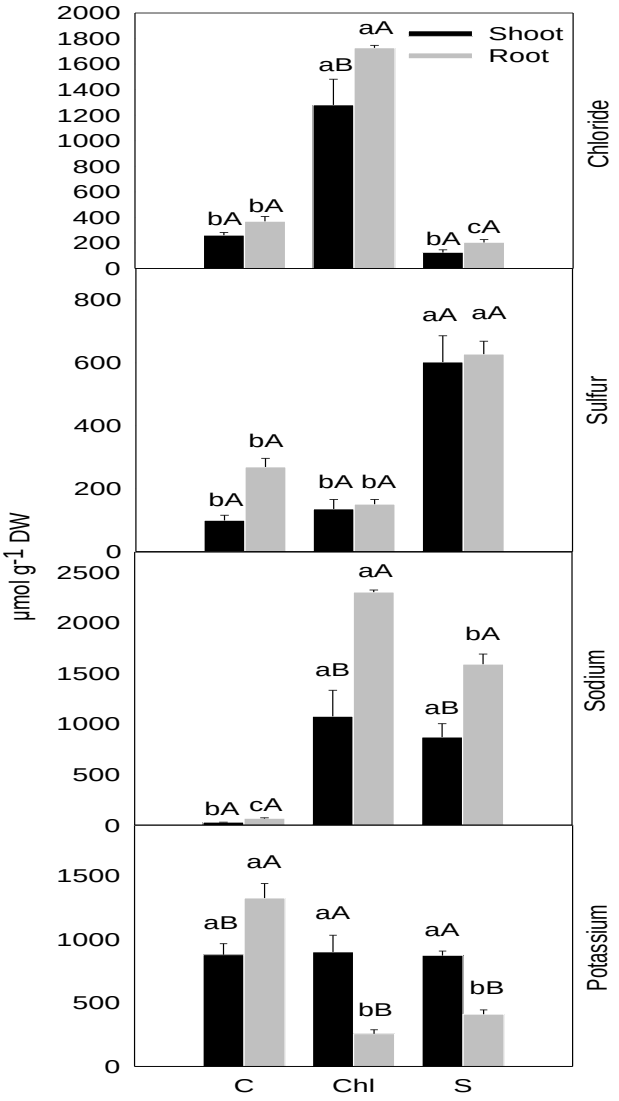

Figure 2. Mineral nutrient concentration in shoot and root of IKP rice cultivar as a result of 48h exposure to two different salt stress: control (C), NaCl 200 mM (Chl) and Na_2SO_4 100 mM (S). Each value is the mean of three replicates per treatment and vertical bar is standard error of mean. Treatments followed by the same lower case letter for a particular plant organ do not differ statistically. Plant organs followed by the same upper case letter in a particular treatment do not differ statistically.

Transcriptome analysis of gene expression

To validate the microarray data, quantitative RT-PCR analysis was used to verify the transcription response of representative genes from microarray results. The four selected genes were expressed in the shoot and root as well as in all treatments: the gene coding for sugar transporter protein (Os10g0360100), the gene coding for Na^+/SO_4^{2-} symporter protein (Os08g0503700), the gene coding for RCI2 protein (Os03g0286900) and the gene coding for serine/threonine protein kinase (Os03g0339900). The expression patterns of the four genes reflected changes observed by microarray analysis in the same proportion but not necessarily with the same expression level (Fig. 3), indicating that our microarray data is reliable.

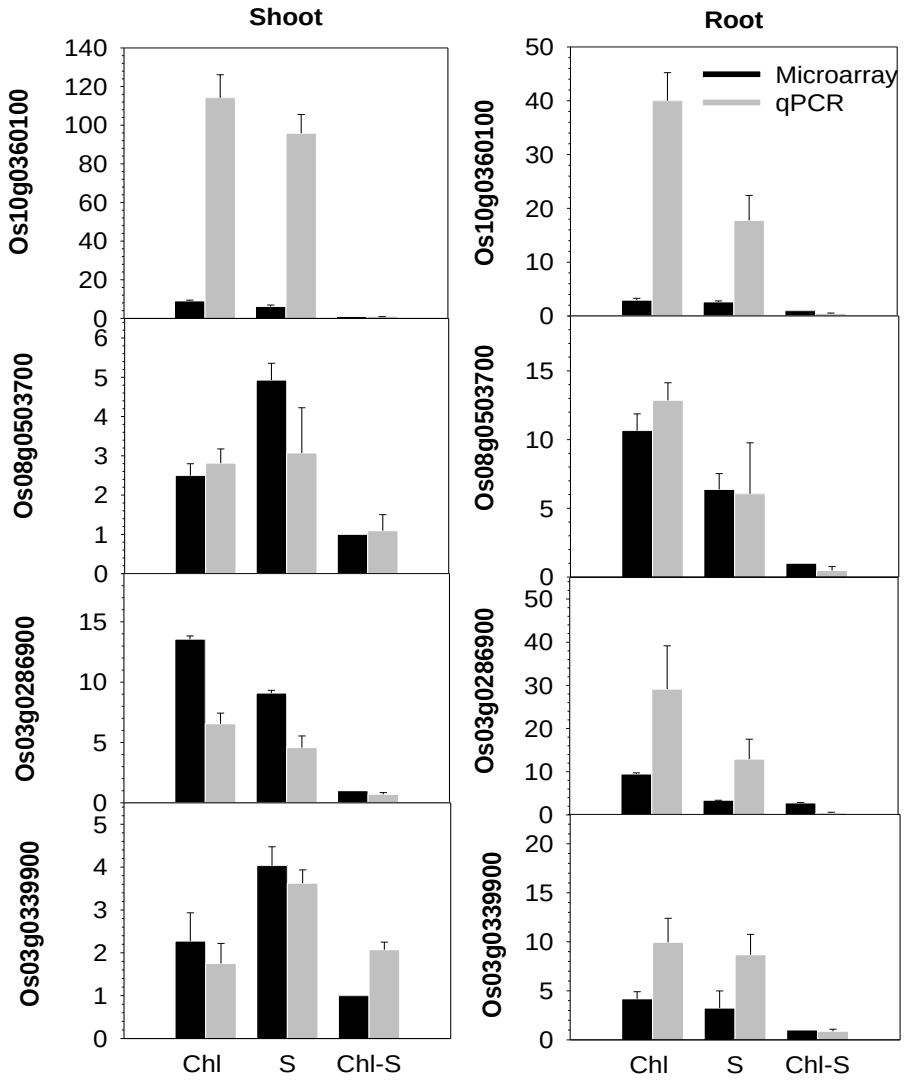

Figure 3. Comparison of gene expression profile by microarray or quantitative RT-PCR analysis. The four genes coding for the rice response to NaCl 200 mM (Chl) and Na_2SO_4 100 mM (S) salinity stresses were analyzed. Quantitative Real Time PCR was performed using gene-specific primers and SYBR Green Real Time Master Mix. The relative gene expression was evaluated using comparative cycle threshold method taking actin as the reference gene.

To analyze the expression profiles of NaCl- or Na_2SO_4-regulated genes in rice, the Agilent 44 K Rice Oligo DNA Microarray, containing 43,735 rice probes were used to identify differentially induced or repressed genes in rice shoots and roots after 48 hours of treatment. The assays were replicated twice on two independent plant cultures.

Following the Gene Ontology enrichment analysis, we categorized the genes in seven biological process groups according to RiceNetDB: photosynthesis, growth, carbohydrate metabolism, response to stress, transport, signal transduction and response to abiotic stimulus (Fig. 4). Their abundance in each group and their differences in regulation were expressed in relation to plant organs and type of salinity stress. Most of the genes affected by chloride or sulfate stress were involved in plant response to stress. In this class, the number of NaCl-induced genes was lower than that induced by sulfate in root. In contrast, the number of down-regulated genes in shoots was higher under Na_2SO_4 than NaCl while an opposite trend was recorded in roots.

A large number of the genes affected by salt stress were also involved in response to abiotic stimulus, signal transduction and transport. Referring to RiceNet Data Base, there is a strong redundancy between genes encoding stress response and those encoding abiotic stress response. Therefore, we found that more than 90% of the genes involved in response to stress were also expressed in response to abiotic stimulus. Regarding the group of genes involved in response to abiotic stimulus, the number of NaCl- or Na_2SO_4-induced genes were similar in shoot and in roots while a number of down-regulated genes was highest in NaCl-treated shoots. Most of genes involved in signal transduction were induced in Na_2SO_4-treated shoots while many genes in this class were down-regulated in NaCl-treated roots. Most genes involved in transport were expressed in root while only few differentially expressed genes from this class were detected in shoot.

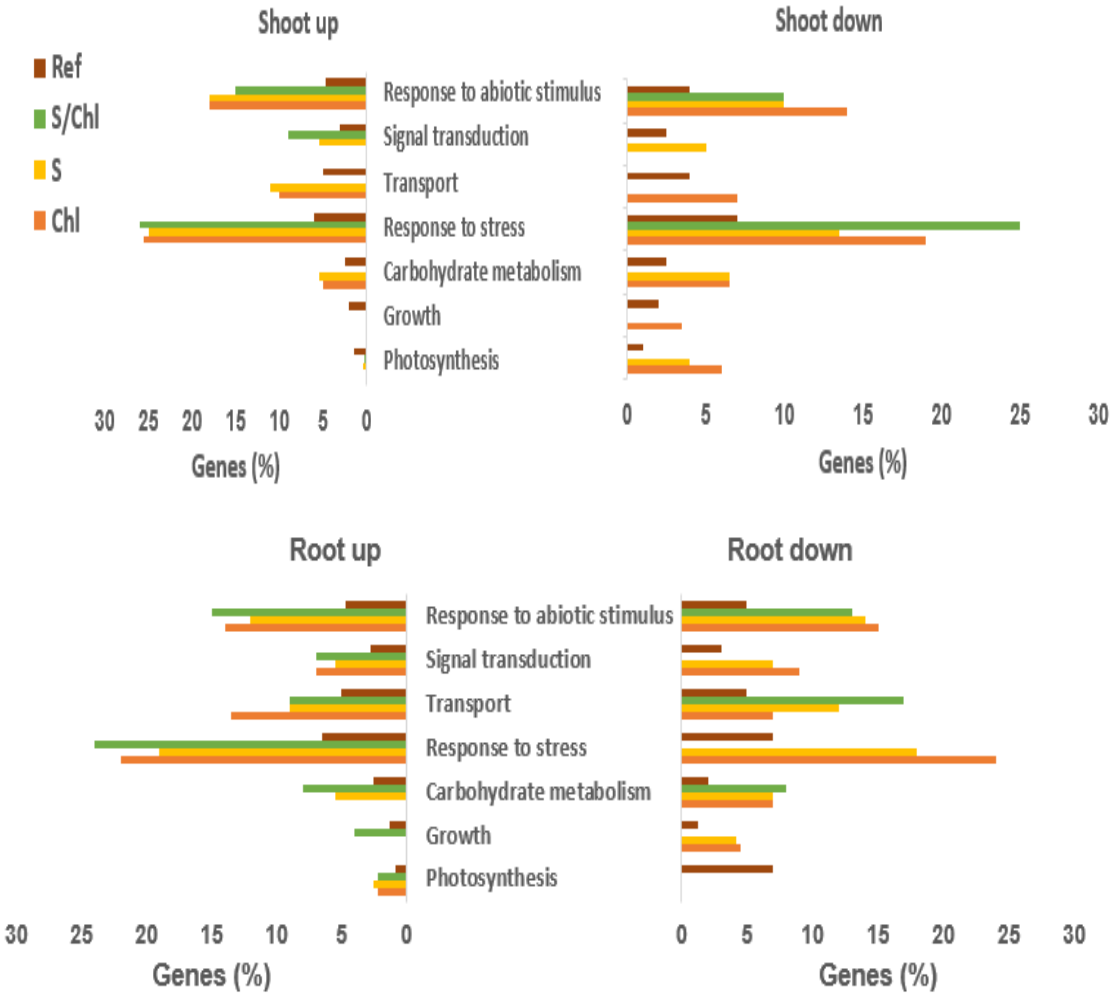

Figure 4. Percentage of up-regulated (left) and down-regulated (right) transcripts observed after NaCl 200 mM (Chl) and Na_2SO_4 100 mM (S) salinity application in shoot (A) and in root (B) of IKP rice cultivar. S/Chl represents the gene expression level under sulfate stress in relation to gene expression level under chloride stress. For a given biological function and considering that the rice plant is not subjected to any stress, Ref represents the ratio between the number of expressed genes coding specifically for this biological function and the total number of rice genes. Transcripts were categorized following RiceNetDB functional catalogue database classification.

Among the seven biological process groups, only the three groups comprising the largest number of expressed genes were retained for further study: genes involved in response to abiotic stimulus, signal transduction and transport. 1745 common genes were identified and selected for further consideration (Fig. 5). Among them, 269 and 354 were induced in shoot in the presence of NaCl and Na_2SO_4 respectively while 200 and 97 were repressed in shoot in the presence of NaCl and Na_2SO_4 respectively. 323 and 368 genes were up-regulated in root under NaCl and Na_2SO_4 respectively while 240 and 327 genes were down-regulated in root under NaCl and Na_2SO_4 respectively. 50.9%, 17.5% and 31.6% of the selected genes were respectively involved in response to abiotic stimulus (Fig. 5A), signal transduction (Fig. 5B) and transport (Fig. 5C). It has to be noticed that only a small portion of genes was shared between the two types of salinity stress. The greatest overlap occurred between the NaCl- and Na_2SO_4-treated roots in case of up-regulated genes involved in response to abiotic stimulus (81 genes). The lowest overlap (1 gene) was observed in many cases as evidenced by the Fig. 5.

As shown in Fig. 6, many genes up or down-regulated by chloride stress exhibited a distinct expression pattern under sulfate stress. The remainder of the chloride response genes exhibited minimal expression changes or a similar expression pattern under sulfate stress. The previous observation was also made between the different plant organs namely shoot and root. In the following presentation of our results, we showed in detail the genes that had displayed a different or similar expression profile depending on the type of stress and plant organ.

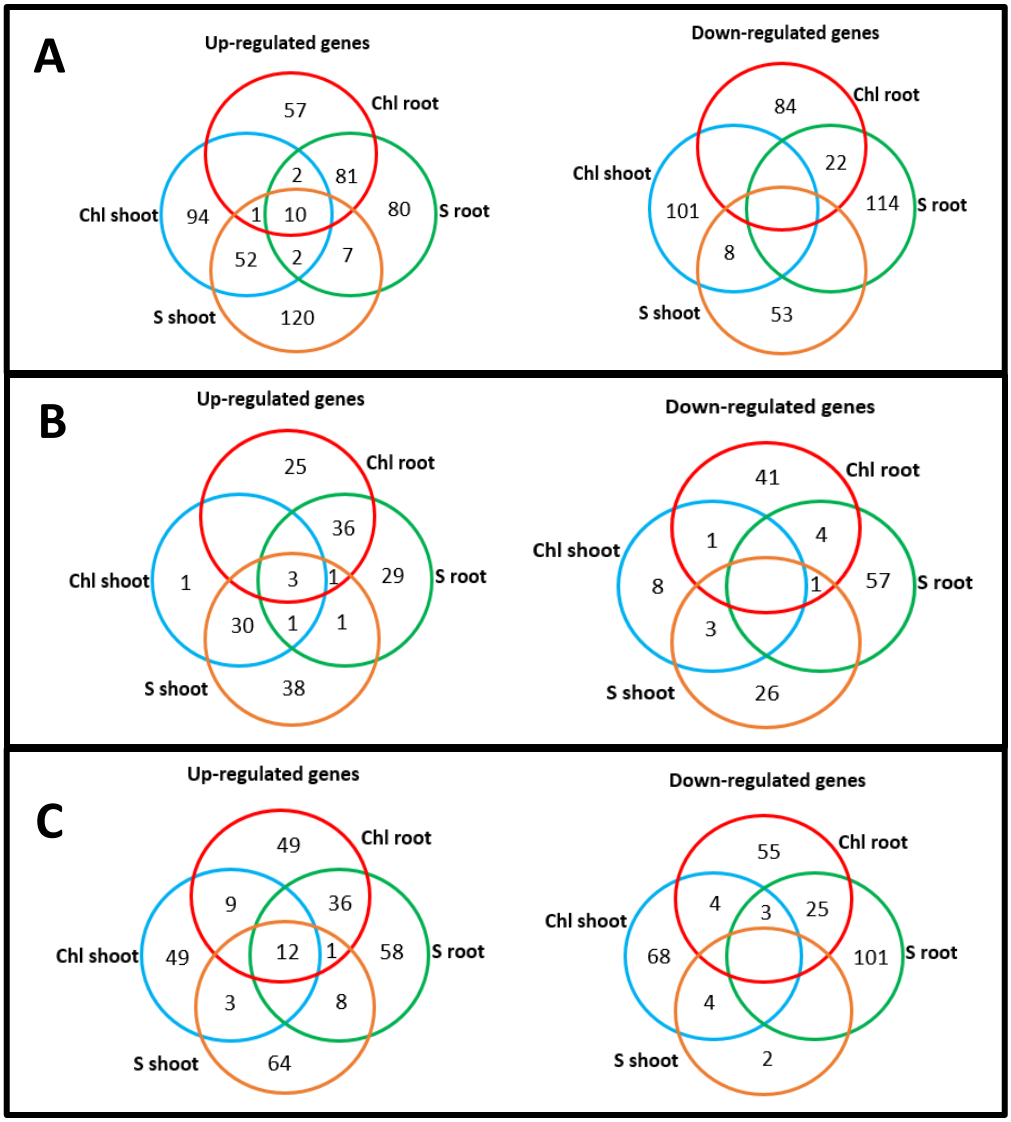

Figure 5. Venn diagrams showing the classification of genes up-regulated or down-regulated by NaCl 200 mM (Chl) or Na_2SO_4 100 mM (S) salinity stresses. A, Expression of the genes involved in response to abiotic stimulus. B, Expression of the genes involved in signal transduction. C, Expression of the genes involved in transport.

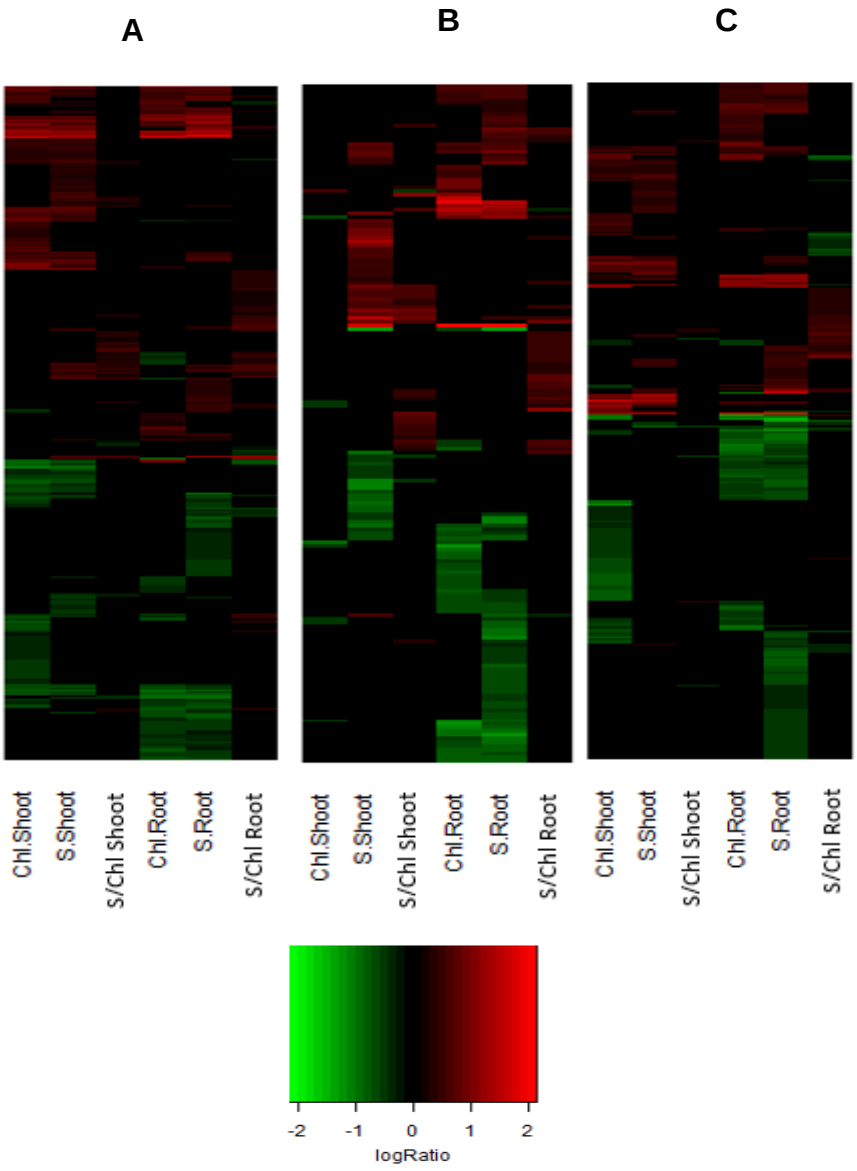

Figure 6. Clustering analysis of chloride (Chl) and sulfate (S) salinities responsive genes involved in response to abiotic stimulus (A), signal transduction (B) and transport (C). S/Chl represents the gene expression level under sulfate stress in relation to gene expression level under chloride stress.

As evidenced by the Tables 2, 3 and 4, many genes expressed under chloride stress exhibited a distinct expression pattern under sulfate stress. The expressed genes were mainly grouped into five categories: (1) genes involved in signal sensing, perception and transduction, (2) transcription control, (3) osmoprotection, (4) chaperone function and (5) transport.

Signal sensing, perception and transduction-related genes

Abiotic stress signaling includes calcium-dependent pathways, ABA-dependent or –independent pathways, and MAP kinase pathways [46]. Among the expressed genes involved in stress signaling pathways via stimulating Ca^{2+} binding proteins, one of the two genes coding for calcium-binding protein (Os02g0753800) (Table 2) was up-regulated in NaCl- and Na_2SO_4-stressed shoot while the other gene (Os03g0743500) (Table 3) was up-regulated in shoot and root under sulfate stress. The gene coding for diacylglycerol kinase1 (DGK1) (Os03g0425300) was induced in NaCl- and Na_2SO_4-stressed root while the genes coding for Calmodulin NtCaM3 (Os03g0319300) and CBL-interacting protein kinase 2 (Os05g0332300) were respectively repressed in chloride and in sulfate-stressed roots (Table 3).

The plant hormones also regulate stress-signaling pathways for maintaining an osmotic adjustment or homeostasis and regulating plant growth and development. The gene coding for ABA response element binding factor (Os01g0859300) (Table 2) was highly up-regulated in Na_2SO_4-stressed shoot while the gene coding for Gretchen hagen 3 (GH3) family protein (Jasmonate signaling) (Os05g0586200) was down-regulated in NaCl- and Na_2SO_4-stressed roots (Table 3). The gene coding for AP2/ERF (APETALA2/ ethylene-responsive factor) protein (Os05g0497200) was repressed in NaCl-stressed shoot but highly induced in Na_2SO_4 -stressed root (Table 3).

As signaling molecules, phospholipids assume important structural roles during stress responses in inducing cytosolic Ca^{2+} spiking [47]. The gene coding for lipid phosphate phosphatase 2 (Os01g0693300) was highly up-regulated in Na_2SO_4-stressed shoot while the gene coding for chloroplast-localized phospholipase D (Os06g0604300) was repressed in Na_2SO_4-

stressed shoot and in NaCl- or Na_2SO_4-stressed root. The gene coding for phospholipase C (Os12g0562400) was repressed in NaCl-stressed root (Table 3).

The protein kinases play a key role in hyperosmotic stress sensing, perception and transduction. Three genes coding for serine/threonine protein kinase were considered in the present study. The first one (Os03g0390200) was induced in Na_2SO_4-stressed shoot, the second one (Os07g0622000) was repressed in chloride-stressed root (Table 2) while the last (Os03g0319400) was repressed in NaCl- and Na_2SO_4-stressed root (Table 3). One of the genes coding for MAP kinase (Os12g0114100) was up-regulated in Na_2SO_4-stressed shoot while the other was down-regulated in NaCl-stressed shoot and root (Table 2).

Table 2. Expression patterns of genes involved in response to abiotic stimulus affected by NaCl 200 mM (Chl) or Na_2SO_4 100 mM (S) stress in the shoot (Sh) or root (R) of IKP rice cultivar. S/Chl represents the gene expression level under sulfate stress in relation to gene expression level under chloride stress.

Gene	Function	Chl Sh	S Sh	S/Chl Sh	Chl R	S R	S/Chl R
	Signal sensing, perception and transduction						
Os02g0753800	Calcium-binding protein, drought stress tolerance	0.55	0.46				
Os01g0859300	ABA response element binding factor		0.69	0.33			
Os03g0390200	Serine/threonine protein kinase, Hyperosmotic stress response		0.40				
Os12g0114100	MAP kinase-like protein		0.34				
Os07g0622000	Serine/threonine protein kinase, Hyperosmotic stress response				-0.54		
	Transcription control						
Os01g0187900	Transcription factor MYBS2	0.39					0.41
Os01g0202500	B-box zinc finger family protein	0.55	0.50				
Os04g0398000	ERF domain containing protein	0.54					
Os07g0688200	Typical P-type R2R3 Myb protein	0.61					
Os09g0106700	Myb proto-oncogene protein	0.78					
Os12g0564100	Myb-like DNA-binding domain containing protein	0.82	0.61				
Os12g0583700	Zinc finger, C2H2-type domain containing protein	0.44	0.52				

Table 2. *Continued*

Gene	Function	Chl Sh	S Sh	S/Chl Sh	Chl R	S R	S/Chl R
Os05g0195700	Similar to Transcription factor MYBS2		0.62	0.39			
Os07g0558100	Similar to Myb-related transcription factor LBM1		0.75	0.44			
Os08g0472000	bZIP transcription factor, Abscisic acid (ABA)-regulated transcription		0.45				
Os09g0456200	BZIP transcription factor ABI5		0.36			0.77	
Os02g0176000	Zinc finger, B-box domain containing protein.			0.34			
Os02g0766700	bZIP transcription factor, Regulation of ABA signaling and biosynthesis, Drought resistance			0.40			0.46
Os03g0171700	bHLH domain containing protein			0.33			
Os08g0408500	ERF domain containing protein			0.40	-0.45		0.49
Os12g0610200	Phytochrome-interacting factor 3 (bHLH8) (AtbHLH008)			0.37		0.44	
Os12g0175400	Similar to OSMYB2					-0.54	
	Chaperone function						
Os04g0589800	Late embryogenesis abundant group 1 family protein.	0.87	1.02			0.79	
Os01g0314800	Late embryogenesis abundant protein 3 family protein.			0.34	-0.53		0.32
Os05g0566800	Cold acclimation protein COR413-TM1						0.31

Table 2. *Continued*

Gene	Function	Chl Sh	S Sh	S/Chl Sh	Chl R	S R	S/Chl R
	Osmoprotection						
Os01g0848200	Delta 1-pyrroline-5-carboxylate synthetase	0.81	0.64				
Os03g0230300	Homologue of SRO, Regulation of stomatal closure	0.77	0.85				0.35
Os03g0286900	RCI2 family protein, Drought resistance	0.96	1.13		0.53	0.98	0.44
Os09g0530300	Cytochrome P450 family protein	0.54					
Os11g0454300	Water-stress inducible protein RAB21	1.37	1.36		1.34	1.50	
Os12g0268000	Cytochrome P450 71A1	0.34	0.42		0.67		
Os07g0687900	WSI76 protein induced by water stress		0.85	0.75		1.27	1.41
Os11g0498600	HVA22 protein		0.53	0.68		0.54	0.73
Os03g0316200	Galactinol synthase		0.56	0.47		0.52	0.71
Os03g0437200	C2H2-type zinc finger protein, Water stress and oxidative stress tolerance			0.43	-0.75	-0.48	
Os10g0550900	Proline oxidase domain containing protein.						0.45
Os03g0643300	Ornithine delta-aminotransferase	0.55	0.56		0.56	0.63	

Transcription control-related genes

Transcription factors (TFs) play an important role in activating stress genes and regulating stress signal transduction [48]. It is well documented that transcription factors belonging to the families of MYB, Zinc finger, bZIP, bHLH, DREB, WRKY and NAC are relevant in salt stress tolerance [49,50]. A large number of TF genes belonging to MYB family were induced in shoot. Os07g0688200 and Os09g0106700 were induced in NaCl-stressed shoot while Os05g0195700 and Os07g0558100 were highly up-regulated in Na_2SO_4-stressed shoot (Table 2). Os12g0175400 and Os02g0685200 (Table 3) were respectively repressed and highly induced in Na_2SO_4-stressed root.

Most of TF genes belonging to zinc finger family were induced in shoot. Os01g0202500 and Os12g0583700 were induced in NaCl and Na_2SO_4-stressed shoot while Os02g0176000 was highly up-regulated in Na_2SO_4-stressed shoot (Table 2). Os08g0130100 was highly induced in Na_2SO_4-stressed shoot and root and repressed in NaCl-stressed root. All genes coding for bZIP TF family were induced under sulfate stress but, under our experimental conditions, were not induced in response to NaCl. Os08g0472000 (Table 2) was induced in Na_2SO_4-stressed shoot and Os01g0808100 (Table 3) was induced in Na_2SO_4-stressed root. Os09g0456200 was induced in Na_2SO_4-stressed shoot and root while Os02g0766700 was highly induced in Na_2SO_4-stressed shoot and root (Table 2).

The genes coding for bHLH TF family were highly up-regulated in Na_2SO_4-stressed shoot (Os03g0171700, Os12g0610200) (Table 2) or up-regulated in Na_2SO_4-stressed root (Os12g0610200) (Table 3). The genes coding for WRKY TF family were highly induced in Na_2SO_4-stressed shoot (Os05g0537100, Os01g0584900) or down-regulated in Na_2SO_4-stressed root (Os02g0265200). The genes encoding the DREB2B protein (Os02g0797100) and OsNAC5 (Os11g0184900) protein were highly induced in Na_2SO_4-stressed shoot (Table 3).

Table 3. Expression patterns of genes involved in signal transduction affected by NaCl 200 mM (Chl) or Na_2SO_4 100 mM (S) stress in the shoot (Sh) or root (R) of IKP rice cultivar. S/Chl represents the gene expression level under sulfate stress in relation to gene expression level under chloride stress.

Gene	Function	Chl Sh	S Sh	S/Chl Sh	Chl R	S R	S/Chl R
	Signal sensing, perception and transduction						
Os01g0859300	ABA response element binding factor		0.51	0.57			
Os03g0743500	Calcium-binding EF-hand domain containing protein		0.38				0.38
Os01g0693300	Lipid phosphate phosphatase 2 (EC 3.1.3.-) (AtLPP2)			0.35			
Os01g0797600	AP2/ERF family protein, Stress signaling			0.3	-0.64		0.40
Os08g0408500	ERF domain containing protein			0.4	-0.45		0.49
Os06g0604300	Chloroplast-localized phospholipase D		-1.14		-0.58	-1.12	
Os03g0425300	DGK1 (DIACYLGLYCEROL KINASE1); calcium ion binding				0.44	0.54	
Os07g0558500	Inositol phosphatase-like protein.					0.40	
Os05g0223000	TCH2 (TOUCH 2); calcium ion binding.	-0.43					0.30
Os05g0497200	AP2/ERF (APETALA2/ethylene-responsive factor) protein	-0.42					0.44
Os03g0319300	Calmodulin NtCaM3 (Calmodulin NtCaM4)				-0.60		
Os03g0319400	Serine/threonine protein kinase, Abiotic stresses				-0.68	-0.61	

Table 3. *Continued*

Gene	Function	Chl Sh	S Sh	S/Chl Sh	Chl R	S R	S/Chl R
Os03g0758300	Cyclic nucleotide-gated ion channel 2 (AtCNGC2)				-0.53	-0.74	
Os05g0586200	Gretchen hagen 3 (GH3) family protein, Jasmonate signaling				-0.78	-0.99	
Os07g0622000	Serine/threonine protein kinase, Hyperosmotic stress response				-0.54		
Os08g0421800	Mitogen-activated protein kinase kinase kinase 1	-0.75			-0.51		
Os08g0446200	Receptor-like protein kinase precursor (EC 2.7.1.37)				-0.60	-0.37	
Os12g0562400	Phospholipase C				-0.55		
Os01g0141000	AP2/ERF and B3 domain-containing protein, Salt stress response					-0.41	
Os05g0332300	Similar to CBL-interacting protein kinase 2.					-0.51	
	Transcription control						
Os02g0797100	Dehydration responsive element binding protein 2B (DREB2B protein)		0.71	0.57			
Os05g0537100	WRKY transcription factor 10		0.49	0.54			
Os08g0472000	bZIP transcription factor, ABA-regulated transcription		0.45				
Os09g0456200	BZIP transcription factor ABI5		0.36			0.77	

Table 3. *Continued*

Gene	Function	Chl Sh	S Sh	S/Chl Sh	Chl R	S R	S/Chl R
Os11g0184900	OsNAC5 protein [imported]-rice		0.66	0.49			
Os01g0584900	WRKY transcription factor 28-like (WRKY5)			0.68			
Os02g0766700	bZIP transcription factor, ABA signaling and biosynthesis, Drought resistance			0.4			0.46
Os08g0130100	Zinc finger, LSD1-type domain containing protein			0.5	-0.38		0.53
Os12g0610200	Phytochrome-interacting factor 3 (bHLH8) (AtbHLH008)			0.37		0.44	
Os01g0808100	BZIP transcription factor.					0.33	
Os02g0685200	MYB transcription factor, Transcriptional activator in mediating stress						0.42
Os02g0265200	WRKY transcription factor 39					-0.32	

Osmoprotection-related genes

In higher plants, proline biosynthesis may proceed either via glutamate, by successive reductions catalyzed by pyrroline-5-carboxylate synthase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) or by ornithine pathway and ornithine δ-aminotransferase (OAT), representing generally the first activated osmoprotectant after stress perception [51]. The gene coding for Δ1-pyrroline-5-carboxylate synthetase (Os01g0848200) was induced in NaCl- and Na_2SO_4-stressed shoot (Table 2). The gene coding for ornithine δ-aminotransferase (Os03g0643300) was induced in both NaCl- or Na_2SO_4-stressed shoot and root. Regarding the proline catabolism process, the gene coding for proline oxidase was highly induced in Na_2SO_4-stressed root.

The genes encoding the galactinol synthase (Os07g0687900, Os03g0316200) were induced to a higher extent under sulfate than chloride stress in shoot and root and the gene coding for SRO protein family (Os03g0230300) implicated in regulation of stomatal closure displayed a similar behavior in shoot.

Chaperone function-related genes

The genes coding for the late embryogenesis abundant protein (LEA) were differently expressed depending on their family. One of the genes from family 1 (Os04g0589800) was induced in NaCl- and in Na_2SO_4-stressed shoot and in the root of Na_2SO_4-stressed plants (Table 2). The gene from family 3 (Os01g0314800) was induced to a higher extent under sulfate than chloride stress both in shoot and root and was repressed in NaCl-stressed root. The gene coding for COR413-TM1 protein (Os05g0566800) was highly induced in Na_2SO_4-stressed root.

Transport-related genes

In this category, the gene products were categorized into three main groups and are presented in Table 4: osmoprotectants, ions and water transporters.

Among osmoprotectant transporters, most genes encoded sugar transport. Among two genes coding for sucrose transporter, one (Os03g0170900) was

induced in NaCl- and in Na_2SO_4-stressed plants at the shoot and root levels, while the other (Os12g0476200) was induced in Na_2SO_4-stressed shoot only. The gene coding for sorbitol transport (Os12g0514000) was also only induced in Na_2SO_4-stressed shoot. Two genes encoding the enzymes initiating the inositol transport were induced under sulfate stress: Os01g0701900 was induced in the shoot while Os07g0558500 was induced in the root. The cell-wall invertase cleaves sucrose for yielding glucose and fructose, which can be taken up by plant cells via hexose transporters. Our results showed that the gene coding for cell-wall invertase (Os04g0413500) was repressed in NaCl-stressed shoot and root as well as in Na_2SO_4-stressed root.

In addition to sugar, the genes encoding the transporter of proline, polyamines and glycolipids were also expressed. The gene coding for proline transporter (Os06g0168700) was up-regulated in NaCl-stressed shoot and root and down-regulated in Na_2SO_4-stressed root. The gene encoding the polyamine transport (Os03g0375900) was repressed in NaCl- and Na_2SO_4-stressed roots while the two genes encoding the glycolipids transport (Os08g0556300 and Os04g0438600) were repressed in Na_2SO_4-stressed root.

Table 4. Expression patterns of genes involved in transport affected by NaCl 200 mM (Chl) or Na_2SO_4 100 mM (S) stress in the shoot (Sh) or root (R) of IKP rice cultivar. S/Chl represents the gene expression level under sulfate stress in relation to gene expression level under chloride stress.

Gene	Function	Chl Sh	S Sh	S/Chl Sh	Chl R	S R	S/Chl R
	Osmoprotectants transport						
Os01g0701900	Phosphatidylinositol transfer-like protein III.		0.51				
Os07g0558500	Inositol phosphatase-like protein.					0.40	
Os01g0700900	Cytochrome P450 monooxygenase.				0.53		
Os03g0375900	Amino acid/polyamine transporter I family protein.				-0.50	-0.63	
Os06g0168700	Similar to Proline rich protein	0.74			0.99		-0.75
Os03g0170900	Sucrose transporter	0.32	0.40		0.38	0.38	
Os10g0360100	Similar to Sugar transporter protein	0.96	0.79		0.47	0.41	
Os12g0476200	Sucrose transporter		0.48				
Os12g0514000	Similar to Sorbitol transporter		0.34				
Os07g0106200	Similar to Hexose transporter	0.60					
Os09g0322000	Similar to sugar transport protein 14	0.49					
Os04g0413500	Cell-wall invertase, Carbon partitioning during early grain filling	-0.56			-0.61	-0.64	
Os07g0559700	Similar to Monosaccharide transporter 3	-0.41			-0.52		0.68
Os07g0561800	Similar to carbohydrate transporter/ sugar porter	-0.58			0	-0.45	
Os11g0508600	Sugar transporter	-0.47			-0.98	-0.39	

Table 4. *Continued*

Gene	Function	Chl Sh	S Sh	S/Chl Sh	Chl R	S R	S/Chl R
	Osmoprotectants transport						
Os07g0131600	Similar to Monosaccharide transporter				0.47	0.70	
Os03g0197100	Similar to Sugar transporter protein				0.61		
Os05g0214300	Similar to Bidirectional sugar transporter SWEET3a				-0.57	-0.77	
Os11g0643800	Similar to Sugar transporter family protein, expressed				-0.45	-0.86	-0.47
Os02g0604300	Nucleotide-sugar transporter family protein					-0.60	
Os04g0490600	Nucleotide-sugar transporter family protein					-0.51	
Os08g0556300	Glycolipid transfer protein domain domain containing protein					-0.53	
Os04g0438600	Glycolipid transfer protein, GLTP domain containing protein						-0.31
	Ions transport						
Os01g0130000	Cation efflux protein family protein.	0.54	0.43				
Os03g0161200	Sulphate transporter.		0.44			1.30	0.67
Os03g0616400	Plasma membrane Ca^{2+}-ATPase.	0.42	0.33				
Os03g0689300	Plasma membrane H+ ATPase (EC 3.6.3.6) (H-ATPase).	0.77	0.58		0.79	0.40	
Os04g0644800	Chloride channel-like protein Osclc.	0.35	0.48		0.44	0.60	
Os07g0108800	Two-pore K+ channel family protein, K+ homeostasis, Osmotic tolerance		0.39				

Table 4. *Continued*

Gene	Function	Chl Sh	S Sh	S/Chl Sh	Chl R	S R	S/Chl R
	Ions transport						
Os07g0666900	Vacuolar Na+/H+ antiporter, Salt tolerance		0.48			0.46	
Os08g0503700	Sodium/sulphate symporter family protein.	0.40	0.69		0.80	1.03	
Os01g0209800	Cationic amino acid transporter (Fragment).	-0.65			-0.78		
Os01g0557500	Cation/proton exchanger 1a.	-0.49			-0.47		
Os03g0203700	Calcium-transporting ATPase 2, plasma membrane-type (EC 3.6.3.8)	-0.59			-0.49		
Os03g0375300	Similar to cationic amino acid transporter.	-0.47					
Os04g0607600	High-affinity K+ transporter (HKT) family protein, Na+ exclusion from leaf blade upon salt stress	-0.76					
Os05g0428700	Potassium channel protein ZMK2.	-0.47					
Os09g0448200	High-affinity potassium transporter.	-0.73					
Os02g0245800	Inward rectifying shaker-like potassium channel, Negative regulation of OsKAT2 channel activity				0.62	0.78	
Os04g0605500	P-type IIB Ca(2+) ATPase, Stress tolerance				0.48	0.53	
Os06g0270200	Similar to Potassium transporter 24.					0.49	0.31
Os06g0701700	Ion transporter , Na+/K+ symport					0.32	0.57
Os12g0638700	Plasma membrane H+ ATPase (EC 3.6.3.6).				0.42		
Os01g0648000	Potassium channel, Potassium uptake, Salt stress tolerance						0.30
Os02g0730300	Potassium transporter HAK3p						0.39
Os03g0147400	Divalent ion symporter domain containing protein.						0.47

Table 4. *Continued*

Gene	Function	Chl Sh	S Sh	S/Chl Sh	Chl R	S R	S/Chl R
	Ions transport						
Os03g0654400	Cationic amino acid transporter-like protein.				-0.82	-0.56	
Os03g0758300	Cyclic nucleotide-gated ion channel 2 (AtCNGC2)				-0.53	-0.74	
Os06g0561200	Potassium/proton antiporter-like protein				-0.62	-0.65	
Os02g0176700	autoinhibited calcium ATPase					-0.39	
Os04g0401700	Potassium transporter, Potassium mediated growth, Salt tolerance					-0.74	-0.34
	Water transport						
Os02g0745100	Aquaporin NIP III subfamily protein, Water transport	-0.45				-0.36	
Os06g0228200	Aquaporin NIP III subfamily protein, Water transport	-0.55			-0.71	-0.88	
Os03g0146100	Tonoplast intrinsic protein (Tonoplast water channel).			0.305			0.55

Regarding the ions transport, the genes encoded mainly the Na^+ and K^+ transport. We distinguished the genes encoding more specifically the Na^+-transport from the genes coding for the enzymes activating the plasma membrane Na^+-transport and most of them were induced. Four genes encoded more specifically the Na^+-transport: the gene coding for vacuolar Na^+/H^+ antiporter protein (Os07g0666900) was induced in Na_2SO_4-stressed shoot and root. The gene coding for sodium/sulphate symporter family protein (Os08g0503700) was induced in NaCl- and Na_2SO_4-stressed shoot and root while the gene coding for Na^+ /K^+ symport (Os06g0701700) was only induced in Na_2SO_4-stressed root. The gene coding for HKT protein family (Os04g0607600) involved in Na^+ exclusion from leaf blade upon salt stress was surprisingly repressed in NaCl-stressed shoot.

All genes coding for the enzymes activating the plasma membrane Na^+-transport were up-regulated: Os03g0616400 was induced in NaCl- and Na_2SO_4-stressed shoots while Os03g0689300 was induced in NaCl- and Na_2SO_4-stressed shoot and root. Os04g0605500 was up-regulated in salt-treated roots, while Os12g0638700 was only up-regulated in NaCl-stressed root.

Most of the genes coding for the K^+-transporters and channels were repressed under chloride stress in shoot (Os05g0428700, Os09g0448200), sulfate stress in root (Os04g0401700) and chloride or sulfate stress in root (Os06g0561200). The gene encoding the negative regulation of OsKAT2 channel activity (Os02g0245800) was induced in NaCl- and Na_2SO_4-stressed roots. The induced genes coding for K^+- transport were only identified in Na_2SO_4-stressed shoot (Os07g0108800) and root (Os01g0648000, Os02g0730300).

Despite their importance in salinity resistance [52], only few genes encoding the salt anions transport were identified. The gene coding for Cl^--transport (Os04g0644800) was induced in NaCl and Na_2SO_4-stressed shoot and root while the gene encoding the SO_4^{2-}-transport was induced in Na_2SO_4-stressed shoot and root.

Three differentially-expressed genes encoding water transporters were detected. Among them, two genes coding for aquaporin NIP III subfamily

protein were repressed: Os02g0745100 was down-regulated in NaCl-stressed shoot and Na_2SO_4-stressed root while Os06g0228200 was down-regulated in NaCl-stressed shoot as well as in NaCl- and Na_2SO_4-stressed roots. Conversely, the gene encoding the tonoplast-water transport (Os03g0146100) was induced in Na_2SO_4-stressed shoot and root.

Discussion

An excess of sulfate salts affects some areas such as the volcanic soils, marine soils, agricultural soils irrigated with saline water or subjected to the anthropogenic inputs from industry [39]. Considering that most studies on salt stress have been conducted with NaCl and that rice plays a major role in human nutrition, it is very interesting to compare the rice response to NaCl and Na_2SO_4 salinities at both physiological and transcriptomic levels. In our study, NaCl was more toxic than Na_2SO_4 for most of physiological parameters studied as found previously in the study dealing with IKP cultivar (Irakoze et al. 2019).

Regarding the results on pH, we deduced that the more the intensity of salt stress increased the more the pH of the culture solution increased. The pH values were relatively higher in Na_2SO_4- than in NaCl-treated solutions and this could be due to a large amount of Na^+ and SO_4^{2-} ions which would remain in Na_2SO_4-treated solutions.

The results on the chemical speciation of the culture solutions revealed that the concentration and activity rates of the salt ions differed widely. Despite the equimolar concentration of Na^+ in the initial saline solution, the Na^+-concentration and activity rates were higher when Na^+ was accompanied by the chloride anion than by the sulfate anion. We also observed that the concentration and activity rates of Na^+ were almost the same as those of Cl^- in the case of NaCl salinity. Surprisingly, the concentration and activity rates of the SO_4^{2-} anion were significantly low and that could partly explain a less intense toxicity recorded for Na_2SO_4-treated plants as observed on several studied parameters. We found that increasing the intensity of salt stress led to a decrease in concentration and activity rates of salt ions.

Numerous studies on molecular mechanisms of rice tolerance to salt stress have been conducted by mainly focusing on NaCl tolerance [53], but none has investigated the difference between NaCl and Na_2SO_4 tolerance. One of the most interesting results of this comparative study of rice transcriptome was the quantitative importance in the difference of gene expression under NaCl and Na_2SO_4 stresses (Figs 4, 5 and 6). Considering the three main biological functions selected for this study, a larger number of genes were expressed under sulfate than under chloride salinity, this difference being the most remarkable in root (Fig. 5). Such a result was, to some extent, unexpected since we used in the present study a short-term exposure to salinity and the doses of NaCl and Na_2SO_4 were chosen in order to apply a similar external osmotic potential. Considering that the biphasic model of salt stress specifies that the first impact of external salt is mainly osmotic [7], strong difference between NaCl and Na_2SO_4 impact for short term exposure was puzzling. This suggests that ionic component is conditioning plant response already after 48h, which is confirmed by accumulation of Cl^-, S and Na^+ in both shoot and roots and confirms previous transcriptomic data mentioning that desiccation and salinity stress differently regulate numerous genes expression [35]. Nevertheless, iso-strength Na^+ solutions were used for NaCl and Na_2SO_4 and differences in term of Na^+ accumulation was significant only for roots and remained limited which suggest that anion accumulation might be, at least partly, responsible for differential gene expression between treatments. Unlike the results of the chapter I where the salt ions accumulated more in the shoots than in the roots, we observed an opposite trend in this study. This could be explained by the short duration of stress exposure which did not allow the translocation of the salt ions absorbed at the root level.

More genes were up-regulated in Na_2SO_4-treated plants whatever the considered organ while more genes were down-regulated in NaCl-treated plants, organs combined. Regarding the gene expression level under sulfate stress in relation to gene expression level under chloride stress (S/Chl), 100% and 87% of genes were induced to a higher extent under Na_2SO_4 than NaCl salinity in shoot and root respectively (Tables 2, 3 and 4). The internal osmotic potential of stressed plant tissues was by far lower in NaCl-treated plants, especially at the shoot level. A similar trend was also recorded for WC, suggesting that modification in the plant water status

does not constitute the sole signal triggering a high level of gene activation in Na_2SO_4-treated plants.

All putative functions of the genes expressed in our study have been detected in the previous studies where rice cultivars were exposed to NaCl stress [15,28,29,32,33,46,47,54,55]. However, many genes up or down-regulated by chloride stress exhibited a distinct expression pattern under sulfate stress. In some cases, it was interesting to note an overlap of up to about half of the genes induced or repressed by NaCl and Na_2SO_4 salinities in IKP cultivar. Hence, these two salinities affected IKP cultivar in overlapping but not in identical ways. This obvious difference in gene expression profile between NaCl and Na_2SO_4 salinities indicated some important processes of metabolic adaptation depending on the type of salinity, which must be considered for the comprehension of the resulting physiological parameters.

Most of the genes involved in signal sensing, perception and transduction were up-regulated in Na_2SO_4-treated shoot while a large number of down-regulated genes were identified in NaCl-treated root. The genes of this class encoded Ca^{2+} -binding proteins, plant hormones, phospholipids and MAP kinases proteins. All genes coding for MAP kinases proteins and phospholipids were repressed in NaCl-treated plants. According to Kader and Lindberg [56] and Mahajan et al. [57], the plants exposed to salt stress initiates the stress signaling pathways for stress tolerance via stimulating various Ca^{2+}-binding proteins including calcineurin B-like (CBL) proteins, CBL-interacting protein kinases (CIPKs), Ca^{2+}-dependent protein kinases (CDPKs) and calmodulins. In Chapter I, the assessment of salinity effect on Ca^{2+} content in rice plant revealed that salinity did not affect the roots unlike the shoots where a significant reduction in Ca^{2+} content was recorded and more particularly in Na_2SO_4-treated plants. Despite this reduction, this study showed that many genes encoding the Ca^{2+}-binding proteins were induced in Na_2SO_4-treated shoots. Since the experimental conditions differed widely in the two studies, the comparison of the results would not make sense. For the next transcriptomic study, we suggest analyzing the Ca^{2+} content in plants since this cation plays a predominant role in signal sensing, perception and transduction. Many of the genes encoding these Ca^{2+}-binding proteins showed NaCl response expression in Nipponbare rice cv. [46,58]. Genes encoding the ABA signaling were only

induced in Na_2SO_4-treated shoot while the genes coding for jasmonate signaling were repressed in NaCl and Na_2SO_4-treated roots. It has been proven that ABA and jasmonates interacted negatively in rice growing under saline conditions [59].

Regarding the genes involved in transcription control, a large number of expressed genes encoded the MYB proteins. Rabbani et al. [54] showed that the MYB family is one of the largest families of transcriptional factors characterized in plants. Most of the gene encoding the MYB and zinc finger TF families were expressed in Na_2SO_4-treated plants while the genes coding for bZIP, bHLH, WRKY, DREB and NAC TF families were exclusively expressed in Na_2SO_4-treated plants. Few genes were expressed in NaCl-treated plants and most of them were down-regulated. Unlike our results where *OsMYB2* was not expressed in NaCl-treated plants, Yang et al. [60] proved that the expression of *OsMYB2* in leaves of Zhonghua 10 rice cv. was up-regulated by NaCl and this gene was localized in the nucleus with transactivation activity. *OsMYB6* gene was also reported to functions as a stress-responsive transcription factor playing a positive role in response to drought and salt stress resistance [61].

OsDREB2B, bZIP (*OsABF2*, *OsABI5*) and WRKY (*OsWRKY45-1*, *OsWRKY45-2*) transcription factors overexpressed in rice leaves exposed to NaCl salinity and conferred a high tolerance to rice plants [35,62]. In our study, *OsNAC5* was only up-regulated in Na_2SO_4-treated shoot while Song et al. [63] observed the induction of *OsNAC5* leaves of Zhonghua 10 rice cv. stressed by NaCl salinity and its overexpression correlated positively with accumulation of compatible solutes such as proline and soluble sugars.

The genes coding for P5CS and OAT involved in proline biosynthesis were overexpressed in NaCl and Na_2SO_4-treated shoot, although proline accumulation was two times higher in plants exposed to NaCl than in those exposed to Na_2SO_4. These two genes were also overexpressed in leaves of rice salt tolerant cv. Lunishree exposed to 255 or 425 mM NaCl but no induction was observed under 85 mM NaCl [64].
In order to limit the water loss at the shoot level, the gene coding for SRO protein regulating the stomatal closure was up-regulated in NaCl and

Na_2SO_4-treated shoot. *OsSRO1a* overexpressed in shoot of IR64 rice cv. exposed to NaCl salinity and interacted with RNA Binding Domain-Containing Protein (*OsRBD1*) to confer a salt stress tolerance in yeast [65]. Galactinol synthase (GolS) is the first committed enzyme in raffinose family oligosaccharide (RFO) synthesis pathway and synthesizes galactinol from UDP-galactose and inositol. Our results showed that the gene coding for galactinol synthase was only induced in Na_2SO_4-treated shoot and root, although no raffinose was detected in response to Na_2SO_4. During NaCl stress, the premature and mature transcripts of two isoforms of *Oryza sativa* IR64 cv. GolS (*OsGolS1, OsGolS2*) accumulated in leaves in a rhythmic manner for very small time-windows interrupted by phases of complete absence [66].

Regarding the transport function, the expressed genes were implicated in osmolytes, ions and water transport. Among osmolytes transporters, most genes encoded sugar transport. Two genes coded for sucrose transport, one was induced in NaCl and Na_2SO_4-stressed shoot and root while the other was exclusively induced in Na_2SO_4-stressed shoot. The transport of sucrose is essential for long distance sucrose transport necessary to meet the cellular energy demands and for osmoprotectant activities during salinity stress. Working on *O. sativa* Nipponbare cv., Ibraheem et al. [67] found that among 5 rice sucrose transporter genes identified, only *OsSUT2* was highly induced in NaCl-treated shoot. *OsSUT1*, *OsSUT4* and *OsSUT5* were only expressed at low levels, while *OsSUT3* showed no significant expression. Transcriptome analyses of *O. sativa* IR64 cv. under NaCl stress revealed that the proline transporter gene (AB073084) was down-regulated in shoot and up-regulated in root after 1h or 24h of exposure to salt stress [55]. Our results showed that proline transporter gene was induced in NaCl-treated shoot and root while this gene was repressed in Na_2SO_4-treated root.

Na^+ transporters play an important role in Na^+ tolerance in plants exposed to salt stress. Among them, we distinguish Na^+/H^+ antiporters from Na^+/H^+ symporters. Our results showed that no gene coding for vacuolar compartmentalization of Na^+ ions was up-regulated in NaCl-treated plants. Some genes were down-regulated while others did not show any expression. Therefore, a large amount of Na^+ would accumulate in the cytosol and inhibit the cell metabolic activity. This observation could

further explain the higher toxicity of NaCl over Na_2SO_4 salinity as evidenced by our previous studies. The gene encoding the vacuolar Na^+/H^+ antiporter was only expressed in Na_2SO_4-stressed shoot and root. In rice, 4 vacuolar Na^+/H^+ antiporters (*OsNHX1-4*) and one endosomal Na^+/H^+ antiporter (*OsNHX5*) have been reported [68]. The expression of *OsNHX1*, *OsNHX2*, *OsNHX3*, and *OsNHX5* was regulated differently in rice tissues and was increased by NaCl stress, probably as an attempt to compartmentalize Na^+ in vacuoles.

HKT is a Na^+/K^+ symporter or Na^+ uniporter present in the plasma membrane of plant cells and rice genome has seven HKT transporter genes and two pseudogenes. In our study, all genes coding for HKT family protein were repressed in NaCl-treated shoot and no gene expression was detected in other treatments. The study on the *OsHKT* expression profile in *O. sativa* cv. Nipponbare exposed to NaCl salinity revealed that the fluxes mediated by HKT transporters were more intense in shoot than in root, and that the functions of *OsHKT3/9* and *OsHKT6* in roots might be of little importance or restricted to very few cells [69]. Furthermore, the functions of the *OsHKT* transporters in root seemed not to be related to the defense from Na^+, because Na^+ did not enhance the expression of any of them, whereas its absence or the absence of K^+ enhanced the expressions of most of them.
As evidenced by our results (Table 4), most of genes involved in K^+ transport were more induced in Na_2SO_4- than in NaCl-treated plants. However, K^+ contents in rice plant organs were not significantly different in both salt treatments. Two alternatives could explain this situation. The first alternative would be that the present transcriptomic study did not identify the expression of all genes encoding K^+ transporters; other useful information would be hidden in this case. The second alternative would be that the identified genes could be involved only in Na^+ transport or in the transport of these two cations but more preferably in Na^+ transport.

It is interesting to notice that regulation of gene expression in response to salt stress was also specific to the considered organ. Most of the expressed genes presented very different expression profiles in shoots and roots, considering the regulation of their expression in relation to their putative function. Within a given gene family, different genes were expressed in

roots and shoots, as recorded for the TF family. The genes coding for bZIP, bHLH or WRKY were exclusively expressed in Na_2SO_4-treated plants and clearly showed a different expression profile depending on the plant organ.

Modification in some gene expression may be followed by modification in the corresponding metabolic products and physiological properties. For example, the gene encoding the Δ1-pyrroline-5-carboxylate synthetase was up-regulated only in NaCl and Na_2SO_4-treated shoots (Table 2). This enzyme catalyzes the biosynthesis of proline. Our results showed that proline significantly accumulated in NaCl and Na_2SO_4-treated shoot while no significant variation was detected in root (Fig. 1D). Another illustrative case concerns the gene encoding the sulfate transport which was induced exclusively in Na_2SO_4-treated shoot and root (Table 4). The Fig. 2 show that the sulfur was only significantly increased in Na_2SO_4-treated shoot and root. This surprisingly suggests that exposure to sulfur excess paradoxically does not imply down-regulation of genes coding for sulfur transporter. A similar situation was recorded for genes coding for Cl^- transporter in NaCl-treated plants. An explanation could be that those transporters are involved in vacuolar sequestration, thus helping plant cell to cope with ion excess [52], or that an inhibition of the mRNA translation lead to an increase in transcript abundance. Kubala et al. [70] previously reported that transcriptomic and proteomic analyses of salt-treated plants lead to contrasting results suggesting that numerous post-transcriptional mechanisms are involved in plant response to salinity.
The gene encoding the cell-wall invertase was down-regulated in both NaCl- and Na_2SO_4-treated plants. Invertases (β-fructofuranosidase) catalyse the hydrolysis of sucrose to glucose and fructose. Furthermore, invertases have a great contribution in carbohydrates portioning and the regulation of the source-sink relationship. As shown in table 1, glucose and fructose were not detected in certain treatments including the plant control and this would have been partly due to the down-regulation of the gene encoding the invertase.

All genes encoding the aquaporin NIP III subfamily protein were repressed in NaCl-treated shoot and root (Table 4). Aquaporins, also called water channels, facilitate the water transport in plant. In addition to transporting water, aquaporins play a key role in osmoregulation process, stress

signaling and ROS detoxification, compartmentalization of NH_3 and urea at vacuolar level as well as in chloroplast transport [71]. Based on our results, NaCl stress greatly reduced the shoot and root water content comparatively to controls, while seedlings exposed to Na_2SO_4 stress exhibited an intermediate behavior (Fig. 1B). Downregulation of water channel coding gene may be involved in the recorded decrease in WC of stressed plants. Morever, the down-regulation of genes encoding the aquaporins would have negatively affected the cell metabolic processes other than water transport.

Our results showed that most of genes involved in response to salt stress were up-regulated in Na_2SO_4-treated plants whatever the considered organ while more genes were down-regulated in NaCl-treated plants, organs combined. This may explain the higher alteration of plant growth and development under chloride than sulfate salinity as evidenced by the results on physiological parameters as well as our previous studies [40]. Although most of the genes involved in saline stress tolerance have been repressed or not expressed in NaCl-treated plants, previous studies show that these genes were induced in other rice cultivars exposed to different doses of NaCl [23,30-32,35]. In these previous studies, the researchers applied low or medium doses of salt stress and sometimes used tolerant rice cultivars. In our study, however, a high dose of salt stress (20 dS/m) applied to a salt-sensitive rice cultivar could justify the down-regulation of most of genes in NaCl-treated plants. Furthermore, the high concentration and activity rates of Na^+ and Cl^- ions recorded in the culture solution as well as their high mobility in plant would have contributed to the genes repression. This proves that the expression profile of the same gene differs depending on the plant cultivar, level and type of saline stress applied. Quinet et al. [44] reported that transcriptomic analysis provides different results considering the duration of stress exposure and we tested only a short duration (48h) of exposure to high stress intensity. It cannot be excluded that exposure to a longer duration will modify the expression of other genes or even the same gene in an opposite way.

Moreover, numerous recent studies consider that alternative splicing is playing a significant role in regulating gene expression in response to environmental stimuli including salinity [30,72] and complementary experiments using RNAseq procedures may thus be useful to afford

additional information regarding comparative effect of NaCl and Na_2SO_4 on gene expression.

Conclusion

Our study reported for the first time the results of rice genes expression patterns in response to sulfate stress. The expressed genes were mainly grouped into five classes of biological function, namely the genes involved in signal sensing, perception and transduction, transcription control, osmoprotection, chaperone function and transport. It has been revealed that many genes up or down-regulated by chloride stress exhibited a distinct expression profile under sulfate stress. The gene expression profile also differed depending on the type of plant organ. NaCl remained more toxic than Na_2SO_4 for some physiological parameters as proved in our previous studies. This could be explained by the high concentration and activity rates of Na^+ and Cl^- ions recorded in culture solution as well as the high mobility of these ions in plant The high stress intensity applied to salt-sensitive rice cultivar might justify the down-regulation of most of genes in NaCl-treated plants. Unlike some genes, the expression profile of a large number of genes corresponded to the observed changes in the metabolic process and physiological properties; underlying the importance of an integrated study to elucidate the global plant response.

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Appendix

S1 [supplemental data 1]: Primers, annealing temperature (Tm) and number of cycles used in quantitative real-time PCR (qPCR).

Gene name	Primers sequences	Tm (°C)	Size (bp)
Actin-1			
LOC4333919	F: 5'-GTATTGTGTTGGACTCTGGTGATGGTGT-3' R: 5'-GATGGATCCTCCAATCCAGACACTGTA-3'	60	587
RCI2 (rare cold-inducible 2) family protein, Drought resistance			
Os03g0286900	F: 5'-GGCTTGGGTGTAAGTTGCAT-3' R: 5'-CCGGGATGTATCCCAGTATG-3'	60	625
Similar to Serine/threonine protein kinase.			
Os03g0339900	F: 5'-TTGGTGCTCAAAAGTTGCAG-3' R: 5'-TTGGGTACAAACCGGAGAAG-3'	60	4428
Sodium/sulphate symporter family protein.			
Os08g0503700	F: 5'-GGAAGAGGGAGGGTGAGAAG-3' R: 5'-GTCGTGGTTGAGTCGTCAGA-3'	60	241
Similar to Sugar transporter protein.			
Os10g0360100	F: 5'-GTGTGGATGACGTGGAAGTG-3' R: 5'-CCACACGACACCAGAATGAC-3'	60	167

F: Forward R: Reverse

Interchapter III–IV

What do we know?

Chapter III gave clear answer to question n°4 and provided evidences that iso-strength concentration of NaCl and Na_2SO_4 have distinct impact on gene expression in roots and shoots of the salt-sensitive cv IKP. Differences between the impact of the two salts were recorded for genes involved in signal sensing, perception and transduction, control of transcription, osmoprotection, chaperone functions as well as solute and ion transport. It has to be noticed, however, that the use of bioinformatics tools may lead to confusion in as much as gene attribution to a given category may, in some cases, be somewhat arbitrary. For example, it is not easy to establish a clear difference between "genes involved to abiotic stimulus" and those involved in "response to stress". As a consequence, it appears that a given gene may be attributed to several categories. Chapter III also demonstrated that most of the genes involved in response to Na_2SO_4 were up-regulated while those whose expression was modified by NaCl were frequently down-regulated.

It is tempting to compare the data obtained in Chapter I and III considering that, in both cases, the salt-sensitive cultivar IKP was exposed to salinity at the seedling stage under similar semi-controlled environmental conditions. However, the modalities of stress imposition were quite different in the two cases: seedlings were abruptly exposed to high salinities for a short period of 2 days in Chapter III while this material was exposed to lower salt concentrations for a longer period of 2 weeks in Chapter I. These two types of strategy led to different pattern of Na^+ distribution within the plant: in response to higher doses, Na^+ accumulated to similar concentrations in shoots of NaCl- and Na_2SO_4-treated plants (Chapter III; Figure 2) while shoot Na^+ concentration was higher in NaCl-treated plants than in Na_2SO_4-exposed ones for lower salinities (Chapter I; Figure 2). Clear differences were also recorded for the roots, with a higher accumulation than in shoots in Chapter III and an opposite trend in Chapter I. This suggests that the root Na^+ sequestration which is commonly considered as a strategy to protect salt-sensitive photosynthetic organs were not operating with a similar

efficiency in the two situation. Since Na^+ may be absorbed by K^+ transporters, this could also explain why K^+ concentration strongly decreased in roots of plants exposed to a short term exposure (Chapter III) but remained unaffected in the shoot while an obvious decrease in the shoot K^+ concentration was recorded in response to a long term exposure. This suggests that salinity interferes with K^+ nutrition first during absorption processes but that an impact on the translocation leading to a shoot K^+ decrease requires a longer period to be observed.

What is our question?

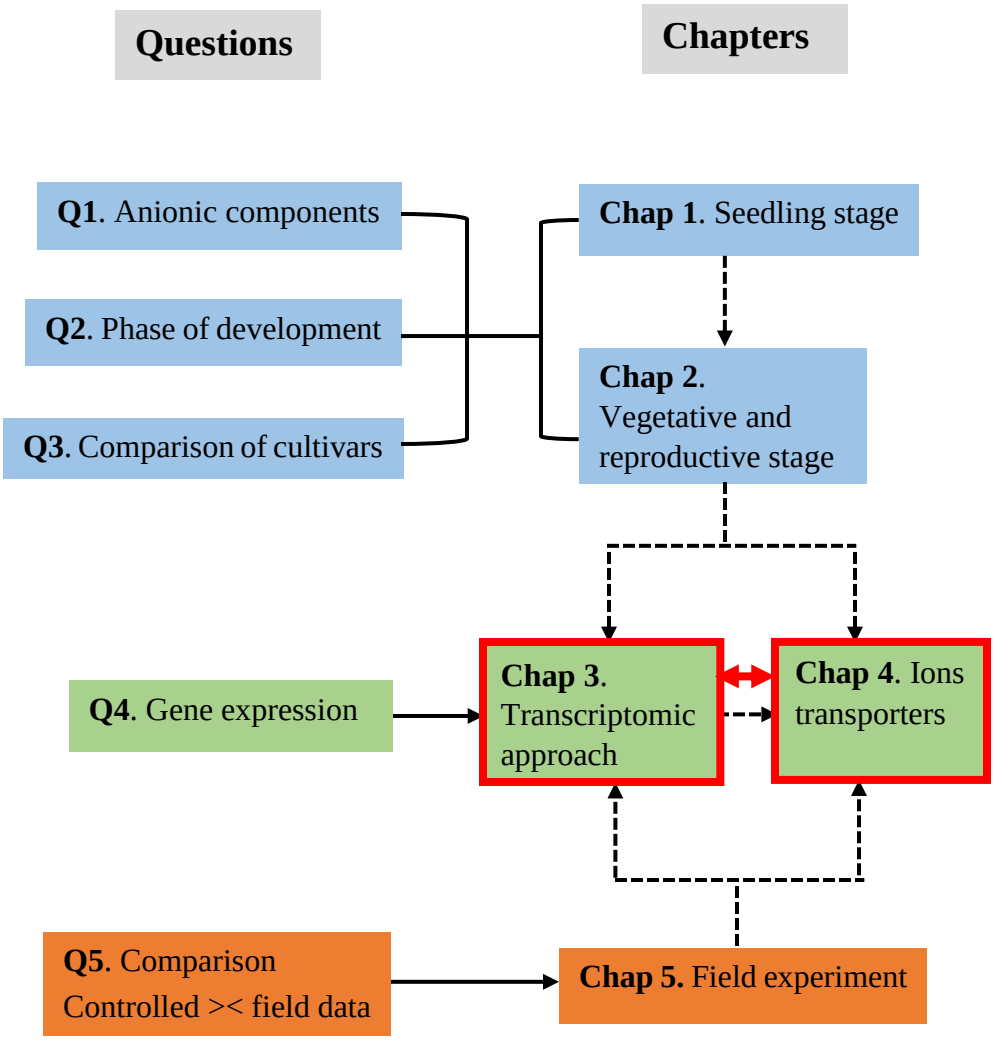
Physiological impacts of Na^+ accumulation (and K^+ deficiency) is not only dependent on the absolute concentration of the considered elements but also depend on their distribution among tissues and between cell compartments. For example, it is widely accepted that Na^+ accumulation in the apoplasm and in vacuoles is quite less toxic for metabolism than accumulation in the cytosol. Reliable distribution of light elements such as Na^+ however requires expensive sophisticated tools, such as synchrotron approaches, which were not affordable and available in the framework of the present study.
An indirect approach to gain information regarding this important aspect is to analyze the expression of genes coding for ion transporters involved in Na^+ compartmentation. The next question is to determine if NaCl and Na_2SO_4 have similar or different effects on gene expression coding for these transporters and if these impacts differ according to the duration of exposure to the considered stressor.

What is our strategy?

The salt-sensitive rice cultivars IKP used in the previous chapters will be cultivated in Chapter IV under nutrient solution. The next experiment intends to provide additional information to the previous transcriptomic approach by focusing on the expression of genes coding for high affinity K^+ transporters (HKT) which play an important role in long-distance Na^+ transport, and for tonoplast localized Na^+/H^+ exchanger involved in vacuolar Na^+ sequestration. The gene coding for *SOS1*, a plasma

membrane-localized antiporter responsible for Na^+ exportation outside the cells will also be considered.

Previous chapters showed that plants behavior differ when exposed for short periods to high stress intensities, or for long period to lower levels of salinity. It is not clear, at this stage, if those discrepancies resulted from differences in stress intensities, duration of exposure, or both. In Chapter IV, we established a compromise and decided to use a medium (although realistic) dose of 100 mM NaCl and 50 mM Na_2SO_4 and to perform a time scale analysis after 3h, 24h and 7 days of exposure.



Synthetic overview of the five research questions addressed during our thesis project and the specific source of their answers in the different chapters of the thesis.

Chapter IV. The differential effects of NaCl and Na_2SO_4 salinities on Na^+-transport regulatory mechanisms in salt-sensitive rice cultivar (*Oryza sativa* L.)

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Abstract

Abiotic stressors, such as high salinity, greatly affect plant growth. In attempt to explore the difference between the mechanisms underlying NaCl and Na_2SO_4 tolerance in rice via excess Na^+ exclusion or sequestration, we analyzed the expression profile of the genes encoding Na^+ transport and we evaluated the salt effect on some physiological parameters. We exposed I Kong Paô (Salt-sensitive rice cultivar) seedlings to 100 mM NaCl or 50 mM Na_2SO_4 (electrical conductivity of c.a. 10 dS m^{-1}) for 3h, 24h and 1 week. The results indicated that both types of salt stress decreased the shoot DW after 1 week and no effect was detected at root level. Salt stress only decreased the water content in shoot and the highest reduction in shoot Ψ_S was observed in NaCl-treated plants after 24h. Both NaCl and Na_2SO_4 induced a strong increase in shoot- and root-Na^+ contents and Na^+ accumulation was higher in NaCl- than in Na_2SO_4-treated plants. K^+ content highly decreased in NaCl-treated roots after 24h but salt stress did not affect K^+ contents in shoots. In order to understand those differences in Na^+ contents, we analyzed transcript levels of genes encoding Na^+ transport proteins in different tissues. In response to salt stress, *OsSOS1* was greatly induced in Na_2SO_4-treated shoot after 24h and showed a similar expression profile in salt-treated shoots after 3h. *OsNHX1* and *OsNHX2* were expressed differently. *OsNHX1* was only induced in NaCl-treated shoots after 3h while it was highly induced in all salt-treated roots after 24h. *OsNHX2* was up-regulated in salt-treated shoots after 3h and no significant expression was detected in root after 3h and 1week. *OsHKT1, OsHKT5, OsHKT7, OsHKT8* and *OsHKT9* expression was not detected in shoot or root for all treatment conditions. *OsHKT2, OsHKT3, OsHKT4* and *OsHKT6* displayed the different expression profiles. We concluded that the gene expression profiles differed depending on the type of applied salt stress, plant organs and saline stress duration.

Key words: NaCl, Na_2SO_4, salinity, Na^+-transport, regulatory mechanisms

Introduction

Food production is highly hampered by various environmental constraints and soil salinity is one of the major abiotic stresses adversely affecting crop productivity. Salinity stress has detrimental effects to many cellular and physiological processes including photosynthesis, nutrient uptake, water absorption, root growth and cellular metabolism, which all contribute to crop yield reduction (Pardo 2010).

Excess sodium ion (Na^+) causes an imbalance in cellular ion homeostasis, resulting in ion toxicity (Assaha et al. 2013). In plant, the excess Na^+ is harmful whereas the potassium ion (K^+) is an essential element. Therefore, cellular Na^+ and K^+ homeostasis plays a fundamental role in plant growth and development. Potassium is involved in osmoregulation, plant-water relation and internal cation/anion balance, enzyme activation and stomatal movement (Rawat et al. 2016). Under saline conditions, increased Na^+-influx frequently induces a decrease in K^+ uptake by plants, which could be related to competitive effect of Na^+ on some K^+ transporters (Maathuis et al., 2014). The adverse effect resulting from this competition is a drastic decrease in K^+/Na^+ ratio within plant tissues (Hasegawa 2013; Almeida et al., 2017).

The mechanisms aiming to alleviate Na^+ toxicity in plant tissues have been proposed by some researchers. They include limiting Na^+-absorption from the soil, reducing the transport of Na^+ to the xylem, storing Na^+ in the lower part of the leaf (such as the sheath) or in the oldest leaves, ensure compartmentation of Na^+ into the vacuole, and cycling Na^+ from shoots to roots (Tian et al. 2010). Several types of transporters mediate the transmembrane movement of Na^+ and K^+ in plants (Yao et al. 2010), and a number of transporters have been involved in leaf-Na^+ exclusion. These include members of the high-affinity K^+ transporters (*HKTs*) which play an important role in long-distance Na^+ transport and plant salt tolerance. Thanks to *HKT* gene family, overaccumulated Na^+ in shoots is loaded in the phloem and unloaded from the phloem in roots. The nine *HKT* genes found in rice are divided into subfamily 1 and subfamily 2 (Waters et al. 2013). Subfamily 1 *HKT* transporters (*OsHKT4*, *OsHKT5*, *OsHKT6*, *OsHKT7, OsHKT8*) are described as Na^+ uniporters, whereas subfamily 2

HKT transporters (*OsHKT1*, *OsHKT2, OsHKT3, OsHKT9*) are thought to allow both Na^+ and K^+ transport and Na^+ uniport at high Na^+ concentrations. The rice Na^+-transporter *OsHKT*1 has been shown to mediate Na^+-influx into roots under K^+ starvation (Horie et al. 2007). Other members of the *HKT* gene subfamily 1 are thought to mediate Na^+ influx in root cells and to regulate the Na^+ distribution between roots and shoots (Horie et al. 2009; Horie et al. 2005).

The regulation of stable K^+ acquisition and distribution in plant is required in order to better control ion homeostasis during salt stress. The tonoplast-localized Na^+/H^+ exchangers (*NHX1* and *NHX2*) and the plasma membrane-localized Na^+/H^+ antiporter (*SOS1*), are expected to play important roles in osmoregulation and in maintaining low cytoplasmic Na^+ concentration in plant cells (Hanin et al. 2016). The authors confirm that most *NHX* proteins are essential for Na^+ detoxification through sequestration into the vacuole, whereas *SOS* signaling pathways are responsible to export Na^+ outside the cell. Roy and Chakraborty (2018) recently found that in Swarnadhan rice cultivar, *OsSOS1*and Os*NHX* genes family did not produce any substantial evidence of overexpression thereby confirming the relative susceptibility of this rice cultivar in response to NaCl stress. Unlike these results, Fukuda et al. (2011) showed that the expression of *NHX1* and *NHX2* was differently regulated in various rice tissues (Nipponbare cv.). Compared with other organs, the transcript levels of *OsNHX1* were higher in panicles, flag leaf sheaths, and seedling shoots and those of *OsNHX2* were higher in flag leaf sheaths, culms, and panicles. Studying the expression profile of *OsSOS1* in two rice cultivars (Sakha 102 and Egyptian Yasmine) under 50 mM NaCl stress, Mekawy et al. (2015) observed a higher level of induced *OsSOS1* expression in the Sakha 102 roots, which might be responsible for the relatively low Na^+ levels in its roots while *OsSOS1* was not induced in Egyptian Yasmine roots. *OsSOS1* was down-regulated in the leaves and sheaths of Egyptian Yasmine cultivar under saline conditions and according to the authors, the *OsSOS1* mediated Na^+ extrusion from the cytosol may not be active in Egyptian cultivar.

An immense amount of research has been devoted to the impact of NaCl on the plant ion nutrition in various plant species, especially in rice which is commonly considered as a salt-sensitive crop (Lutts et al., 1995; Grattan

et al., 2002). In field conditions, however, other salts may contribute to salinity and this is especially the case for Na_2SO_4 (Kimura et al., 2004; Fort et al., 2014; Reich et al., 2017; Aghajanzadeh et al., 2018). Although sodium sulfate also exposes the plant to Na^+ excess, there is no exhaustive proofs that the counter-anion has no influence on Na^+ transporters and previous study performed with rice cell line even suggested that NaCl and Na_2SO_4 had different impact on Na^+ accumulation within cells (Lutts et al., 1996a). We previously studied the differential effects of NaCl and Na_2SO_4 salinities on genes expression patterns via a transcriptomic approach performed on the rice cultivar I Kong Pao (Irakoze et al., unpublished). We found that many genes encoding transporters putatively involved in Na^+ absorption were differently expressed in shoot and/or in root depending on the type of saline stress applied. For example, the gene coding for vacuolar Na^+/H^+ antiporter protein (*OsNHX2*) was only induced in Na_2SO_4-stressed shoots and roots. The gene coding for *HKT* family protein (*OsHKT7*) involved in Na^+ exclusion from leaf blade upon salt stress was repressed in NaCl-stressed shoot and no expression was detected in other treatments. The gene coding for Na^+ /K^+ symport (*OsHKT1*) was only induced in Na_2SO_4-stressed roots. We also observed that all genes coding for the enzymes activating the plasma membrane Na^+-transport were up-regulated.

A transcriptomic study gives an overview of the gene expression profile in the plant, but some specific details remaining hidden. In addition, it is highly important to determine the influence of the anionic component of salt stress on the expression profile of the genes encoding Na^+ transport at different time points while the data available in the literature on this aspect are almost absent, most published studies focusing on NaCl stress only. Quinet et al. (2012) indeed demonstrated that transcriptomic response of rice to mineral toxicities not only depend on the stress intensity but is also a direct function of stress duration: according to this study, less than 3% of identified genes were similarly affected in rice exposed to iron toxicity for 3 days or for 3 weeks. It appears justified to deepen our previous transcriptomic approach by focusing on gene expression coding for Na^+ transporters after different duration of exposure to NaCl or to Na_2SO_4 in order to improve our knowledge on the kinetic of plant response.

Therefore, the objective of this study was to elucidate differences in the mechanisms of salinity tolerance by comparing the expression profiles of

some genes encoding putative Na^+ transport proteins in shoots and roots of the IKP salt-sensitive rice cultivar exposed to 100 mM NaCl or 50 mM Na_2SO_4 (EC of c.a. 10 dS m^{-1}) for 3h, 24h or 1week. The gene expression analysis was performed by the semi-quantitative RT-PCR method.

Materials and methods

Plant culture and growing conditions

Seeds of the rice cultivar I Kong Pao (IKP; salt-sensitive) were obtained from IRRI (International Rice Research Institute; Philippines). Seeds were germinated on two layers of filter paper (Whatman no. 2) moistened with 10 mL of sterile deionized water in a growth chamber at 25°C under a 12 h daylight period (120 µmol m^{-2} s^{-1}). Ten-day-old seedlings were transferred into a phytotron and fixed on polystyrene plates floating on Yoshida nutritive solution(Cock et al. 1976). Illumination was provided by Sylvania fluorescent tubes (F96T12/CW/VHO) for 12 h d^{-1} at a photon flux density (PAR) of 300 µmol m^{-2} s^{-1}. Daytime humidity was between 60% and 80%, and the temperature was maintained at 29 °C during the day and 26 °C during the night. For each treatment, seedlings were distributed among five tanks (six seedlings per tank) containing 1.3 L of solution. The solutions were renewed weekly and pots were randomly rearranged in the phytotron. After two weeks of acclimatization in control conditions, seedlings were stressed by adding 100 mM NaCl (Chl) or 50 mM Na_2SO_4 (S) (electrical conductivity of *c.a.* 10 dS m^{-1}) to the nutrient solution for 3 hours, 24 hours and 1 week. No salt was added to the control treatment.

The pH values recorded in the culture solutions salinized with 50 mM Na_2SO_4 and 100 mM NaCl were 6.32 and 5.88 respectively. In addition, a chemical speciation of the culture solutions salinized with NaCl and Na_2SO_4 was performed using Visual Minteq software. The identified species are presented in appendix 2 of this document. The concentration and activity rates of Na^+, Cl^- and SO_4^{2-} are presented in results section.

Estimation of ion and relative water content, dry weight and osmotic potential

After 3 hours, 24 hours and 1 week, 10 randomly-chosen plants per treatment were harvested. Roots were rinsed for 30 s in deionized water to remove ions from the free spaces. Roots and shoots were separated and weighed. After drying the samples at 70°C during 72 hours in an oven, dry weight (DW) was estimated. The water content (WC) was calculated as: WC= [(Fesh Weight-Dry Weight)/Fresh Weight] x100.

For shoots and roots, 20 mg DW were 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^+ and Na^+) by flame emission using Atomic Absorption Spectrophotometer (Thermo scientific S series model AAS4) and anion (sulfur) were quantified by liquid chromatography (HPLC-Dionex ICS2000). 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 10000 rpm for 10 minutes at 25°C was subsequently filtered and then quantified via liquid chromatography (HPLC-Dionex ICS2000).

For the determination of osmotic potential (Ψ_S), the last fully expanded leaf and the roots were removed from the plant and cut in small segments. The samples were then separately introduced in Eppendorf tubes perforated with four small holes, frozen in liquid nitrogen for 30 s and warmed again at room temperature (three cycles of gel/thaw) in order to break the cellular membrane (Lutts et al. 1999a). Tubes were then encased in the second intact Eppendorf tubes and centrifuged at 8,000 *g* during 15 min at 4°C. The osmolarity of collected tissular sap was assessed with the Wescor vapor pressure osmometer (W5500) and converted to Ψ_S according to Van't Hoff equation.

Gene expression analyses

Shoot and root were separated from the seedlings of IKP salt-sensitive cultivar and immediately frozen in liquid nitrogen. For each treatment, total RNA was isolated from 100mg of pooled plants taken from each of the three biological replicates using the RNeasy Plant Mini Kit (Quiagen, Germany). The RNA quality and concentration were assessed 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 GoScript^{TM} Reverse Transcription Mix, Oligo (dT) Protocol Kit (Promega Benelux b.v., Leiden, The Netherlands) by following the manufacturer's instructions.

At least three independent PCR amplifications were conducted for each gene using the primer pairs, annealing temperatures, and number of cycles presented in appendix (S1) of this chapter. Expression differences were analyzed by gel densitometry using ImageJ software and expressed as relative values compared to *Osactin* and *OsEF1α* expression [Avarage (peak size of target gene/peak size of *Osactin*;peak size of target gene/peak size of *OsEF1α*)]. Gene expression analyses were technically repeated three times on one independent culture and gave similar results. This study involved twelve genes namely *OsSOS1*, *OsNHX1*, *OsNHX2*, *OsHKT1*, *OsHKT2*, *OsHKT3*, *OsHKT4*, *OsHKT5*, *OsHKT6*, *OsHKT7*, *OsHKT8* and *OsHKT9*.

Statistical analysis

Each experiment was repeated three times and led to statistical similar results. Normality tests were performed and no further transformation of the raw data was required. An analysis of variance (ANOVA) was performed on all data set using JMP Pro 14 software; variance analysis with one factor (stress type), with two crossed fixed factors (stress type x stress time) or with three crossed fixed factors (stress type x stress time x organ type). Differences between means were scored for significance according to Tukey's test.

Results

The pH values recorded in this experiment were within the acceptable range for good plant growth (5.5-6.5). These pH values were approximately close to the pH values recorded in Chapter III. For those two chapters, the pH values were relatively higher in Na_2SO_4- than in NaCl-treated solutions.

Considering that the identified species did not vary depending on the stress intensity, the data presented in appendix 2 still valid for all studies conducted in hydroponic culture. Regarding NaCl stress, the concentration and activity rates of Na^+ in culture solution were 96.79% and 75.18% respectively. The concentration and activity rates of Cl^- were 96.94% and 75.3% respectively. In Na_2SO_4-treated solutions, the concentration and activity rates of Na^+ were 92.48% and 70.5% respectively while the concentration and activity rates of SO_4^{2-} were 82.97% and 27.97% respectively.

Physiological parameters

For most studied physiological parameters, the impact of salt stress depended on the type of salt and duration of exposure as well as the considered plant organ. Salinity applied for a short term had no impact on shoot and root dry weights but NaCl and Na_2SO_4 significantly decreased the shoot biomass to the same extent after one week of treatment (Fig. 1). Conversely, no salt stress impact was detected at the root level. Shoot dry weight was higher than root dry weight for all treatments.

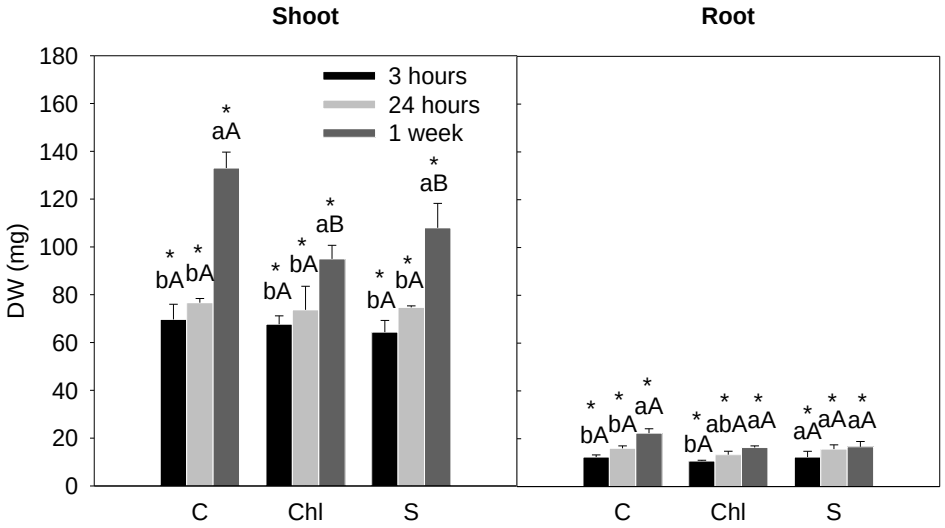

Figure 1. Shoots and roots DW of rice seedlings from IKP cv. cultivated during 3 hours, 24 hours or 1 week in control conditions (C) or in the presence of 100 mM NaCl [chloride salinity (Chl)] or 50 mM Na_2SO_4 [sulfate salinity (S)]. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Stress duration followed by the same lowercase letter for a particular treatment do not differ statistically. Treatments followed by the same uppercase letter for a particular stress time do not differ statistically. Plant organs followed by the asterisk (*) for a particular stress time in each treatment differ statistically.

The water content in shoot significantly decreased already twenty-four hours after the application of salt stress and the reduction in water content intensified during the following days (Fig. 2). No significant differences were observed between chloride and sulfate salinities. Comparatively to control plants, the salt stress did not significantly decrease the water content in the roots.

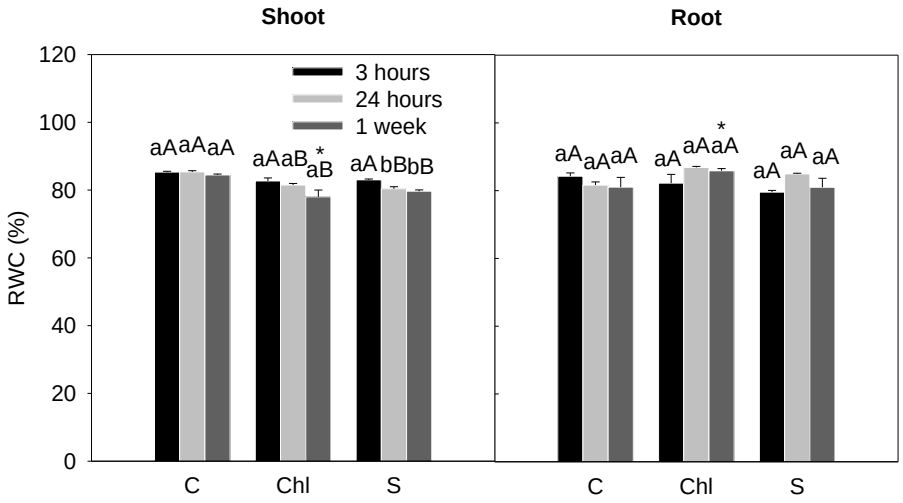

Figure 2. Shoot and roots RWC of rice seedlings from IKP cv. cultivated during 3 hours, 24 hours or 1 week in control conditions (C) or in the presence of 100 mM NaCl [chloride salinity (Chl)] or 50 mM Na_2SO_4 [sulfate salinity (S)]. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Stress duration followed by the same lowercase letter for a particular treatment do not differ statistically. Treatments followed by the same uppercase letter for a particular stress time do not differ statistically. Plant organs followed by the asterisk (*) for a particular stress time in each treatment differ statistically.

The osmotic potential decreased more significantly in shoot of salt-treated plants than in root. The most noticeable reduction in shoot Ψ_S was observed after twenty-four hours in NaCl-treated plants, although a significant decrease was observed already after a 3h-exposure (Fig. 3). Na_2SO_4 salinity also significantly decreased the Ψ_S twenty-four hours after saline stress application and, overall, the impact of chloride salinity was higher than the impact of sulfate. Unlike the salt stress effect on shoot Ψ_S, a decrease in root Ψ_S was detected only one week after salt application and both types of salinity had a similar effect.

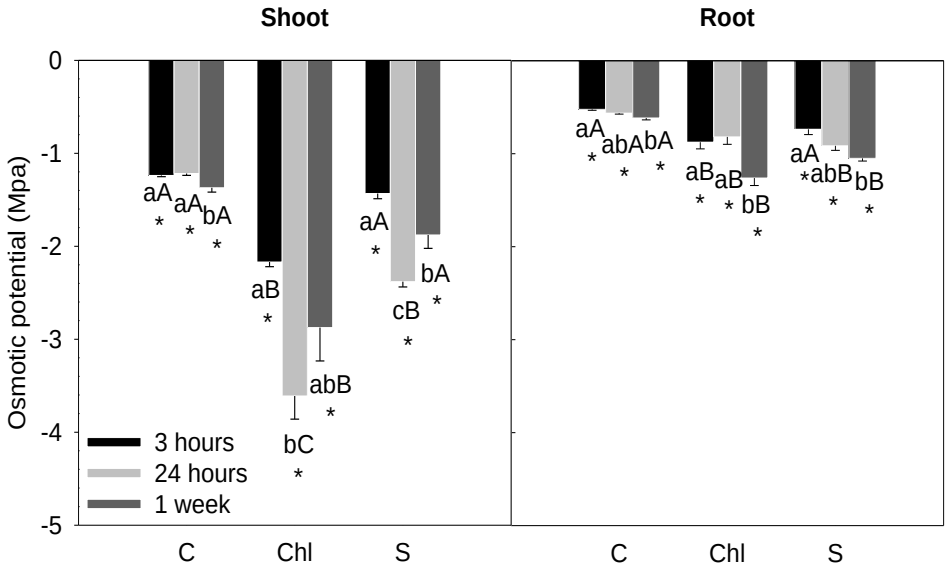

Figure 3. Shoot and root Ψ_s of rice seedlings from IKP cv. cultivated during 3 hours, 24 hours or 1 week in control conditions (C) or in the presence of 100 mM NaCl [chloride salinity (Chl)] or 50 mM Na_2SO_4 [sulfate salinity (S)]. Each value is the mean of six replicates per treatment and vertical bars are standard errors of the mean. Stress duration followed by the same lowercase letter for a particular treatment do not differ statistically. Treatments followed by the same uppercase letter for a particular stress time do not differ statistically. Plant organs followed by the asterisk (*) for a particular stress time in each treatment differ statistically.

The two types of salinity induced a strong increase in shoot- and root-Na^+ contents already after 3h of treatment (Fig. 4). As far as shoots are concerned, Na^+ accumulation was obviously higher in plants exposed to NaCl salinity than in those exposed to Na_2SO_4 while no significant differences were observed between the two types of salinity at the root level. Overall, Na^+ accumulated to a higher extent in shoot than in root of NaCl-treated plants while no significant differences were detected between the two plant organs in Na_2SO_4-treated plants. In control plants, K^+ concentration was higher in the roots than in the shoot. Salinity had no significant impact on the shoot K^+ concentration, whatever the considered

salinity or duration of exposure. In contrast, salt stress significantly reduced root K^+ concentration and the recorded decrease was more marked for Na_2SO_4-treated plants after 24h than in plants exposed to NaCl. Both NaCl and Na_2SO_4 salinities increased the Na^+/K^+ ratio in shoot and the former induced a more pronounced effect while the later exhibited an intermediate behavior after 24h and 1 week. Regarding the roots, both types of salinity affected the Na^+/K^+ ratio in the same way and the highest ratio was observed after one week of saline stress.

As expected, chloride and sulfur accumulations were observed mainly in NaCl- and Na_2SO_4-treated plants, respectively (Fig. 5). Cl^- and S concentrations in both shoot and root significantly increased over time but the recorded increase was already significant after 3h of treatment. The two considered elements exhibited a higher accumulation in the shoot than in the root.

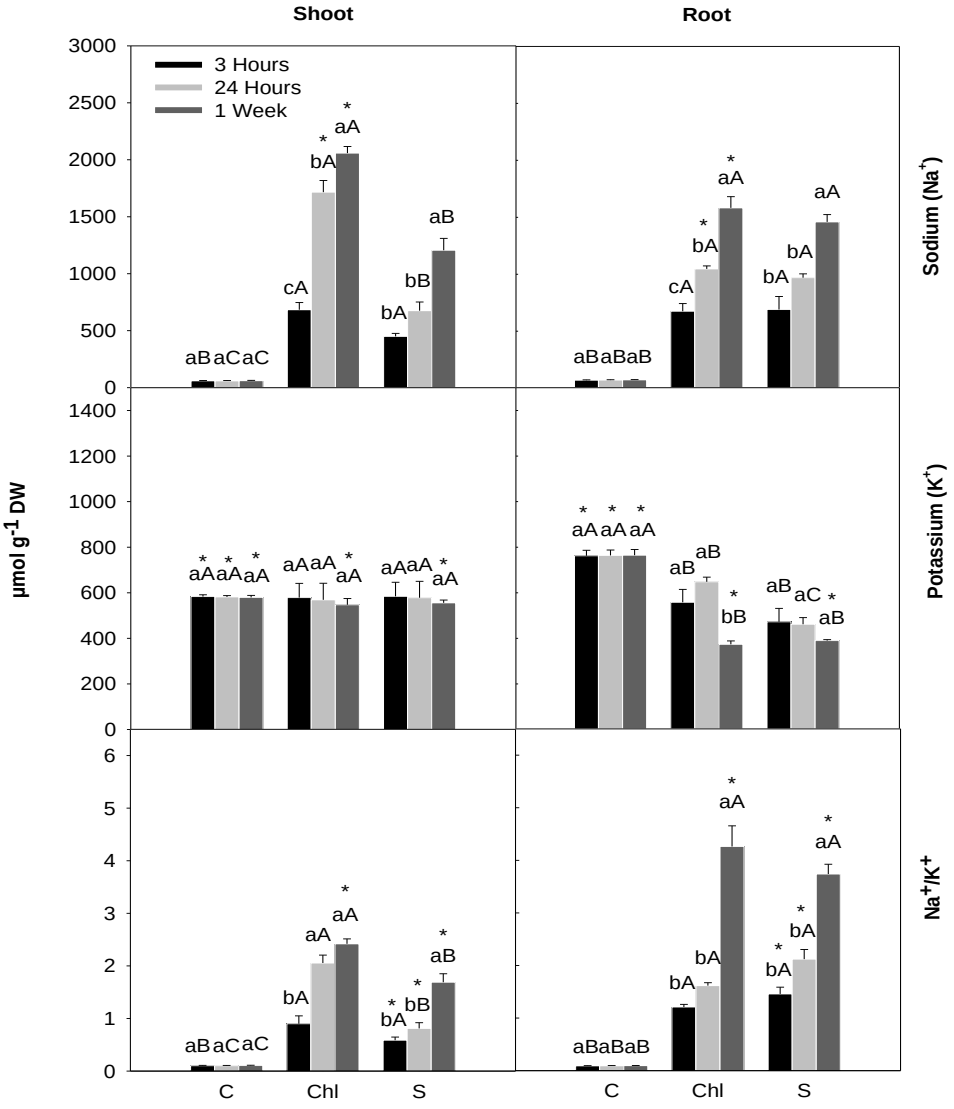

Figure 4. Cation concentrations in shoot and root of rice seedlings from IKP cv. cultivated during 3 hours, 24 hours or 1 week in control conditions (C) or in the presence of 100 mM NaCl [chloride salinity (Chl)] or 50 mM Na_2SO_4 [sulfate salinity (S)]. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Stress duration followed by the same lowercase letter for a particular treatment do not differ statistically. Treatments followed by the same uppercase letter for a particular stress time do not differ statistically. Plant organs followed by the asterisk (*) for a particular stress time in each treatment differ statistically.

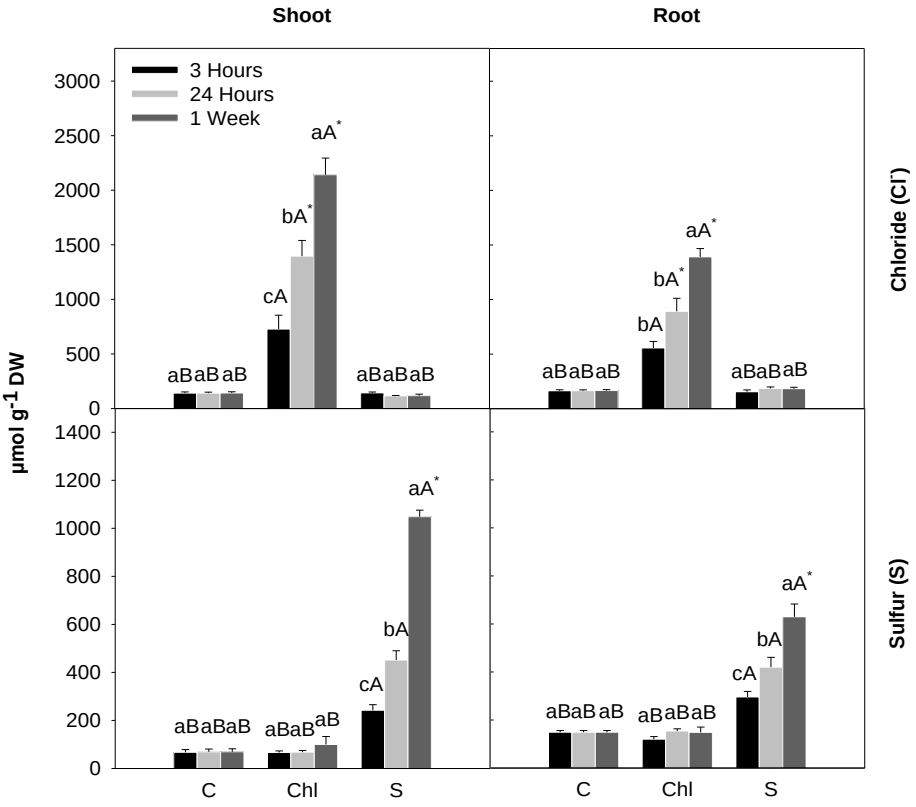

Figure 5. Anion concentrations in shoot and root of rice seedlings from IKP cv. cultivated during 3 hours, 24 hours or 1 week in control conditions (C) or in the presence of 100 mM NaCl [chloride salinity (Chl)] or 50 mM Na_2SO_4 [sulfate salinity (S)]. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Stress duration followed by the same lowercase letter for a particular treatment do not differ statistically. Treatments followed by the same uppercase letter for a particular stress time do not differ statistically. Plant organs followed by the asterisk (*) for a particular stress time in each treatment differ statistically.

Analyses of Na^+ transport-related genes expression profile in rice shoot and root under NaCl and Na_2SO_4 stresses

OsSOS1 was highly induced in the shoot of control plant after one week (Fig. 6). Comparatively to control plants, *OsSOS1* was more expressed in the shoot of NaCl-treated plants after 3h and 24h exposure but it was highly

down-regulated after one week with more than 6-fold reduction. After 24h, the highest *OsSOS1* expression was detected in shoot treated by sulfate salinity while *OsSOS1* showed a similar expression pattern after 3h in plants exposed to NaCl and Na_2SO_4. *OsSOS1* expression also drastically decreased in the shoot of Na_2SO_4-treated plants after one week of treatment. At the root level, the highest expression of *OsSOS1* was detected 24h after saline stress application and no significant differences were observed between both types of salinity. The salt stress also significantly increased the expression of *OsSOS1* in salt-treated root after 3h and expression level was similar after one week.

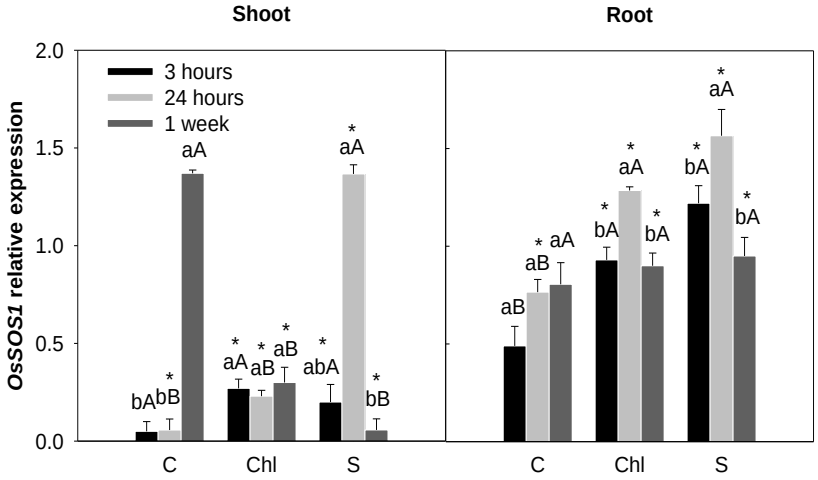

Figure 6. Semi-quantitative RT-PCR expression analysis of *OsSOS1* coding for sodium ion transport in shoot and root of rice seedlings from IKP cv. cultivated during 3 hours, 24 hours or 1 week in control conditions (C) or in the presence of 100 mM NaCl [chloride salinity (Chl)] or 50 mM Na_2SO_4 [sulfate salinity (S)]. *OsActin* and *OsEF1α* transcripts were used as a PCR control. Relative expression level was analyzed by gel densitometry. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Stress duration followed by the same lowercase letter for a particular treatment do not differ statistically. Treatments followed by the same uppercase letter for a particular stress time do not differ statistically. Plant organs followed by the asterisk (*) for a particular stress time in each treatment differ statistically.

As far as *OsNHX1* was concerned, we observed a similar expression profile in the shoots of control and Na_2SO_4-treated plants (Fig. 7). In both cases, gene expression increased over time. In NaCl-treated plants, *OsNHX1* was highly induced already after 3h in the shoot while the highest expression in root was detected after 24h. In plants exposed to control conditions and to Na_2SO_4, the minimal values of expression for *OsNHX1* were recorded after 24h. Hence, the gene expression profile was very different between shoot and root of the Na_2SO_4-treated plants. In shoot as in root, chloride and sulfate salinities exhibited the different expression profiles of *OsNHX1* depending on stress duration.

OsNHX2 was up- and down-regulated respectively after 3h and 24h in NaCl-treated shoots. The gene expression increased after 3h in Na_2SO_4-treated shoots and then remained constant. The similar expression pattern of *OsNHX2* was observed in shoot under the two types of salinity, except after 24h where the expression was higher under sulfate than chloride salinity. In roots, *OsNHX2* was down-regulated after 24h of NaCl stress and the gene expression level was the same after 3h and 1 week for NaCl- and Na_2SO_4-treated roots. The gene expression profile between the two plant organs (shoot and root) was significantly different under NaCl after 24h and 1 week while a significant difference was only observed after 24h for Na_2SO_4 treatment.

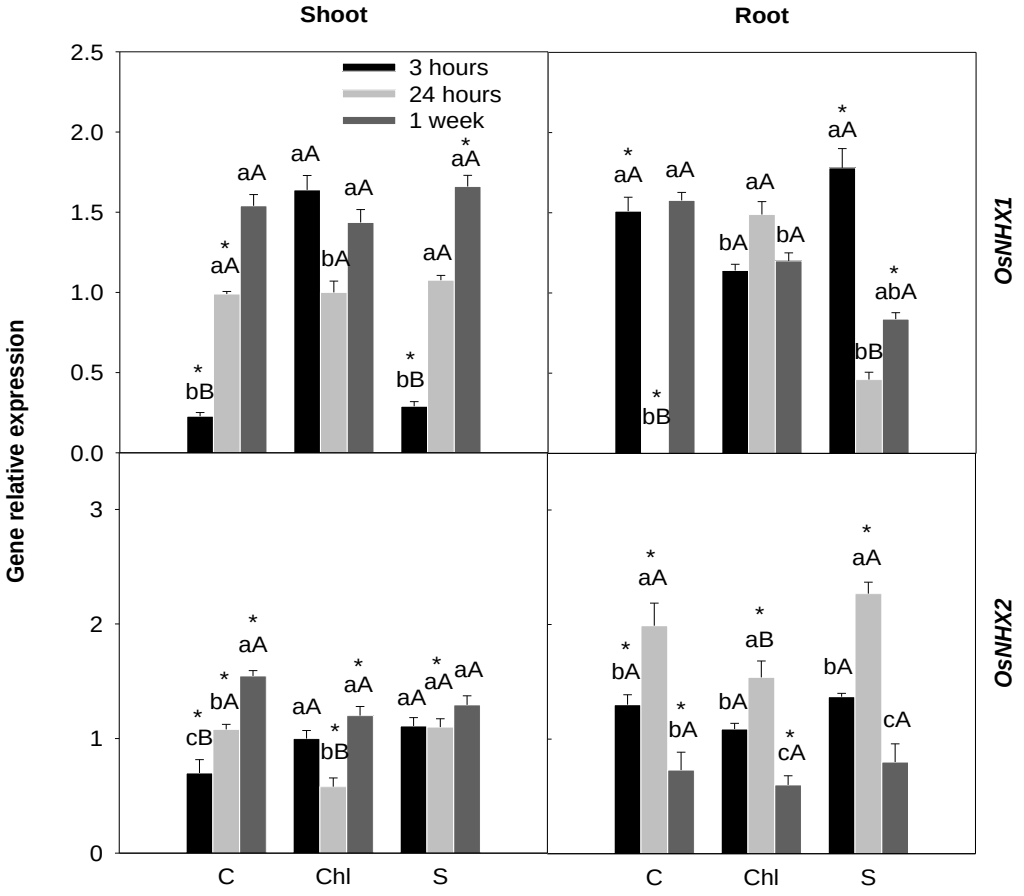

Figure 7. Semi-quantitative RT-PCR expression analysis of *OsNHX* genes family coding for sodium ion transport in shoot and root of rice seedlings from IKP cv. cultivated during 3 hours, 24 hours or 1 week in control conditions (C) or in the presence of 100 mM NaCl [chloride salinity (Chl)] or 50 mM Na_2SO_4 [sulfate salinity (S)]. *OsActin* and *OsEF1α* transcripts were used as a PCR control. Relative expression level was analyzed by gel densitometry. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Stress duration followed by the same lowercase letter for a particular treatment do not differ statistically. Treatments followed by the same uppercase letter for a particular stress time do not differ statistically. Plant organs followed by the asterisk (*) for a particular stress time in each treatment differ statistically.

We did not observe the amplification of five genes, namely *OsHKT1*, *OsHKT5, OsHKT7, OsHKT8* and *OsHKT9*. The amplification was not observed in shoots and roots, whatever the treatment or duration of stress exposure. The amplification of *OsHKT2* was not detected in NaCl-treated shoots after 24h as well as in Na_2SO_4-treated shoots after 3h and 1week. *OsHKT2* was up-regulated in NaCl-treated shoots after 3h and significantly repressed in all salt-treated roots.

OsHKT3 amplification was not observed in roots whatever the treatment as well as in Na_2SO_4-treated shoots. *OsHKT3* was up-regulated in NaCl-treated shoots after 24h unlike in control shoots where the gene did not show any amplification after 24h. No *OsHKT3* amplification was observed after 3h and one week in NaCl-treated shoots, in contrast to control plants. *OsHKT4* was more indiuced in shoots than in roots for all treatment. Its expression was significantly lower in NaCl- than in Na_2SO_4-treated shoots after 3h and 24h while a reverse trend was observed after 1 week. No *OsHKT4* amplification was detected in NaCl-treated roots after 24h and 1 week as well as in Na_2SO_4-treated roots after 3h and 24h.

As observed for *OsHKT3* and *OsHKT4*, *OsHKT6* was less expressed in root than in shoot for all treatments. *OsHKT6* was down- and up-regulated respectively after 24h and 3h in salt-treated shoots. In roots, no *OsHKT6* expression was detected after 1 week for all treatments but a high expression was observed after 24h under NaCl salinity.

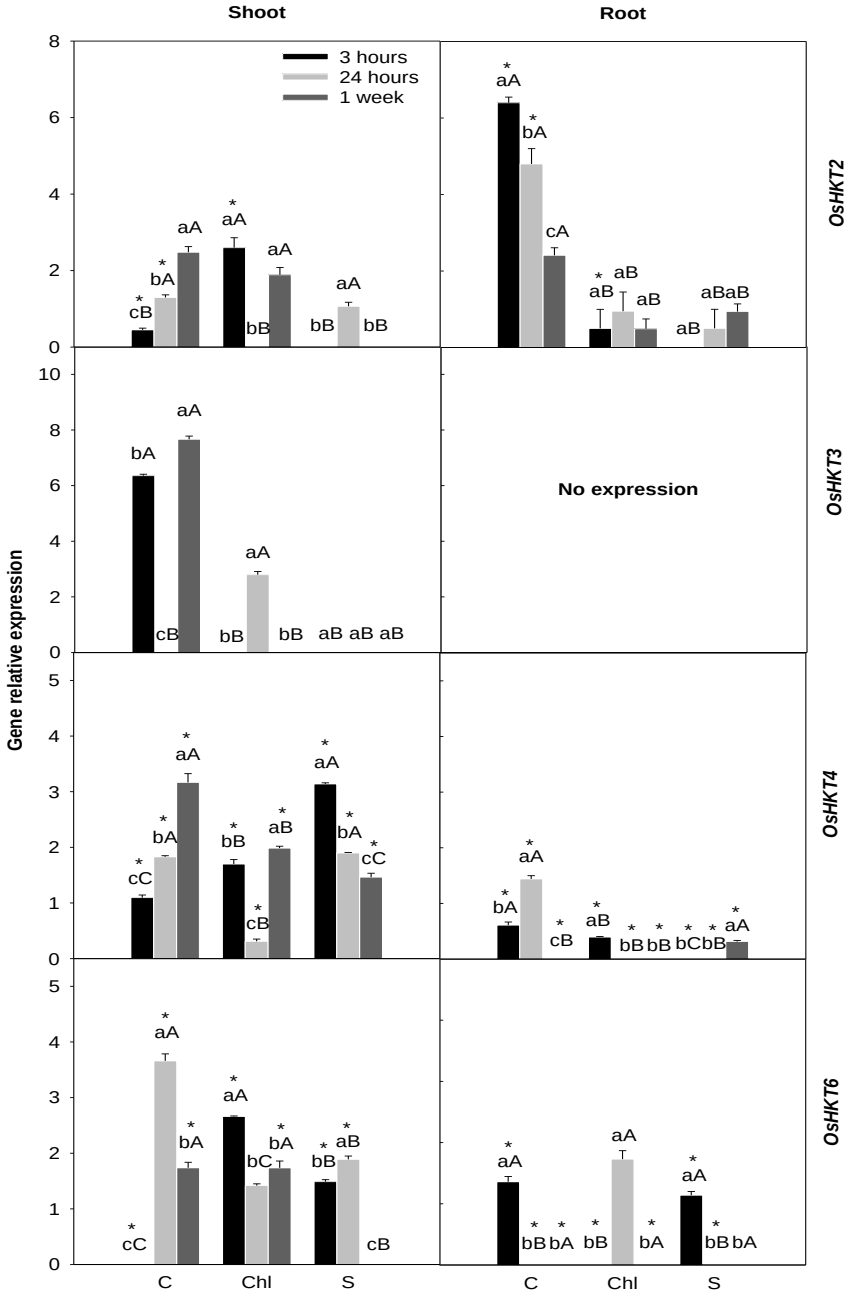

Figure 8. Semi-quantitative RT-PCR expression analysis of *OsHKT* genes family coding for sodium ion transport in shoot and root of rice seedlings

from IKP cv. cultivated during 3 hours, 24 hours or 1 week in control conditions (C) or in the presence of 100 mM NaCl [chloride salinity (Chl)] or 50 mM Na_2SO_4 [sulfate salinity (S)]. *OsActin* and *OsEF1α* transcripts were used as a PCR control. Relative expression level was analyzed by gel densitometry. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Stress duration followed by the same lowercase letter for a particular treatment do not differ statistically. Treatments followed by the same uppercase letter for a particular stress time do not differ statistically. Plant organs followed by the asterisk (*) for a particular stress time in each treatment differ statistically.

Discussion

The pH values were relatively higher in Na_2SO_4- than in NaCl-treated solutions and this could be due to a large amount of Na^+ and SO_4^{2-} which would remain in Na_2SO_4-treated solutions.

The results on the chemical speciation of the culture solutions revealed that the concentration and activity rates of the salt ions differed widely. Despite the equimolar concentration of Na^+ in the initial saline solution, the Na^+-concentration and activity rates were higher when Na^+ was accompanied by the chloride anion than by the sulfate anion. We also observed that the concentration and activity rates of Na^+ were almost the same as those of Cl^- in the case of NaCl salinity. Surprisingly, the concentration and activity rates of the SO_4^{2-} anion were significantly low and that could partly explain a less intense toxicity recorded for Na_2SO_4-treated plants as observed on several studied parameters.

Physiological parameters

Rice is the main food crop grown worldwide and salt stress is one of the major abiotic stress factors restricting not only its growth but also its yield. Under salt stress, reduction in biomass production is a common feature in crop plants. In this study, salinity caused a significant reduction in shoot DW after 1 week (Fig. 1). Amirjani (2011) and Haq et al. (2009) also

showed that NaCl highly decreased the shoot DW of rice. The root biomass was not affected by the salt stress and Irakoze et al. (2019b) previously demonstrated that neither chloride nor sulfate salinities reduced the root biomass of the IKP cultivar. Such a decrease was logically not observed for short-term exposure, and even control plants did not exhibit a significant increase in biomass for such short periods.

It is commonly considered that the first constraint induced by salinity at the plant level is due to a decrease in the external water potential inducing an osmotic stress on plant tissues (Hasegawa, 2013; Maathuis et al., 2014; Almeida et al., 2017). The present work however demonstrated that even a short-term exposure (3h) to NaCl or Na_2SO_4 was sufficient to induce sodium accumulation not only in roots but also in shoots of salt-treated plants. Accumulation up to 500 µmol.g^{-1} DW might be regarded as a toxic level for normal cell metabolism (Lutts et al., 1996b) which suggests that an ionic component of salt stress may also result from short-term exposure to high salt level.

Decrease in Ψ_S should be regarded as a strategy to overcome, or at least limit the impact of osmotic component of salinity. Such an osmotic adjustment was already recorded after 3h and 24h in salt-treated shoots unlike in root where a great reduction in Ψ_S was observed after one week. We deduce that, under saline stress, the rice plant establishes osmotic adjustment mechanisms earlier in the shoots than in the roots. An interesting aspect is to analyze the possibility that a burst in Na^+ occurring very quickly after stress imposition may be precisely involved in this rapid osmotic adjustment, allowing the plant to wait until sufficient soluble organic compounds are produced to assume this function (Munns and Lutts, personal communication). Indeed, the shoot Ψ_S was more strongly reduced under NaCl than Na_2SO_4 and it could be related to a higher Na^+ accumulation in NaCl-treated shoot. Considering the toxicity of Na^+, such a solution should remain transient, except if optimal compartmentation such as vacuolar sequestration occurs to limit Na^+-induced metabolic disorders. The method used to quantify Ψ_S did not allow us to discriminate between individual cell compartments but is a measurement of global Ψ_S at the whole organ level (including possible dilution with apoplastic fluid) but

our data nevertheless suggest that the classical “biphasic model” needs to be revised.

Lutts et al. (1996a) also found that NaCl salinity significantly reduced Ψ_S in roots as in leaves of IKP cultivar. According to the authors, Ψ_S was slightly higher in the youngest leaves than in the oldest ones. Although osmotic adjustment in the shoot was observed after 24 hours, the WC began to decrease at the same time in all salt-treated shoots. This confirm that osmotic adjustment is necessary but not sufficient to ensure the maintenance of an optimal plant water status. A poor stomatal regulation in salt-sensitive cultivars (Lutts et al. 1999a) might explain the low WC recorded in IKP. It has to be noticed, however, that in the present case, the decrease in shoot WC remained limited from a relative point of view and should not strongly impact shoot metabolism in contrast to Na^+ accumulation, reinforcing our view that the water stress component of salinity does not necessarily precede the ionic component.

The sodium ion accumulation in shoots was higher in the case of chloride stress compared to sulfate stress (Fig. 4). This observation suggests that Na^+ uptake is influenced by the nature of anion excess present in the nutrient solution. Given the negative electrical membrane potential difference at the plasma membrane (−140 mV), a rise in extracellular Na^+ concentrations will establish a large Na^+ electrochemical potential gradient that will favor the passive transport of sodium from the environment into the cytosol (Blumwald 2000). Low- and high-affinity K^+ carriers such as *AKT* and *HKT* genes families respectively, could mediate a significant Na^+ uptake with an increase in external Na^+ concentrations. Outward-rectifying ion channels could also play a role in mediating the influx of Na^+ into cells. These channels, which open during the depolarization of the plasma membrane could mediate the efflux of K^+ and the influx of Na^+ (Roberts and Tester 1995; Wegner and De Boer 1997; Wegner and Raschke 1994). As evidenced by Irakoze et al. (2019b), our results showed a higher Na^+ accumulation in shoots than in roots and significant Na^+ accumulation began from 24h after the saline stress application.

Both types of salinity have significantly reduced the K^+ contents in roots. Na^+ and K^+ having similar physiochemical properties, a high external Na^+ concentration negatively affects the K^+ acquisition at the uptake sites. Moreover, *KUP* (Potassium Uptake Permease) and *HAK* (High Affinity K) transporters are blocked by Na^+ under sodium-based saline stress despite being extremely selective for K^+. In contrast, salinity had no impact on the shoot K^+ concentration, and this was not an expected result. It clearly suggests that, for the duration of the present experiment (max. 1 week), the plants were able to restrict salt-induced decrease in K^+ to the root system and that K^+ absorption, and root-to-shoot K^+ translocation are not necessarily affected by salt at the same time, and not necessarily in the same way. For a longer stress treatment, it is highly probable that a decrease in the shoot K^+ concentration should be recorded as the result of a decrease in K^+ absorption.

As expected, Cl^- and S highly accumulated in NaCl- and Na_2SO_4-treated plants respectively. As observed for Na^+, Cl^- and S^{6+} showed a higher accumulation in shoots than in roots. Distribution of Na^+ and Cl^- however appear to be different. Indeed, the inside negative plasma membrane potential, and high apoplastic Na^+ concentration establish a thermodynamic potential whereby Na^+ influx across the plasma membrane is typically passive while the efflux is active while an opposite trend appears for Cl^- with an active influx and a passive efflux (Teakle and Tyerman, 2010).

Analysis of genes expression

To assess the effect of chloride and sulfate salinities on the expression pattern of *OsSOS1, OsNHX1, OsNHX2, OsHKT1, OsHKT2, OsHKT3, OsHKT4, OsHKT5, OsHKT6, OsHKT7, OsHKT8* and *OsHKT9*, the transcript levels of these genes were quantified using semi-quantitative RT-PCR in the shoots and roots of the salt-sensitive rice cv. IKP after 3h, 24h and 1 week of salt stress. The present study indicates that the expression of the *HKT* and *NHX* gene families and of *OsSOS1* in tested rice tissues all changed over time during salt stress. Their expression profile also differed depending on the type of saline stress. Although these genes are involved in Na^+ transport, their expression profiles were not always related to the amounts of sodium ion accumulated in plant tissues.

Shi et al. (2002) hypothesized a long-distance Na^+ transport model, in which under moderate stress (25 mM NaCl), *SOS1* functions to load Na^+ into the xylem in roots for Na^+ transfer and storage in leaf mesophyll vacuoles. According to the authors, under severe Na^+ stress (100 mM NaCl), *SOS1* is proposed to function in unloading Na^+ from the root xylem to reduce Na^+ damage of leaves that might be caused by exceeding the capacity of Na^+ sequestration in leaf cell vacuoles. In our study conducted under severe Na^+ stress, the highest expression of *OsSOS1* was detected in shoot treated by sulfate salinity after 24h and *OsSOS1* showed a similar expression pattern in salt-treated shoots after 3h. In root, *OsSOS1* was significantly up-regulated after 3h and 24h under both saline stresses. Overall, the gene was more induced in root than in shoot despite the highest Na^+ accumulation in shoot. The induced gene in root could facilitate Na^+ loading into the xylem, and Na^+ is controllably delivered to the shoot and subsequently compartmentalized into the vacuoles, presumably through the *OsNHX1* activity, leading to high shoot Na^+ levels and lower Na^+ levels in the root. *OsSOS1* was more repressed in NaCl- than Na_2SO_4-treated shoot and this could explain in part the great toxicity of NaCl over Na_2SO_4 in rice as evidenced by Irakoze et al. (2019). The high expression of *OsSOS1* in roots during the first 24 hours after salt application shows the rice plant's ability to establish early the tolerance mechanisms to Na^+ toxicity. The main function of *SOS1*, however, is not restricted to its dual impact on xylem loading. As reported by Maathuis et al. (2014), *SOS1* expression is prominent in the root cell tips, where cells are predominantly not vacuolated. *SOS1* is a Na^+/H^+ antiporter present in the plasma membrane and it mediates Na^+ efflux to the apoplast against the electrochemical potential through secondary active transport that is driven by the H^+ gradient across the plasma membrane and results from H^+-ATPase activity (Hasegawa, 2013; Almeida t al., 2017). At the epidermis, *SOS1* is probably involved in Na^+ re-excretion to the soil while Na^+ efflux from root cortical cell also contribute to Na^+ exclusion and the role of *SOS1* at the shoot level is less clear. It may thus not be excluded that *SOS1* is induced in specific cell layers, and that extraction of mRNA from the whole organ somewhat masked the plant response. After one week of stress exposure, *OsSOS1* was higher induced in shoot of plant control than in shoot of salt-treated plants. Studing the relative expression pattern of

OsSOS1 in suarnadhan rice cultivar exposed to 10, 100 and 200 mM of NaCl during 24h and 72h, Roy and Chakraborty (2018) showed that *OsSOS1* was higher induced in control plants than in NaCl-treated plants. According to the authors, the down-regulation of *OsSOS1* in NaCl-treated plants confirmed the relative susceptibility of the rice cultivar in response to NaCl stress. Other studies also proved a great induction of *OsSOS1* in control plants (Hossain et al. 2017; Yanshou et al. 2009).

The vacuolar Na^+, K^+/H^+ antiporters, *OsNHX1* and *OsNHX2*, plays an important role in the compartmentalization of highly accumulated cytosolic Na^+ and K^+ into vacuoles (Fukuda et al. 2004). The compartmentalization of Na^+ into vacuoles not only reduces the toxicity of Na^+ in the cytosol but also contributes to osmotic regulation. Our results showed the different expression profile between *OsNHX1* and *OsNHX2*. Similar to the results of Roy and chakrabarty (2018), these two genes were highly induced in plant control. In shoot as in root, chloride and sulfate salinities induced a different expression profiles of *OsNHX1* depending on stress duration. In shoot, a significant expression of *OsNHX1* was only detected in NaCl-treated plants after 3h while its significant expression in root was observed in all salt-treated plants only after 24h. Studying the expression pattern of *OsNHX1* in leaves of salt-tolerant (FR13A) and salt-sensitive (BRRI dhan29) rice cultivars under NaCl salinity, Hossain et al. (2018) found that the gene was up-regulated 1h up to 72h of saline stress in FR13A while it was induced in BRRI dhan29 at 72h saline stress. In our study and comparatively to control plants, *OsNHX2* was significantly up-regulated only after 3h in salt-treated shoot while no significant induction was observed in root. Overall, the *OsNHX1* and *OsNHX2* expression profiles were not much related to Na^+ accumulation in IKP salt-sensitive rice cultivar. According to Hossain et al. (2017), the vacuolar compartmentalization is evident under salt stress in the salt-tolerant rice cv. while apoplastic sequestration of cytosolic Na^+ is dominant in the salt-sensitive cultivar. Rice genome contain at least five *NHX* members belonging to the type I which are vacuolar located while *OsNHX5* belonging to the type II is found at endosome, Trans-Golgi Network and prevacuolar compartment. Thus, considering other members of the *NHX* family may provide useful data for an improved understanding of rice behavior in salt conditions considering the importance of these transporters

in Na^+ sequestration. Zeng et al. (2018) demonstrated that two *NHX*-type transporters isolated from *Helianthus tuberosus* (*HtNHX1* and *HTNHX2*) may improve the tolerance of rice to NaCl but that only the expression of *HtNHX2* and not *HtNHX1* increased grain yield under K^+-limited salt stress. Moreover, most *NHX* isoforms are able to transport both K^+ and Na^+ and usually have similar Km for these substrates. According to Maathuis et al. (2018), this implies that, unless the cytoplasmic Na^+ concentration is considerably higher than that of K^+, *NHX* exchangers mainly mediate K^+:H^+ exchange rather than Na^+:H^+ exchange.

According to Jabnoune et al. (2009), the nine *HKT* genes found in rice are strongly expressed in tissues (root epidermis and cortex, xylem and phloem, vascular bundle regions) playing crucial role in ion uptake, long distance transport and redistribution in the rice plant. In this study, we found that *OsHKT1, OsHKT5, OsHKT7, OsHKT8* and *OsHKT9* were not amplified in shoots or roots for all treatment conditions (Fig. 8). Garciadeblás et al. (2003) proved that *OsHKT1* specifically mediates Na^+ uptake in rice roots when the plants are K^+ deficient. Horie et al. (2007) also found that *OsHKT1* transporter directly mediated K^+-starvation-induced Na^+ influx into rice roots and these findings provided genetic evidence that Na^+ compensates for some roles of K^+ by acting as a substitute-nutrient in K^+-depleted plants. In our study, the culture solution was not deficient in K^+ and this could explain the non-expression of *OsHKT1*. In saline tolerant landraces, however, Jayabalan et al. (2019) recently consider that the sodium transporter gene *OsHKT1;5* mays also be responsible for shoot exclusion that confers salinity tolerance in both the vegetative and reproductive stage of rice.
In other plants, however, *HKT1* transporter may be involved in the control of Na^+ transport independently of the K^+ status. In some barley accession, *HvHKT1;1* was found to be localized at the cell plasma membrane of root stele and epidermis. Knock-down of *HvHKT1;1* led to a higher accumulation of Na^+ in both roots and shoots while overexpression reduced Na^+ accumulation (Han et al., 2018). Jaime-Pérez et al. (2017) reported a major role for *HKT1;2* in the control of Na^+ homeostasis and salinity tolerance in tomato. Similarly, Zhang et al. (2018) focused on *ZmHKT1* from maize, which encode a plasma-membrane-localized Na^+-selective transporter, and is preferentially expressed in root stele. According to these

authors, *ZmHKT1* promotes leaf Na^+ exclusion and salt tolerance by withdrawing Na^+ from the xylem sap.

OsHKT7 and *OsHKT8* were only expressed in salt-tolerant rice cultivar and operated harmoniously in a two-staged Na^+ exclusion model in rice (Cotsaftis et al. 2012; Ren et al. 2005). The root-to-shoot Na^+ transfer mechanism represents the first stage of Na^+ exclusion model, which is controlled by *OsHKT8*, whereas sheath-to-blade Na^+ transfer mechanism, which is regulated by *OsHKT7*, represents the second stage. The expression profile of *HKT* genes family depends on the rice cultivar. Similarly to our results, Garciadeblás et al. (2003) found that *OsHKT5* was a non-functional gene in cv. Nipponbare while the expression of *OsHKT9* was marginal.

Amplifications were detected for four genes belonging to *HKT* gene family (*OsHKT2, OsHKT3, OsHKT4* and *OsHKT6*). For these genes, we also observed their great induction in control plants. Analysing the expression level of the *HKT* gene family in shoots and roots tissues from weedy rice (JYGY-1 and JYFN-4) and rice (Nipponbare and 9311) genotypes, Yuhua et al. (2018) showed a higher induction of some *OsHKT* genes in plant control than in NaCl-treated plants. Compared to gene expression profile in control plant, *OsHKT2* was significantly down-regulated in salt-treated roots after 3h, 24h and 1 week. According to Kader et al. (2006), *OsHKT2* was down-regulated in the root tissue of BRRI Dhan29 salt-sensitive rice cultivar up to 24 h after the stress (to nearly 50% of the control expression), after which a slight up-regulation was evident 48 h and 72 h after the stress. Playing an important role in maintaining cytosolic Na^+ homeostasis in rice, *OsHKT2* induction mostly occurred in the phloem, in the transition from phloem to mesophyll cells, and in the mesophyll cells of the leaves. As observed for all genes concerned by our study, the previous studies proved that the expression profiles of *OsHKT3, OsHKT4* and *OsHKT6* differed depending on rice cultivars, plant organs, saline stress duration and the composition of culture solution. (Abdulhussein et al. 2018; Campbell et al. 2017; Garciadeblás et al. 2003; Jabnoune et al. 2009; Singh et al. 2016; Wang et al. 2015b).

Although our results and the results of some other studies showed a great induction of *OsSOS1*, *NHX* and *HKT* gene families in control plants, the

general rule is that these genes should not be up-regulated in a plant not exposed to Na^+ toxicity. The key role of the Na^+ transporters is to regulate the excess Na^+ in the plant as has been proved by many researchers. Considering that our control plants were not exposed to Na^+ excess, the results of this study should be taken with more caution. To confirm our results, we suggest to repeat this study on plants from three or more independent cultures.

Furthermore, the amplification of some *HKT* genes were not observed in this study although many other studies showed the induction of these genes in rice plants exposed to NaCl salinity. As we used a single pair of primers during the gene expression analysis, it becomes impossible to confirm that these genes were not expressed. In this case, we also suggest to repeat this study using several pairs of primers for each gene.

Conclusions

In the present study, we demonstrated that the anionic component of salt stress affected differently the physiological parameters and the expression profiles of the genes encoding the Na^+ transport in rice plant. The present work demonstrated that even a short-term exposure (3h) to NaCl or Na_2SO_4 was sufficient to induce sodium accumulation not only in roots but also in shoots of salt-treated plants. This suggests that an ionic component of salt stress may also result from short-term exposure to high salt level. We also found that the genes encoding the Na^+ transport regulation were induced in control plants, which were not subject to sodium toxicity, and therefore, we suggest taking our results more carefully before confirmation. The gene expression profiles differed depending on plant organ and saline stress duration. Most of *HKT* genes family were not amplified thereby confirming the relative susceptibility of IKP rice cultivar in response to salt stress. We finally observed that the changes in gene expression levels were not always related to Na^+ transport regulation.

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Appendix: S1 [Supplemental data 1]**.** List of primers and amplification conditions used for semi-quantitative RT-PCR expression analysis.

Gene name	Primer sequences	*T*m	No. of cycles	Size
	Expressed genes			
OsActin	F (5’-GTATTGTGTTGGACTCTGGTGATGGTGT-3’) R (5’-GATGGATCCTCCAATCCAGACACTGTA-3’)	60	35	587
OsEF1α	F (5’-TTTCACTCTTGGTGTGAAGCAGAT-3’) R (5’-GACTTCCTTCACGATTTCATCGTAA-3’)	60	35	103
OsSOS1	F (5'-CTCCGTGCTCATAGAATCGC-3') R (5'-ATACTCACTCAAGTGGGTCAATACC-3')	55	38	207
OsNHX1	F (5'-GTTCAAGAGTTACAACAAAGCACG-3') R (5'-CAGCGGGAATACAAAAGCAG-3')	55	37	200
OsNHX2	F (5'-ACCAAGACGAAACACCCCTAC-3') R (5'-AACCCAGCAACTACTCCAAGAA-3')	55	38	196

S1. *Continued*

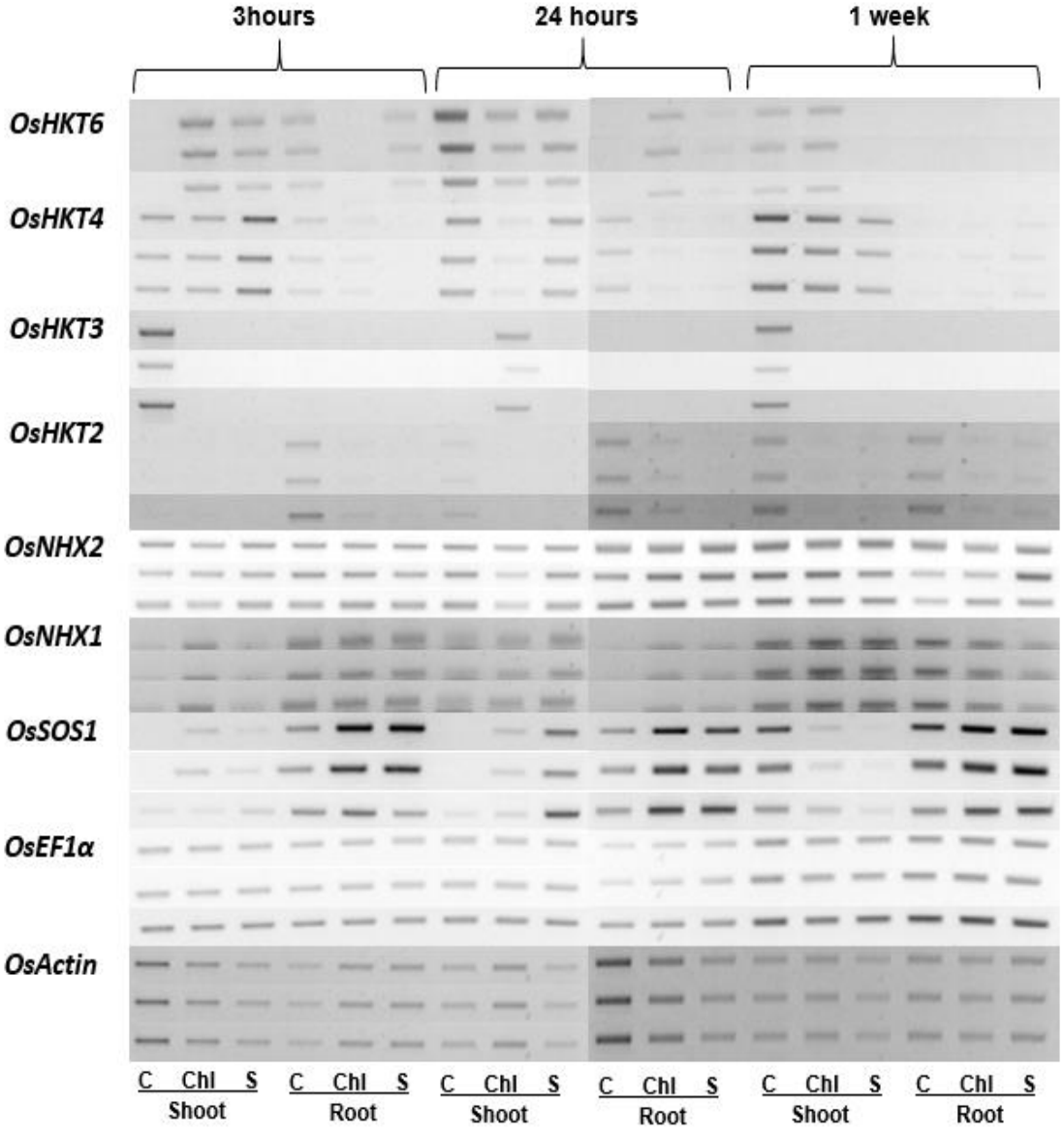
Gene name	Primer sequences	*T*m	No. of cycles	Size
	Expressed genes			
OsHKT2	F (5′-GAGTCGTCTCAGAAATGA-3′) R (5′-TTCTACGATTCAAAAGGC-3′)	55	40	349
OsHKT3	F (5'-TCTTCTGCTGCTTGAACTGGG-3') R (5'-ATCGCATACTGATC GCTTCTG-3')	57	40	621
OsHKT4	F (5’-GGAAGTCAATTTCTTCTGATCCA-3’) R (5’-TCATTTCAGGATGAACTCCTTG-3’)	55	40	239
OsHKT6	F (5’-CTCCAAACTTTCCGCACATT-3’) R (5’-TTGACTTGTCTCGTGGCTTG-3’)	55	40	190
	Unexpressed genes			
OsHKT1	F (5’-CATACTCGTTGGCTCGTTGC-3’) R (5’-GGTTGCTTGGCTTCAGGAAC-3’)	55-57-60	40	232

S1. *Continued*

Gene name	Primer sequences	*T*m	No. of cycles	Size
	Unexpressed genes			
OsHKT5	F (5'-TCCTTTGTTGGTTTCCTTGC-3' R (5'-GCTTCTGATTGGAAGCCTTG-3')	55-57-60	40	344
OsHKT7	F (5'-TCGAGATGGAGGTGTTCTCC-3') R (5'-AGATGGCGAGCACGATGTAG-3')	55-57-60	40	306
OsHKT8	F (5'-TGCCACCTTACACCACTTTCG-3') R (5'-TGCCATACGCACTGATAACCTC-3')	55-57-60	40	237
OsHKT9	F (5'-CTTGGTTTTGTTGCCTTGGT-3') R (5'-TCATGGATGGAGTTTGTGGA-3')	55-57-60	40	322

F: Forward
R: Reverse

S2 [Supplemental data 2]**.** Expression analysis of different genes encoding Na^+ transport by semi-quantitative RT-PCR amplification of RNA from IKP shoot and root after different time points under control conditions (C), 100 mM NaCl (Chl) or 50 mM Na_2SO_4 (S). *OsActin* and *OsEF1α* were used as internal control genes.



Interchapter IV – V

What do we know?

Chapter IV confirmed that the mineral status of the tested plants may be very quickly affected by salt exposure. This is especially the case for Na^+ which already accumulated at the shoot level 3h after exposure. Such as rapid accumulation was already noticed several years ago by another study published by our laboratory (see Lefèvre et al., 2001. Plant Science 161:943-952) but in this last case; Na^+ accumulation remained quite lower than in our study (176 µmol.g^{-1} DW versus 400 µmole.g^{-1} DW). If we compare ion accumulation in chapter III and Chapter IV, we notice that a 24h exposure to 100 mM NaCl in Chapter IV brings a higher Na^+ concentration than 48h exposure to 200 mM, which is surprising. This suggests that unidentified environmental conditions changes between the two set of experiments and that this modification had an impact on the plant behavior in terms of ion absorption and translocation. An alternative would be that exposure to a very high dose of 200 mM NaCl induced a very important osmotic stress which immediately reduced stomatal conductance, reducing transpiration and thus delaying Na^+ accumulation by the transpiration stream. In contrast to Na^+, K^+ presented a similar pattern in Chapter IV and III, with a stable content in the shoot and a decrease in the roots and this clearly suggests that Na^+ accumulation and K^+ decrease often considered to happen concomitantly are not necessarily simultaneous events.

Chapter IV also confirmed that genes coding for ion transporters may be very quickly regulated in response to salinity. Activation of *SOS1* at the root level already after 3h of exposure suggests that even a salt-sensitive rice cultivar such as IKP may activate processes to re-excrete absorbed Na^+ to the external medium if expressed at the epidermal layer, or retrieve Na^+ from the xylem sap if expressed at the level of the parenchyma cells at the vascular tissues.

The obtained data in Chapter IV however need to be confirmed and should be considered with caution. This is especially clear if we observe gene

regulation in the control plants: activation of *SOS1*, as a major Na^+ excretion transporter, or *NHX* genes as transporters involved in vacuolar sequestration is surprising in control plants since there is no Na^+ to re-excrete or to compartmentalize in these plants.

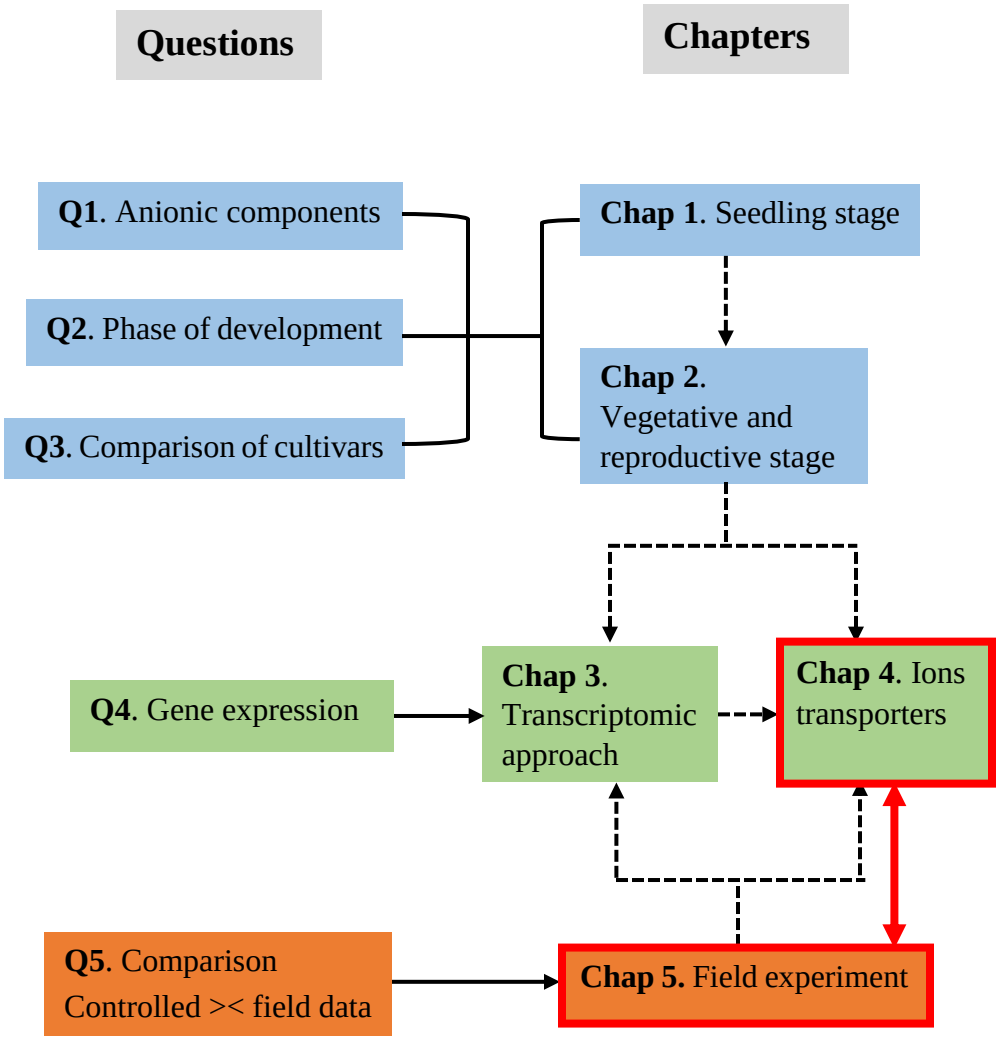
What is our question?

All the previous chapters were conducted in nutrient solution and semi-controlled environmental conditions. Chapter II, III and IV also considered a single salt-sensitive cultivar IKP. Moreover, until now, only Chapter II considered yield-related parameters.
One of the major motivation of the present thesis is to contribute to afford solution for the management of the Na_2SO_4-affected area of the Rusizi lowland in Burundi. It is therefore extremely important to integrate our research in experimental conditions close to the field environment and this implies to consider cultivars displaying contrasting levels of salinity-resistance, grown on a soil substrate under non-controlled environmental conditions. The next chapter thus intends to provide additional information to question 5: "Are the data obtained for the two types of salinity in controlled conditions and hydroponic culture comparable to those obtained in field conditions on a soil substrate? Moreover, it should help us to answer to question n°3: "Could the "NaCl-resistant" rice cultivars be considered as "Na_2SO_4-resistant" as well?"

What is our strategy?

In order to work in a well-adapted environment close to field conditions, the next experiments will be conducted in the experimental station of IRRI in Bujumbura (Burundi). Soils spiked with precise doses of NaCl or Na_2SO_4 will be used to control the nature and the amounts of tested soils and a specific attention will be paid to soil properties in the next chapters.
The salt-sensitive cultivar IKP used in the previous chapters is not well adapted to environmental conditions of Burundi and in the framework of a collaboration with IRRI researchers, we decided to focus on the rice cultivar V14 which is more suitable for local conditions and extensively cultivated in the concerned area. Comparison will be established with the salt-resistant cv Pokkali, already used in Chapter I. Since no porometer was

available for this field experiments, we quantified stomatal density and stomatal index rather than stomatal conductance; other parameters (ion and protein content) will be quantified in Belgium on sample harvested in Burundi.



Synthetic overview of the five research questions addressed during our thesis project and the specific source of their answers in the different chapters of the thesis.

Chapter V. Comparative effects of NaCl and Na_2SO_4 salinities on soil chemical properties and yield-related parameters of two contrasting rice cultivars (*Oryza sativa* L.) grown in clay soil conditions in Burundi.

Submitted in Pedosphere

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Abstract

The interactions between root-zone environments and plants responses to increased osmotic pressure or specific ion concentrations in the field are complicated by many soil processes, which have been less studied for Na_2SO_4 salinity. The aim of the present study was to compare the effect of NaCl and Na_2SO_4 salinities on the soil chemical properties as well as on rice physiological- and yield-related parameters. Pot experiments were conducted using a vertisol soil and two electrolyte solutions namely NaCl and Na_2SO_4 inducing two electrical conductivity levels (EC: 5 or 10 dS m^{-1}) of the soil solutions were afforded during the growth of two contrasted cultivars (V14: salt-sensitive and Pokkali: salt-resistant). The control treatment was watered by salt-free tap water (EC: 0.06 dS m^{-1}) and water content in the pot soils was maintained at field capacity for all treatments. Our results showed that soil pH highly increased under Na_2SO_4 salinity while soil EC increased as the level of saline stress increased. The soil sampled in Rusizi lowlands showed a medium CEC values, which corresponded to low soil fertility and only the high level of sulfate salinity significantly reduced the soil CEC values. Salinity induced an increase in Na^+ concentrations on solid soil complex and in soil solution. NaCl reduced the stomatal density in salt-sensitive cultivar and the plants treated by high level of salinity exhibited a higher stomatal index. The total protein contents in rice grain were higher in V14 than in Pokkali cultivar. Saline stress significantly affected all yield-related parameters and we concluded that NaCl was more toxic than Na_2SO_4 for most of the studied parameters while Pokkali exhibited a higher tolerance to saline stress than V14.

***Key Words*:** chloride, sulfate, salinity, rice, soil chemical properties, yield

Introduction

Word agriculture faces many constraints like producing 70% more food for the growing population by 2050 (FAO 2009) and the crop productivity does not increase along with the food demand. Reduced plant productivity is often attributed to various abiotic stresses including the soil salinity which affects more than 6% of the word's land (Parihar et al. 2015). Approximately one-half of the total irrigated arable lands could be adversely affected by salinity and this constraint leads to a great reduction in global agricultural production as irrigated soils contribute to roughly one third of the global food production (Munns 2002; Munns and Tester 2008). In Burundi, more than 15% of the Rusizi plain area has been severely affected by salinity and 182 rice fields of about 0.5 ha each have been completely abandoned in 2010 (Nijimbere 2014), the result being a drastic reduction in rice production at the national level. Rice (*Oryza sativa* L.) is the principal source of cereal foods for more than two third of the Burundian population and more than 50 per cent of which is grown in Rusizi plain (Gahiro 2011).

According to Rengasamy (2006), there are three major types of salinity based on soil and groundwater processes found all over the world: (1) groundwater associated salinity, (2) transient salinity and (3) irrigation salinity. These components of the soil salinization process are observed in the clay soils of the Rusizi lowland in Burundi. Nijimbere (2014) showed that the primary source of the salts ions in Rusizi soils was related to the volcanism and hydrothermalism of its watershed, the widely dominant salt ions being Na^+ among cations and SO_4^{2-} among anions unlike the largest salinized part in the world where the dominant anion remains Cl^-.

The salinity effects on plant growth and development are complex. High accumulation of salt ions in soil solution hinders water uptake by plant roots and result in cellular dehydration. In addition, a portion of excess salt ions enters the plant through the plasma membrane and accumulates in plant cell at a toxic level; this results in inhibition of many metabolic activities of the cell. Components of the final rice grain yield are severely affected by root-zone salinity. Grain weight produced per plant, total number of spikelets per plant, spikelet fertility, mean 1000-grains weight,

plant height, panicle length, tillers production and number of panicles per plant are significantly reduced by NaCl salinity (Ndayiragije and Lutts 2007; Zhu et al. 2004). Despite its detrimental effects on agricultural production in some parts of the world and particularly in the Rusizi lowlands, plant responses to Na_2SO_4 salinity only received minor attention. Our recent comparative study of NaCl and Na_2SO_4 effects on rice yield parameters revealed that sulfate salinity also reduced most of the aforementioned yield components, NaCl remaining more toxic than Na_2SO_4 (unpublished results). Irakoze et al. (2019a) also proved the higher toxicity of NaCl over Na_2SO_4 on most of rice physiological-related parameters.

Plants have the ability to cope with salinity stress. Salt tolerance is the ability of plants to grow and complete their life cycle on a substrate that contains high concentrations of soluble salts. For rice grown in saline conditions, the increase in total proteins content of the grains as well as the reduction in leaf stomatal density were considered as salinity stress tolerance markers (Baxter et al. 2011; Ouyang et al. 2010; Ramani and Apte 1997; Yunita et al. 2018). However, very few studies have been conducted on these two resistance mechanisms adopted by a plant under saline stress. Furthermore, no comparative study has explored the rice responses to NaCl and Na_2SO_4 through regulation of stomatal density or via the increase in protein synthesis.

Surprisingly, most studies on salinity effects on plant growth and production have been conducted in simplified conditions such as solution culture, hydroponics or sand culture. However, the interactions between root-zone environments and plant responses to increased osmotic pressure or specific ion concentrations in the field are complicated by many soil processes such as soil water dynamics, soil structural stability, solubility of compounds in relation to pH and pE (electron concentration related to redox potential), nutrient and water movement in soil (Rengasamy 2010). Salinity may cause dispersion of the soil particles in relation to flocculation processes resulting from the replacement of calcium and magnesium adsorbed on the soil exchange complex by sodium (Almaida Machado and Serralheiro, 2016). Furthermore, under field soil conditions, soil water content fluctuates and hence, the salt concentration in the soil solution

around roots also fluctuates (Rengasamy et al. 2003). Despite these constraints, conducting an experiment in conditions close to field realities should allow to produce a new set of data allowing to better understand the effects of the soil salinity on the plants.

The aim of the present study was to compare the effect of NaCl and Na_2SO_4 on the clay soil chemical properties as well as on rice physiological- and yield-related parameters. We conducted pot experiments in greenhouse using the clay soil sampled in Rusizi lowlands (Burundi). The plants from two contrasted rice cultivars (V14: salt-sensitive and Pokkali: salt-resistant) were exposed to soil salinized by three different iso-strenght Na^+ nutrient solutions (EC: 0; 5 or 10 dS m^{-1}) at the young seedling stage, which is commonly considered as one of the most salt-sensitive development stage in *Oryza sativa* (Lutts et al. 1995). Salinity was maintained until harvest.

Material and methods

Soil

Non-salinized clay soil (EC of 0.17 dS m^{-1}) collected from a vertisol surface layer (0-20 cm) in a farmer's field in Rusizi plain (29°19'-21' E; 3°13'-15' S) was used in this study. The soil was air-dried for two weeks, sieved passing through a 2-mm mesh and homogenized. Fertilizers to provide the equivalent of 75 kg N, 30 kg P and 30 kg K ha^{-1} were uniformly mixed in soil before filling the pots. Fertilizers were in three different forms, namely urea for N ($CO(NH_2)_2$), Diammonium phosphate for P and N ($(NH_4)_2HPO_4$) and KCl for K. 4kgs of fertilized soil was placed in a plastic pot (22 cm x 22 cm x 15 cm).

Experimental set-up

The experiment was conducted from November 2018 to April 2019 in a greenhouse of IRRI-Burundi. The temperature in the greenhouse with natural light varied between 25-30°C (day time) and 20-25°C (night time). Seeds of the rice cultivars Pokkali (salt-tolerant) and V14 (salt-sensitive)

were respectively obtained from IRRI (International Rice Research Institute; Philippines) and ISABU (Institut des Sciences Agronomiques du Burundi; Burundi).V14 cultivar was chosen because it is more suited to the agro-ecological conditions of Burundi than the cultivar IKP. In addition, being the most cultivated cultivar in the Rusizi plain due to its high productivity and organoleptic qualities, the IRRI researchers (with whom we collaborated in this study), wanted to confirm its sensitivity once again. Seeds were germinated on two layers of filter paper (Whatman N° 2) moistened with 10 ml of sterile deionized water in growth chamber at 28°C under a 12 hr daylight period. Ten day-old seedlings of the two cultivars were transferred into the greenhouse. Four seedlings were transplanted into each pot and afterwards thinned to two plants per pot.

After two weeks, the rice seedlings were subjected only once to saline irrigation treatments by using the saline solutions equivalent to 50 and 100 mM NaCl (EC: 5 and 10 dS m^{-1} NaCl) or 25 and 50 mM Na_2SO_4 (EC: 5 and 10 dS m^{-1} Na_2SO_4). The salinity was developed by dissolving NaCl or Na_2SO_4 in tap water having an initial electrical conductivity of 0.06 dS/m. 1400 ml of the saline solution were added to the soil providing concentrations of 10 mmol NaCl/100 g soil (treatment Chl5d), 20 mmol NaCl/100 g soil (treatment Chl10d), 5 mmol Na_2SO_4/100 g soil (treatment S5d) and 10 mmol Na_2SO_4/ 100 g soil (treatment S10d). The control treatment was watered by salt-free tap water. Each treatment was replicated for three times in a randomized complete block design. Thereafter, irrigation was applied manually to approximately 90% of pot water-holding capacity using the tap water.

The following growth and yield parameters were measured throughout the experiment: stubble height, heading time, panicle length, straw fresh weight, tillers number per plant, fertile tillers number per plant, grains number per panicle, grains weight per plant, % solid grains per panicle and 1000-grains weight. We referred to the flowering tillers as fertile tillers. The panicle length, the grains number per panicle, the % solid grains per panicle and the 1000-grains weight were recorded on main stem.

Soil analyses

The analyzes of the soil chemical parameters were performed on a soil sample which was not used in the experiment as well as in the soils recovered at the end of the complete life cycle of the rice plant (after the harvest of the rice grains). Soil electrical conductivity (EC) and pH (H_2O) were measured in a 1:5 soil/water suspension ratio with a shaking time of about 2 min using a pH meter coupled to the conductivity metter (Seven MultiTM, METTLER TOLEDO, Switzerland). Exchangeable cations and cation exchange capacity (CEC) have been determined by the Metson method (extraction of cations by NH_4-acetate 1M, pH 7; washing excess extractant with ethanol; extraction of NH_4^+ by KCl 1M) (Ciesielski et al. 1997). The content of the soil solution in soluble cations and anions was measured in 1:2 soil/water suspension ratio. The suspensions were left to equilibrated for 12 hours and then stirred for 15 min before switching to centrifugation at 10,000 *g* for 10 min. Decanted solutions were filtered on paper Whatman n ° 41. Cations concentration (Ca, Mg, Na, K) were quantified by the atomic emission spectrophotometry ICP-AES (ICAP 6500 Thermo Scientific) while the non-carbonated anions (Cl, S) concentrations were quantified by high-performance liquid-phase ion chromatography (HPLC-Dionex ICS2000). Carbonate concentrations were determined by potentiometric titration (Rodier 2005).

Estimation of ion content in plants

Twenty mg dry weight (DW) of shoots were digested with nitric acid (68%) at 80°C. After complete evaporation, residues were dissolved with HNO_3 (68%) + HClcc (1:3, v/v). The solution was filtered using a layer of Whatman (85mm, Grade 1). The filtrate was used to determine the cations concentration (Na^+ and K^+) by flame emission using Atomic Absorption Spectrometer (Thermo scientific S series model AAS4, Thermo Fisher Scientific Waltham, MA, USA). Chloride was specifically extracted according to Hamrouni et al. (2011). Anions (S and Cl^-) were quantified by liquid chromatography (HPLC-Dionex ICS2000, Dionex Corporation, Sunnyvale, California, U.S.A.).

Measurement of stomatal density and stomatal index

At the beginning of the maturity stage, the flag leaf was sampled; the abaxial epidermis of the leaf was cleaned and smeared with nail polish more precisely in the mid-area between the central vein and the leaf edge. After estimating that the nail polish was well dried, we gently detached the thin film (approximately 5mm x 15mm) from the leaf surface and the film was then spread on a glass slide, covered with a cover slip and pressured by tweezers. Numbers of stomata and epidermal cells for each film strip were counted under a photomicroscope system (Euromex Microscope Holland; 400x). The counting of stomata and epidermal cells number was carried out on the three flag leaves for each treatment. The leaf stomatal index was estimated using the formula: stomatal index = [number of stomata/ (number of stomata + number of epidermal cells)] x 100%.

Total protein analysis

Total protein contents in rice grains were determined by the kjeldahl method. A Büchi Digestion System (Flawil, Switzerland) and Büchi Distillation unit, K-355, were used for the analysis. 1g of finely ground rice grains was used in Kjeldahl procedure. After weighing the samples, they were transferred into digestion flask containing 10g of catalyst (mix of 9.65 g of K_2SO_4, 0.15 g of $CuSO_4$ x $5H_2O$ and 0.2 g of Se) and 25 mL of concentrated H_2SO_4. After more or less 2h of digestion, 80 mL of NaOH base (mass fraction $w = 0.33\%$) was added to each flask. Ammonium hydroxide was trapped as ammonium borate in a boric acid solution by distillation method. The HCl-based titration method was used to determine the amount of total nitrogen. Determination of the moisture content in the rice grains led to the actual estimation of the total nitrogen content, and the factor 6.25 was used to calculate the total protein contents.

Statistical treatment

Statistical analyses were performed using JMP Pro 14 software. Data were treated by variance analysis and means were compared using Tukey's HSD all-pairwise comparisons at the $p = .05$ level as a post-hoc test. The graphs were plotted using SigmaPlot 10.0 software.

Results

Soil chemical properties

Soil chemical properties considered in this study are pH, EC, CEC, exchangeable cations and water-soluble ions contents, exchangeable sodium percentage (ESP) and sodium adsorption ratio (SAR) (Table 1, table 2 and table 3). Overall, the type of rice cultivar is not expected to affect the change in soil chemical properties.

The Table 1 shows that the soil pH significantly increased under sulfate salinity comparatively to control soil by passing from neutral (6.9) to alkaline values (8.2) while such an increase was not observed in the chloride salt treatments. The soil EC significantly increased as the level of salt stress applied increased. At the end of the experiment, the EC values were almost 10 times lower than the EC values of saline solution initially applied. For a given salt concentration in the initial solution, no difference was recorded for the final soil EC values between chloride and sulfate treatment.

The CEC parameter represents the total capacity of soil to hold exchangeable cations, which is part of the soil reservoir providing nutrient elements for plant growth. Compared to higher values reported for other vertisols (Hazelton and Murphy 2016), the soil taken from the Rusizi field had a medium CEC values that correspond to low soil fertility (HillLab 2019). In general, saline stress did not significantly affect the soil CEC, with the exception of high level of Na_2SO_4 salinity (10 dS m^{-1}) which significantly reduced the CEC values, although such a decrease was recorded for soils cultivated with Pokkali, only.

Table 1. Hydrogen potential (pH), Electric Conductivity (EC) and Cation Exchange Capacity (CEC) of uncultivated and unsalted soil (Soil), unsalted soil and cultivated with control plant (C), cultivated soil and salinized by 5 dS/m (5d) or 10 dS/m (10d) of NaCl (Chl) or Na_2SO_4 (S).

Cultivar	Treatment	pH	EC (dS/m)	CEC (meq/ 100g soil)
	Soil	6.9 ± 0.09^{bA}	0.17 ± 0.003^{cA}	22.67 ± 1.16^{aA}
	C	7.0 ± 0.05^{bA}	0.21 ± 0.022^{cA}	21.62 ± 0.13^{aA}
Pokkali	Chl5d	7.4 ± 0.2^{bA}	0.72 ± 0.116^{bA}	21.14 ± 0.15^{aA}
	Chl10d	7.5 ± 0.2^{bA}	0.97 ± 0.027^{aA}	19.34 ± 0.68^{aA}
	S5d	8.2 ± 0.04^{aA}	0.69 ± 0.019^{bA}	22.34 ± 1.05^{aA}
	S10d	8.2 ± 0.01^{aA}	1.03 ± 0.017^{aA}	15.27 ± 1.14^{bA}
	Soil	6.9 ± 0.09^{bA}	0.17 ± 0.003^{cA}	22.67 ± 1.16^{aA}
	C	7.1 ± 0.03^{bA}	0.25 ± 0.050^{cA}	18.50 ± 0.60^{aA}
V14	Chl5d	7.3 ± 0.07^{bA}	0.65 ± 0.038^{bA}	17.19 ± 1.15^{aA}
	Chl10d	7.0 ± 0.14^{bA}	0.98 ± 0.033^{aA}	17.60 ± 0.84^{aA}
	S5d	8.1 ± 0.06^{aA}	0.61 ± 0.078^{bA}	19.32 ± 1.10^{aA}
	S10d	8.0 ± 0.15^{aA}	0.91 ± 0.021^{aA}	20.53 ± 6.14^{aA}

Notes: ±Standard error of means. Treatments followed by the same lowercase letter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a particular treatment do not differ statistically.

Table 2. Exchangeable cations content of uncultivated and unsalted soil (Soil), unsalted soil and cultivated with control plants (C), cultivated soil and salinized by 5 dS/m (5d) or 10 dS/m (10d) of NaCl (Chl) or Na_2SO_4 (S).

Cultivar	Treatment	Exchangeable cations (meq/100g soil)				
		Na	K	Ca	Mg	ESP (%)
Pokkali	Soil	0.8 ± 0.02^{cA}	0.9 ± 0.05^{bA}	20.3 ± 0.4^{aA}	10 ± 0.2^{cA}	3.5 ± 0.2^{cdA}
	C	0.3 ± 0.06^{cA}	1.8 ± 0.05^{abA}	20.4 ± 0.3^{aA}	15.7 ± 0.4^{aA}	1.4 ± 0.3^{dA}
	Chl5d	1.3 ± 0.5^{cA}	2.8 ± 0.5^{aA}	19.9 ± 0.6^{aA}	12.9 ± 0.9^{bA}	6.1 ± 2.1^{cdA}
	Chl10d	3.4 ± 0.3^{bA}	2.4 ± 0.2^{aA}	19.3 ± 0.3^{aA}	12 ± 0.4^{bcA}	17.6 ± 1.7^{bA}
	S5d	3.0 ± 0.15^{bA}	2.0 ± 0.02^{abA}	20.0 ± 0.3^{aA}	14 ± 0.5^{abA}	13.4 ± 1.3^{bcA}
	S10d	5.5 ± 0.2^{aA}	2.3 ± 0.04^{aA}	20.2 ± 0.4^{aA}	13.9 ± 0.8^{abA}	$36.\pm5^{aA}$
V14	Soil	0.8 ± 0.02^{dA}	0.9 ± 0.05^{cA}	20.3 ± 0.4^{aA}	10 ± 0.2^{cA}	3.5 ± 0.2^{dA}
	C	1 ± 0.009^{dA}	2.4 ± 0.09^{aA}	18.3 ± 1.3^{abA}	15 ± 0.4^{aA}	5.4 ± 0.2^{dA}
	Chl5d	2 ± 0.2^{cA}	2.0 ± 0.24^{abA}	18.4 ± 0.6^{abA}	12 ± 0.2^{bA}	11.6 ± 1.2^{bcA}
	Chl10d	1.4 ± 0.2^{cdB}	2.2 ± 0.1^{abA}	16.9 ± 0.18^{abA}	9.9 ± 0.2^{cA}	$8.\pm1.4^{cdB}$
	S5d	3.1 ± 0.3^{bA}	1.7 ± 0.13^{bA}	16.7 ± 0.7^{bB}	13 ± 0.3^{abA}	16.1 ± 1.4^{abA}
	S10d	4.2 ± 0.9^{aA}	2.1 ± 0.06^{abA}	18 ± 0.8^{abA}	13 ± 0.6^{bA}	20.5 ± 1^{aB}

Notes: ±Standard error of means. Treatments followed by the same lowercase letter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a particular treatment do not differ statistically.

The exchangeable cations and water-soluble ions contents of the soil without added salt (control) and the soil salinized by NaCl or Na_2SO_4 are respectively presented in tables 2 and 3. The exchangeable Na and water-soluble Na contents significantly increased as the level of salt stress applied increased and the highest values were recorded under sulfate stress. After converting the Na concentration in the soil solution from mmol L^{-1} to meq/ 100 g of soil (dilution ratio of 2 mL for 1 g of soil which gives a conversion factor of 0.2), we observed that sodium ions were equally distributed in exchangeable forms and free ions in soil solutions.

Table 3. Water soluble ions content of uncultivated and unsalted soil (Soil), unsalted soil and cultivated with control plants (C), cultivated soil and salinized by 5 dS/m (5d) or 10 dS/m (10d) of NaCl (Chl) or Na_2SO_4 (S).

Cultivar	Treatment	Water soluble ions (mmol/L)							
		Na	K	Ca	Mg	Cl	S	CO	SAR
Pokkali	Soil	4 ± 0.03^{dA}	0.5 ± 0.02^{bA}	1.9 ± 0.005^{bcA}	0.8 ± 0.007^{dA}	1.5 ± 0.03^{cA}	1.4 ± 0.06^{cA}	3.7 ± 0.2^{cA}	3.4 ± 0.02^{dA}
	C	2.2 ± 0.2^{dA}	1.3 ± 0.06^{abA}	1.6 ± 0.03^{cA}	1.1 ± 0.04^{cdA}	1.7 ± 0.7^{cA}	0.8 ± 0.04^{cA}	9.1 ± 0.5^{abA}	1.9 ± 0.1^{dA}
	Chl5d	7.5 ± 1.3^{cA}	3.3 ± 1.23^{aA}	2.8 ± 0.7^{abcA}	1.6 ± 0.3^{abcA}	16 ± 4.9^{bA}	1.2 ± 0.16^{cA}	6.6 ± 1.4^{bcA}	5.3 ± 1^{cA}
	Chl10d	17 ± 0.8^{bA}	2.8 ± 0.3^{abA}	3.2 ± 0.28^{abA}	1.8 ± 0.06^{abA}	27 ± 1.6^{aA}	1.2 ± 0.02^{cA}	6.8 ± 0.7^{abcA}	11 ± 0.8^{bA}
	S5d	14 ± 0.6^{bA}	1.7 ± 0.02^{abA}	2.4 ± 0.16^{abcA}	1.5 ± 0.02^{bcA}	2.8 ± 0.5^{cA}	7.4 ± 0.3^{bA}	10.5 ± 0.6^{aA}	10.5 ± 0.7^{bA}
	S10d	27 ± 0.3^{aA}	2.3 ± 0.14^{abA}	3.4 ± 0.15^{aA}	2.2 ± 0.03^{aA}	2.7 ± 0.5^{cA}	17 ± 0.6^{aA}	10.1 ± 0.7^{abA}	16.4 ± 0.3^{aA}
V14	Soil	4 ± 0.03^{dA}	0.5 ± 0.02^{cA}	1.9 ± 0.005^{bA}	0.8 ± 0.007^{cA}	1.5 ± 0.03^{cA}	1.4 ± 0.06^{cA}	3.7 ± 0.2^{cA}	3.4 ± 0.02^{dA}
	C	3 ± 0.07^{dA}	1.7 ± 0.37^{abA}	1.8 ± 0.09^{bA}	1.1 ± 0.04^{bcA}	1.2 ± 0.1^{cA}	0.8 ± 0.07^{cA}	9.5 ± 0.3^{aA}	2.5 ± 0.02^{dA}
	Chl5d	8 ± 0.7^{cA}	1.6 ± 0.3^{bcA}	1.7 ± 0.1^{bA}	0.9 ± 0.02^{cB}	13 ± 2.4^{bA}	0.8 ± 0.03^{cA}	7.8 ± 0.05^{bA}	7.3 ± 0.5^{cA}
	Chl10d	10 ± 1.1^{bcB}	2.8 ± 0.3^{aA}	3.4 ± 0.3^{aA}	1.7 ± 0.12^{aA}	27 ± 1.3^{aA}	1.4 ± 0.03^{cA}	2.6 ± 0.48^{cB}	6.3 ± 0.9^{cB}
	S5d	13 ± 0.7^{bA}	1.5 ± 0.1^{bcA}	1.7 ± 0.1^{bA}	1.1 ± 0.06^{bcA}	1.5 ± 0.3^{cA}	7.5 ± 1.5^{bA}	8.4 ± 0.3^{abA}	10.5 ± 0.5^{bA}
	S10d	19 ± 0.7^{aB}	1.9 ± 0.07^{abA}	2.4 ± 0.17^{bA}	1.4 ± 0.14^{abB}	1.1 ± 0.09^{cA}	14 ± 0.6^{aB}	8.4 ± 0.2^{abA}	13.7 ± 0.1^{aA}

Notes: ±Standard error of means. Treatments followed by the same lowercase letter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a particular treatment do not differ statistically.

The ESP (table 2) and SAR (table 3) parameters are conventionally used to determine the level of soil salinity and sodicity. Soils that have SAR values ≥13 or ESP>15% and pH>8.5 are considered as sodic soils. Our results showed that both SAR and ESP values increased significantly in salt-treated soils and more particularly in soils salinized by the high level of sulfate salinity. Overall, the exchangeable K and water-soluble K contents were not impacted by the type or level of saline stress applied. The water-soluble Ca and Mg contents increased significantly as the level of salt stress applied increased. As expected, the water-soluble chlorine and sulfate contents were respectively higher in NaCl- and Na_2SO_4-treated soils. Carbonate contents were higher in unsalted soil and cultivated with control plants (C) as well as in soil salinized by Na_2SO_4 comparatively to control soil. The lowest carbonate contents were recorded in the soil salinized by NaCl.

Physiological- and yield-related parameters

During the time-course of the experiment, all plants remained alive and set seeds, whatever the cultivar or the salt treatment. The two types of salinity induced a significant increase in shoot Na^+ content and Na^+ accumulation was obviously higher in plants exposed to NaCl salinity than in those exposed to Na_2SO_4 at the highest salinity level (Figure 1). Sodium accumulation was higher in Pokkali (salt-tolerant cultivar) than in V14 (salt-sensitive cultivar). The high level (10 dS m^{-1}) of NaCl or Na_2SO_4 salinity significantly decreased K^+ concentration in shoot comparatively to control plants but no difference between cultivars was recorded. As expected, sulfur and chloride highly accumulated in Na_2SO_4- and NaCl-treated plants respectively. Cl^- accumulation was significantly higher in V14 than in Pokkali cultivar at the highest level of NaCl stress (10 dS m^{-1}).

To check whether the salt stress would have an impact on the development of stomata and epidermal cells, we compared the stomatal density and stomatal index in three flag leaves of Pokkali and V14 cultivars at the beginning of the maturity stage. In the absence of salt, stomatal density was

lower in Pokkali than in V14. NaCl and Na_2SO_4 salinities induced a decrease in the stomatal density (Figure 2), but only at the high level of saline stress for Pokkali. For the V14 cultivar, stomatal density decreased as the salt stress level increased. In addition, for this salt-sensitive cultivar, the recorded decrease in leaf stomatal density was higher under the high level of chloride than sulfate stress. High level of NaCl salinity significantly increased the leaf stomatal index for both salt-sensitive and salt-tolerant cultivars. For Na_2SO_4 treatment, a slight increase of the leaf stomatal index was observed only for the Pokkali cultivar at the highest dose.

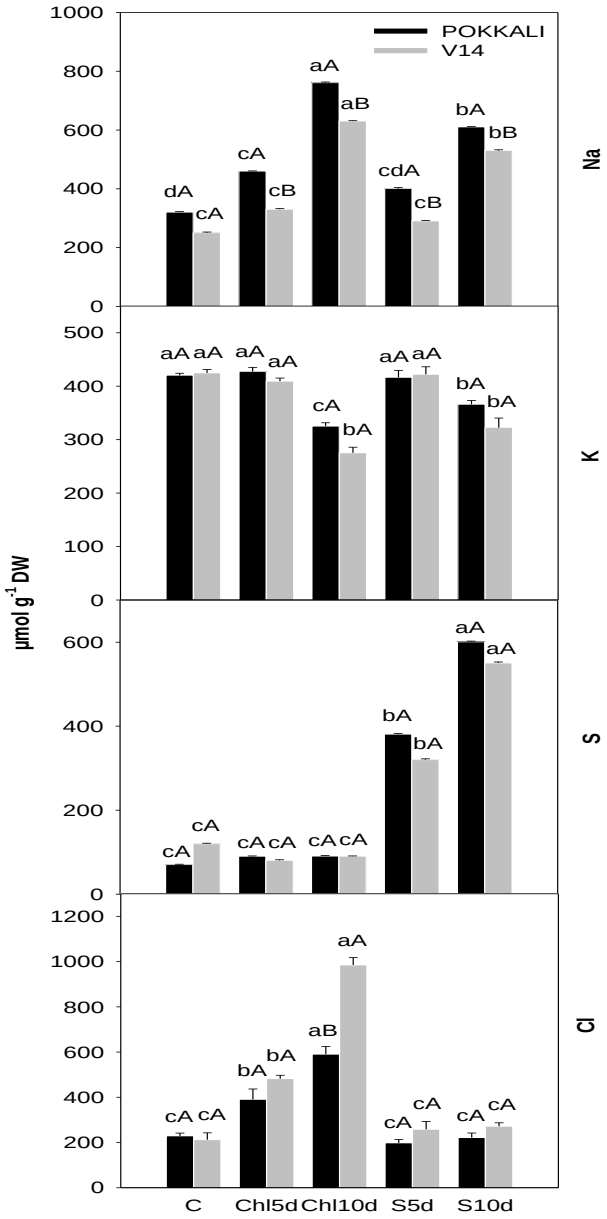

Fig. 1. Mineral nutrients concentration in shoot of control plant (C) and plant stressed by 5 dS/m (5d) or 10 dS/m (10d) of NaCl (Chl) or Na_2SO_4 (S). Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments followed by the same lowercase letter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a particular treatment do not differ statistically.

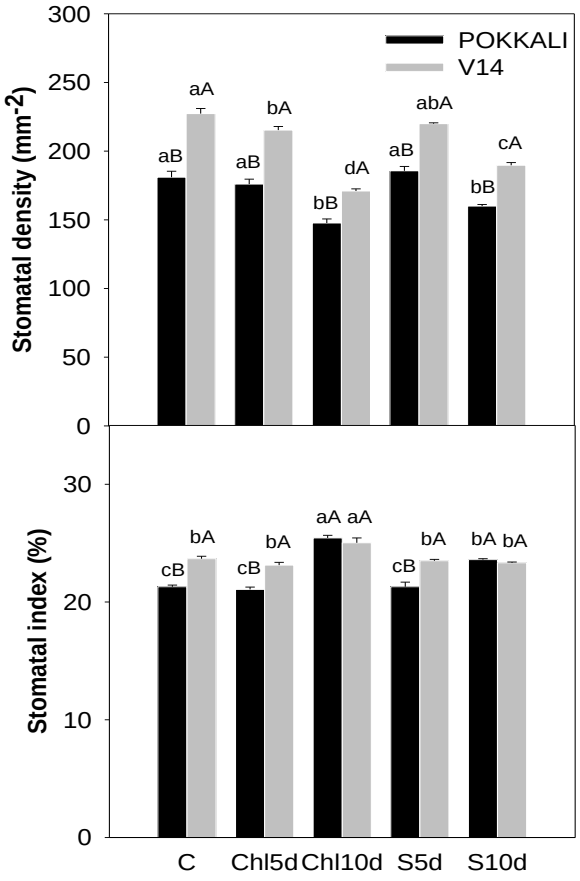

Fig.2. Comparisons of stomatal density and stomatal index in the leaves of control plant (C) and plant stressed by 5 dS/m (5d) or 10 dS/m (10d) of NaCl (Chl) or Na_2SO_4 (S). Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments followed by the same lowercase letter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a particular treatment do not differ statistically.

Total protein contents of rice grains are given in Figure 3. The highest level of chloride stress significantly increased the total grain protein in both cultivars, and the value recorded for V14 was higher than for Pokkali. As far as Na_2SO_4 is concerned, the recorded increase was significant for V14 only.

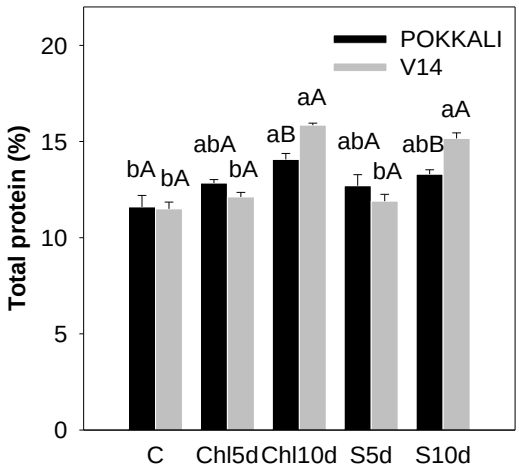

Fig.3. Total protein content in the rice grain of control plant (C) and plant stressed by 5 dS/m (5d) or 10 dS/m (10d) of NaCl (Chl) or Na_2SO_4 (S). Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments followed by the same lowercase letter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a particular treatment do not differ statistically.

As far as yield-related parameters are concerned (Table 3), the two considered cultivars displayed contrasting properties in the absence of salt: Pokkali exhibited a higher stubble height, panicle length, straw fresh weight par plant, grain number per panicle than V14 while an opposite trend was recorded for tillers number per plant and fertile tillers per plant. In contrast, the heading time, the 1000 grain weight and the grain weight produced per plant were similar. Most yield-related parameters were affected by salinity. Considering the two rice cultivars, the deleterious impact of NaCl was higher than the impact of Na_2SO_4 for the mean number of tillers and fertile tillers produced per plant, grains number and solid grains percentage per panicle, grains weight and straw fresh weight per plant. Pokkali was more tolerant than V14 for most parameters like stubble height, grains number and solid grains percentage per panicle, 1000-grains weight, grains weight and straw fresh weight per plant. The heading time was reduced by high level of NaCl and Na_2SO_4 in all cultivars. Overall, a more deleterious effect was recorded under the high level of saline stress while a moderate level showed an intermediate behavior.

Table 4. Yield parameters measured on control plant (C) and plant stressed by 5 dS/m (5d) or 10 dS/m (10d) of NaCl (Chl) or Na_2SO_4 (S)

Yield parameters measured	**Cultivar**									
	Pokkali					V14				
	C	Chl5d	Chl10d	S5d	S10d	C	Chl5d	Chl10d	S5d	S10d
Stubble height	157±3.5^{aA}	154±4.6^{aA}	124±3.2^{bA}	154±3^{aA}	136±3^{bA}	73±1.7^{aB}	70±1.2^{abB}	60±0.58^{dB}	68±1.15^{bcB}	64±0.7^{cdB}
Tillers number/plant	25±0.7^{aB}	16±2.6^{abB}	7±3.3^{bA}	20±2^{aB}	16±1.2^{abB}	45±1.5^{aA}	38±0.6^{bA}	6±1.9^{dA}	45±1.5^{aA}	30±1.5^{cA}
Fertile tillers number/plant	25±0.7^{aB}	16±2.6^{abB}	5±2.3^{cA}	20±2^{abB}	14±0.9^{bcB}	45±1.5^{aA}	35±1.2^{bA}	3±1^{dA}	39±2^{abA}	24±0.9^{cA}
Heading time (days)	110±1.7^{aA}	107±0.1^{abA}	106±1.2^{abA}	109±0.7^{aA}	104±0.1^{bA}	107±2^{aA}	108±2^{aA}	95±0.9^{bB}	107±0.3^{aA}	96±1.9^{bB}
Panicle length (cm)	28±0.6^{aA}	26.7±0.9^{aA}	20±0.6^{bA}	27.7±0.9^{aA}	22.3±0.9^{bA}	21±0.09^{aB}	18.5±1^{aB}	14±0.5^{bB}	19.3±0.3^{aB}	15.2±0.8^{bB}
Straw fresh weight/plant (g)	87.3±2^{aA}	74.7±1.9^{bA}	38±3.2^{cA}	76.3±1.9^{bA}	67.7±0.9^{bA}	76±0.7^{aB}	69±1^{bA}	22.7±1.5^{dB}	71±0.6^{bA}	37±1.5^{bA}
Grains number/panicle	195±4.7^{aA}	186±3.8^{aA}	72.7±8^{cA}	192±5^{aA}	128±10^{bA}	160±5^{aB}	139±2^{bB}	92.3±4.4^{dA}	146±1.5^{abB}	118±1.9^{cA}
% Solid grains/panicle	85±1.8^{aA}	55.4±5^{bA}	35.6±3.7^{cA}	84.3±1.8^{aA}	58.7±3.1^{bA}	81.3±3^{aA}	51±0.6^{cA}	12.6±1.2^{eB}	64.3±1.2^{bB}	41.7±1.5^{dB}
1000-grains weight (g)	19.5±1^{aA}	18.4±0.4^{aA}	12.6±0.5^{bA}	17.4±0.4^{aA}	14±0.6^{bA}	21.6±0.8^{aA}	16.4±0.5^{bcA}	11.4±0.9^{dA}	19±0.6^{abA}	14±0.9^{cdA}
Grain weight/plant (g)	23±1.2^{aA}	17±0.58^{bA}	10.7±0.9^{cA}	18.3±0.9^{bA}	15.3±0.7^{bA}	24.7±0.3^{aA}	16.3±0.3^{cA}	6.3±0.3^{eB}	19.3±0.3^{bA}	10.7±0.9^{dB}

Notes: ±Standard error of means. Treatments followed by the same lowercase letter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase letter in a particular treatment do not differ statistically.

Discussion

In this study, we found that applied saline stress significantly affected the soil chemical properties as well as physiological- and yield-related parameters. The significant effects of salinity on soil chemical properties occurred in particular for the exchangeable and soluble Na^+ ion and for the soil pH. The application of salt resulted in increased saturation of sodium on exchangeable sites, and the conditions akin to sodic soils (EC<4 dS m^{-1}, ESP>15%, except that the pH was <8.5) were observed in some salt-treated soils.

Although the effects of soluble salts and exchangeable sodium are currently known, the sensitivity thresholds remain questionable (Suarez 2001, Skaggs et al. 2001). Regarding the harmful effect of exchangeable sodium on the soil structure, the 15% threshold is considered to be the critical level characterizing the structural instability of the soil (U.S Salinity Laboratory 1954). However, some studies showed that this threshold is variable according to soil parameters such as the soil texture, the clay mineralogy as well as the content and the nature of the organic compounds (Shainberg et al. 1984, Lebron et al. 2002). Mclntyre (1979) considered a threshold of 5% exchangeable sodium to define a sodic soil while Saidi et al. (2004) found that the acceptable threshold values of SAR and ESP necessary to maintain a stable structure are respectively 2 and 5%. We could hypothesize that sodisation process affected all salt-treated soils because during the plant watering in our experiment and soil watering after the experiment, we found that the amount of water required to reach the soil water saturation point was significantly lower in all salt treatments compared to the amount of water used in control treatments. This would have been due to the decrease in soil porosity and therefore, to the alteration of the soil physical properties.

The increase of soil pH was observed only under sulfate salt treatment. This could be explained by the adsorption of SO_4^{2-} anions onto soil particles and the subsequent release of OH^- desorbed in the soil. Previous studies had showed that the sulfate anion could be adsorbed in several types of soil (Bolan et al. 1987; Norio et al. 2018; Sokolova et al. 2008; Alves et al. 2004). Knowing that only the soil pH between 5.5 and 6.5 are suitable for agriculture, the pH values between 7.3 and 8.2 resulting from soil

salinization are detrimental for plant growth and development. It has been proven that the high soil pH values negatively affect the plant through the ion imbalance of soil solution due to low solubility and high adsorption of certain nutrients like N, P, K, Ca, Cu, Zn, Fe and Mn (Marlet and Job 2006; Qadir et al. 2006).

As expected and proven by Rengasamy (2010a), the soil EC increased significantly as the level of applied saline stress increased. Unlike the hydroponic set experiment where the electrical conductivity of the culture solution remained stable (Irakoze et al. 2019), the soil solution EC values at the end of our experiment were almost 10 times lower than the EC values of saline solution initially applied. The high reduction of the soil solution EC could be related to the gradual migration of salt ions in plant and to the solid soil complex. Mizanur and Roxana (2005) applied in the soil a saline solution of 13 dS m^{-1} NaCl and recorded only an EC of 2 dS m^{-1} at the end of the experiment. Under field conditions in Rusizi plain, an EC as low as 1 dS m^{-1} highly decreased the rice yield and the growth of the rice plant was completely hindered at 2.96 dS m^{-1} (Nijimbere 2014). The detrimental effects on plant were related not only to the salts ions concentration in the soil solution but also to the soil dispersion generated by sodium saturation on solid complex.

The soil taken from the Rusizi field had a medium CEC values. Studying the chemical and physical properties of the soil sampled in the Rusizi plain, Nijimbere (2014) showed that the low soil CEC was related to a small amount of clay and to low organic matter contents (19,4% of clay and 0,5% of organic carbon).

The accumulation of salt ions in the soil solution and on the solid soil complex are largely responsible for the degradation of soil structure as well as osmotic stress, nutritional imbalance and ion toxicity limiting plant production (Li et al. 2016). In our study, we observed that exchangeable Na and water-soluble Na highly accumulated in the soil salinized by NaCl or Na_2SO_4. According to Naher et al. (2011), the attractive forces which bind clay particles together are disrupted when too many sodium ions get between the clay particles and the soil eventually disperse. The sodium-induced dispersion cause the reduction in air and water infiltration, reduced hydraulic conductivity and surface crusting. Warrence et al. (2002) proved

that the clay soil dispersion due to sodium accumulation hindered the plants establishment, roots penetration in the soil, and adequate water and nutrients absorption. Overall, the previous effects negatively affected the plant yield and survival.

In addition to sodium accumulation, we also observed a high concentration of chloride and sulfate in soil salinized by NaCl and Na_2SO_4 respectively. Previous studies highlighted the deleterious effects of Cl^- and SO_4^{2-} accumulation on the soil structure (Li et al. 2016; Nijimbere 2014). Potassium fertilization was obviously responsible for the significant increase in K^+ ion observed in cultivated soils compared to uncultivated soil. We observed a high concentration of Ca^{2+} and Mg^{2+} ions on the exchange complex in contrast to the Na^+, which highly accumulated in the soil solution. This observation could be explained by the high binding capacity of divalent cations on exchangeable sites compared to the monovalent cations.

Considering the physiological- and yield-related parameters, the present study revealed the high tolerance of Pokkali compared to V14 as well as the high toxicity of NaCl compared to Na_2SO_4. The sodium ion accumulation was higher in NaCl- than in Na_2SO_4-treated shoot (Figure 1) and this observation suggests that Na^+ uptake is influenced by the nature of anion excess present in the soil solution. The higher accumulation of Na^+ in Pokkali confirms its ability to tolerate salt stress during the whole cycle. The mechanism for managing excess accumulated sodium could be related to Na^+-sequestration in the vacuole, but also, at the whole plant level to accumulate Na^+ in the oldest leaves which exhibit the lowest photosynthetic rate in mature plants, thus preserving the youngest leaves contributing to grain filling (Lutts *et al.,* 1999). Pokkali is a well-known tolerant cultivar and molecular targets putatively involved in adaptation of this cultivar to salinity, such as gene involved in MAPK signaling, have been recently identified (Shankar *et al.,* 2016). Since it suffers from agronomic drawback, such as a poor harvest index, it has also been used for crosses with salt-sensitive cultivar to produce salt-tolerant semi-dwarf hybrids (Mirdar Mansuri *et al.,* 2019).

The Cl^- anion also accumulated in NaCl-treated and to a higher extent in salt-sensitive V14 than in salt-tolerant Pokkali. Chloride toxicity received less attention than Na^+ toxicity in physiological studies devoted to salt stress, but recent data clearly demonstrate that Cl^- excess induces a wide range of physiological disorders in non-halophyte (Wu and Li, 2019). Jeschke and Pate (1991) also showed that Cl^- unlike Na^+ was not substantially retained in the root of *Ricinus communis* L, but deposited more or less uniformly in stem, petiole and leaf lamina tissues. The chloride anion accumulated in the large quantities in shoot since the exclusion of Na^+ did not absolutely resulted in Cl^- exclusion. Studying the effects of chloride versus sulfate anions on nutrient-ion absorption by several plant species, Kretschmer et al. (1953) showed the higher concentration of Cl^- than SO_4^{2-} in shoots and according to Gauch and Wadleigh (1945b), the anionic imbalance observed could be explained by the low mobility of SO_4^{2-}. Chloride fluxes were reported to play important role in pollen tube elongation: although this question is still a matter of debate (Messerli et al. 2004), a high chloride content in reproductive organs of V14 exposed to the highest dose of NaCl might explain the very low percentage of solid grains recorded per panicle in these plants (Table 3). Alternatively, accumulation of Na^+ in pollen grain could also explain the low male fertility recorded in some rice genotypes (Zhu *et al.* 2000).

Concerning the stomata, we found that their density was higher in V14 (sensitive cultivar) than in Pokkali (tolerant cultivar) for all treatment (Figure 2). According to Yunita et al. (2018), the lower density of stomata on the leaves indicates tolerance of the genotype to salt stress and it fits perfectly with our observation. Stomatal density was affected by salinities and the greatest reduction was observed in leaves of the sensitive cultivar grown under high level of NaCl salinity. Previous studies on rice showed that a change in stomatal density depended on rice cultivar as well as the type and level of stress applied (Bahaji et al. 2002; LESTARI 2006; Wankhade et al. 2010; Yunita et al. 2018). Ouyang et al. (2010) found that the receptor-like kinase *OsSIK1* improved the salt stress tolerance in rice (*Oryza sativa* L.) plants by regulating the stomatal density in the abaxial and adaxial leaf epidermis. Studying the responses of the tomato (*Lycopersicon esculentum* Mill.) plants to exposure to different salt forms and rates, Yokas et al. (2008) showed the strong reduction of stomatal

density in NaCl-treated plants compared to those in Na_2SO_4- and $CaCl_2$-treated plants. Differences in stomatal density can result from either a general effect on non-stomatal epidermal cell density or a specific reduction in stomatal development (Boccalandro et al. 2009). We therefore calculated the stomatal index. Plants treated by the high level of salinity exhibited a higher stomatal index compared to control plant, revealing that the high salt stress change the proportion of epidermal cells that differentiate into stomata. In the present study, stomatal parameters were recorded in the flag leaf whose function is mainly to perform photosynthesis for grain filling so that a differential impact of salinity on the size of stomatal and epidermal cells could be regarded as the best compromise to increase water use efficiency in terms of CO_2 fixation relative to transpiration, smaller stomata being more efficiently regulated than big one. Besides, Shankar *et al*. (2016) also reported in the salt-tolerant Pokkali a NaCl-induced stimulation of genes involved in wax and terpenoids metabolism in close link with cuticular synthesis reducing the non-stomatal transpiration.

Starch is the main grain reserve in rice accumulating up to 50−90% of dry weight with protein contributing 5−12% to the total (Chen et al. 2012). Our results showed that salinity stress significantly affected the total proteins content in rice grains (Figure 3). Baxter et al. (2011) found that the NaCl treatment resulted in significantly higher protein contents in the milled rice and according to the authors, the increase in protein content was mainly attributed to increase of glutelin, with lower contributions from albumin. Similarly, the study on rice (*Oryza sativa* L.) cv. Nipponbare response to salinity stress also proved that rice grain proteins were increased by NaCl stress with a major contribution from glutelin and prolamin (Thitisaksakul et al. 2015). The increase in protein content in rice grown under saline conditions could be related to rice plant response to salinity stress since Ramani and Apte (1997) found that salinity stress induced the synthesis of around 40 polypeptides previously shown to be associated with stress in rice. Under high level of salt stress, the total protein content was higher in V14 than in Pokkali. Only poor differences were recorded between NaCl and Na_2SO_4 for the grain protein content which contrasts with the data provided by Wu *et al.* (2016) who mentioned in the halophyte Quinoa a

highest grain protein content for plants exposed to Na_2SO_4 than for those exposed to NaCl.

Saline stress significantly affected most yield-related parameters measured in this study (Table 3). NaCl was more toxic than Na_2SO_4 for most of the studied parameters while Pokkali exhibited a higher tolerance to saline stress than V14. Similar results were observed in our hydroponic experiment where the yield components of salt-sensitive I Kong Pao cultivar were more affected by NaCl than Na_2SO_4 salinity (unpublished results). Other studies conducted on different rice cultivars grown in hydroponics or soil conditions also showed the detrimental effect of salinity stress on yield-related parameters (Hasanuzzaman et al. 2009; Khan and Abdullah 2003; Ndayiragije and Lutts 2007; Zhu et al. 2004). Salinity stress significantly reduced the 1000-grains weight in Pokkali as well as in V14 cultivar. According to Abdullah et al. (2001), the reduction in seed set are primarily due to reduced translocation of soluble carbohydrates to primary and secondary spikelets, accumulation of more sodium and less potassium in all rice floral parts and inhibition of the specific activity of starch synthetase in developing rice grains. Unlike previous studies that showed the effect of NaCl on plant flowering delay heading (Grieve et al. 1993; Khatun et al. 1995; Lutts et al. 1995), our results revealed the significant effect of the saline stress on the heading time shortening in V14. A thorough study of the salinity effect on the rice flowering in relation to fluctuation of all environmental parameters could explain this contradiction.

Overall, a strong similarity was observed between our results on some yield parameters and those of another study previously conducted in hydroponic conditions (unpublished results). However, a perfect comparison remains difficult to establish between the two studies as the rice cultivars used and the applied saline stress levels differed. Tavakkoli et al. (2010b) showed that the response of barley to saline stress differed between hydroponic and soil systems. The growth reductions were greater under hydroponics than in soil at similar electrical conductivity values, and the uptake of Na^+ and Cl^- was also greater. Considering the low EC values observed in our study, the detrimental effect of saline stress on physiological- and yield-related parameters could be due to salt ions highly accumulated in plant as well as

other soil chemical properties like pH and Na^+ accumulation on solid soil complex. The unsuitable soil pH for agriculture would result in low availability of mineral nutrients to rice plant. Moreover, the sodium-induced soil dispersion could be assigned to reduction in air and water infiltration, decrease of hydraulic conductivity and reduced plant rooting. This hypothesis reinforces the view of Grattan *et al.* (2002) reporting that an electrical conductivity in the field of 1.9 $dS.m^{-1}$ may reduce grain yield while current guidelines for plant analyzed in hydroponic culture mentioned 3 (or above) $dS.m^{-1}$ as threshold value.

Conclusion

This study reports that salinity significantly affected the soil chemical properties like pH, EC, ESP and SAR. We observed the salinity-induced increase in Na^+ concentrations on solid soil complex and in soil solution. Although the detrimental effect of salt stress on soil properties was higher in Na_2SO_4- than in NaCl-treated soils, chloride salinity highly hampered the physiological- and yield-related parameters. This would be due to osmotic stress and ion toxicity more recorded in NaCl- than in Na_2SO_4-treated plants. The present study revealed the higher toxicity of NaCl over Na_2SO_4, and the higher level of salinity resistance of cv. Pokkali comparatively to local cultivar V14.

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GENERAL DISCUSSION

General discussion

The purpose of this study was to evaluate the putative influence of the anionic components of salt stress on the rice physiological and yield-related parameters. It also aimed to provide information on the tolerance mechanisms adopted by this species to cope with NaCl or Na_2SO_4 salinities. Our experimental strategy involved hydroponics culture under controlled conditions on the one hand and a soil experimentation under semi-controlled conditions on the other hand. This double approach is fully justified by the fact that plant behavior under field conditions may drastically differ from the data obtained in controlled conditions, firstly because different substrates are used in the two cases (soil *versus* nutrient solution), and secondly because environmental parameters (temperature, relative humidity, light intensity,…) may have a strong impact on ion absorption and translocation (Thapa et al. 2017).

Chloride and sulfate salinities altered differently the physiological, biochemical, molecular and yield-related parameters of rice plant. Furthermore, the tolerance mechanisms to saline stress adopted by rice plant differed depending on the type and level of applied salt stress. The observed variability also depended on the considered rice cultivar as well as stress duration. An understanding of sulfate contribution to the toxicity of soil salinity will be important to understand and increase salt tolerance of crops under conditions in which not only NaCl, but additionally sulfate salts are also present. Therefore, the results of this study could be very useful for the establishment of sound improvement programs aimed at increasing rice's resistance to Na_2SO_4 salinity. This may be one of the suitable pathways in the recovering process of salinized area in the Rusizi plain in Burundi.

1. Does the anionic component of salt stress have a specific impact on rice behavior for a same Na^+ external concentrations?

According to Munns and Tester (2008), soil salinity stresses the plants in two ways. (1) High concentrations of salts in the growing medium make it harder for the roots to extract water and this is referred to as the osmotic or

water-deficit effect of salinity. (2) High concentrations of salts within the plant cause ions imbalance or disturbances in ion homeostasis and toxicity.

The osmotic component is considered to impair the plant functioning before the ion component. A gradual accumulation of ions in the plant is considered to require time before reaching toxic levels (Munns et al., 2020a). Our data nevertheless provide evidence that this "biphasic model" should not be regarded as a "dogma". We indeed recorded a significant accumulation of toxic ions after a short term of exposure to either NaCl or Na_2SO_4 (see Chapter IV) and this is similar to data provided by Lefevre et al. (2001) who demonstrated that toxic amounts of Na^+ and/or Cl^- could be reached in rice plants before any modification of the plant water status. It may also be argued that under natural field conditions, salt stress does not appear "abruptly" (as frequently applied in laboratory studies, including our approach) but rather develops progressively at a specific rate which depends on several factors, including the soil water content. As a consequence, plants are rarely exposed to a sudden burst of electrical conductivity and osmotic stress. Even if they are (as might occur very saline soils), the fact that the external osmotic constraint is important does not necessarily imply that the internal water content drops instantaneously, and this mean that even the water stress component of salt stress is progressive. Hence, beside the bi-phasic model, we suggest that both components of stress develop simultaneously but that the rate of development may differ as a function of both external (salt doses) and internal (transpiration,…) factors.

To cope with osmotic stress, the plant adopts two main mechanisms, namely stomatal closure aiming to reduce water losses by transpiration and osmotic adjustment allowing to maintain water absorption despite a decrease in the external Ψs. To achieve osmotic adjustment, the plant concentrates the osmolytes in the cellular cytoplasm to equilibrate with toxic ions accumulated in the vacuoles (Shabala et al., 2020). Among putative soluble organic compatible compounds, proline is a major osmolyte, which is synthesized by most if not all plants in response to salinity stress (and a wide range of other stresses as well) and thereby help in maintaining the osmotic status of the cell to ameliorate the salt stress effect (Tuteja 2007). Comparatively to control plants, the results of this

study showed that proline was more overaccumulated in NaCl- than in Na_2SO_4-treated plants. A similar trend was reported by Lutts et al. (1996a) at the callus level. Correlatively, our results showed that osmotic potential was lower for NaCl-treated plants than for Na_2SO_4-treated ones and this should help the NaCl-treated plants to maintain the water potential gradient required for water absorption.

Regarding proline, it has to be noticed that the accumulation of this osmolyte did not necessary improve the water status of rice plants as the water content decreased more in plants treated with chloride than in plants treated with sulfate salinity. Although the relative rate of proline accumulation is important under stress conditions, it is interesting to calculate its real absolute contribution to osmotic adjustment. Our results showed that the contribution of proline in osmotic adjustment was between 0.55 and 1.5%; a notoriously low contribution. This calculation consider that proline accumulated only in the cytosol, which represent around 10% of the cell volume. Although this assumption is commonly accepted, it has, to the best of our knowledge, never been exhaustively demonstrated. On the contrary, Gagneul et al. (2007) demonstrated that most of proline accumulated in the vacuoles of halophyte plant species *Limonium latifolium*.

Beside acting as an osmolyte, Joseph et al. (2015) proved that proline plays major roles as antioxidative defense molecule and signaling molecule during stress. In our case, the proline level may be more regarded as a symptom of stress intensity signal than an indicator of stress tolerance. In line with our hypothesis, Ghars et al. (2008) showed that the accumulation of proline itself does not confer a higher tolerance to salt stress in *Thellungiella halophila*. It could be considered that proline is part of a signaling pathway and that higher proline content just reflects that the plant experiences higher stress intensity (Kaur and Asthir 2015; Kavi-Kishor and Sreenivasulu 2014; Zhang and Becker 2015). This does not necessarily imply that proline has no positive impact. In rice, Lutts et al. (1999) hypothesized that proline accumulation results from glutamate dehydrogenase activation allowing to detoxify NH_4^+ through integration in glutamine so that the highest accumulation of proline in salt-sensitive cultivars should be regarded as the consequence of an overaccumulation of

ammonium independently of any attempt of osmotic adjustment. Besides, one may also argue that a moderate proline accumulation is sufficient in the most tolerant cultivars to trigger adaptation: in this sense, tolerance would be related to the kinetics of the response rather than to the nature of the response.

In the specific case of salinity, accumulation of toxic ions may also contribute to lowering osmotic potential and our results confirmed this hypothesis. Indeed, depending on rice cultivar and anionic component of salt stress, the contribution rate of Na^+, Cl^- and S^{6+} in osmotic adjustment were found to be between 48-80.1%, 58.6-97% and 6.1-8.8% respectively, considering a vacuolar accumulation. It should be noticed that the total contribution rates of salt ions in osmotic adjustment were higher for NaCl than for Na_2SO_4 salinity. Although proline and salts ions showed a higher contribution to osmotic adjustment in NaCl- than Na_2SO_4-treated plants, we observed that the water content was drastically decreased in IKP salt-sensitive rice cultivar stressed by chloride salinity, confirming that osmotic adjustment is necessary but not sufficient to ensure the maintenance of an optimal plant water status. Moreover, only the salt ions compartmentalized in the vacuole would play a key role in osmotic adjustment since their accumulation in the cytoplasm is detrimental to cellular metabolism. This type of calculation may be valid for Na^+ and Cl^-, which are not supposed to be integrated in organic compounds (except a very small percentage for Cl^-) but it is probably not valid for S which is integrated in numerous organic molecules (Jez 2019; Kopriva and Kopriva 2005; Chan et al., 2019). Although vacuolar accumulation of Cl^- is a cheap process from a metabolic point of view (Wege et al., 2017), Na^+ sequestration appears rather expensive and has an important metabolic cost (Munns et al., 2020a; 2020b; Rubio et al., 2020).

An efficient stomatal regulation should complete the osmotic adjustment mechanism, even though this regulation is complicated in a salt-sensitive cultivar and has the major drawback to decrease CO_2 assimilation. Stomatal regulation depends on essential element availability at the guard cell level (mainly K^+ and Ca^{2+}) and phytohormones. It has nevertheless to be mentioned that despite the highest osmotic adjustment and the lowest stomatal conductance in NaCl-treated plants comparatively to Na_2SO_4-

exposed one, the shoot water content often decreases in responses to NaCl but not in response to Na_2SO_4. Even if osmotic adjutsment occurs to maintain the water potential gradient in favor of water absorption by the plants, it indicates the direction of water flux but not the water velocity and we need perhaps to consider that salinity had an impact on the root hydraulic conductivity (Kaneko et al., 2015) and aquaporins (Rios et al., 2017) to alter the water movement.

In our study, the salt ions were more accumulated in NaCl- than in Na_2SO_4-treated plants. Throughout this study, salt-treated plants were exposed to similar Na^+ concentration and accumulated anions thus appeared to influence Na^+ translocation in the plant. This view is supported by the fact that accumulated Cl^- in NaCl-treated plants was by far higher than sulfate accumulation in Na_2SO_4-treated ones. We noticed that, in most of our experiments, Na^+ accumulation was higher in NaCl-treated plants than in Na_2SO_4-treated ones. Hence, it is difficult to determine if the higher toxicity recorded for NaCl comparatively to Na_2SO_4 is due to the specific anion impact or to the fact that Na^+ was more concentrated in the first case than in the later one. Nevertheless, an argument to hypothesize a specific anion effect came from our transcriptomic approach: gene regulation was quite different for the two salts, and this support a role for anion accumulation since a quantitative difference for Na^+ accumulation should probably lead to quantitative difference in similar transcript but not to so many differences in terms of transcript identity.

The negative effects of salinity on plant growth and performance are generally attributed to the toxicity of Na^+, which at high cytosolic concentrations may disrupt K^+ homeostasis, negatively affecting functioning of a large group of enzymes (Kronzucker and Britto 2011). Although anions such as Cl^- and SO_4^{2-} have often been considered as essential elements in the plant metabolic process, their excessive concentration in the cellular cytoplasm impairs the normal plant functioning. It has been observed that in some species, Cl^- may even be more toxic than Na^+ (Luo et al. 2005; Tavakkoli et al. 2010a) while recently, Khare et al. (2015) found that though Cl^- was relatively less toxic than its counter-cation in rice, its effects could not be totally ignored. It is clearly established that Na^+ interact with a wide range of physiological and

metabolic process in plant cell, but we do not have exhaustive information on the fact that Cl^- is able to act on the same set of cellular targets. Regarding S, we were able to quantify the global S content in our plant sample, but we were unable to assess if this sulfur is present in organic compounds, or still accumulated as SO_4^{2-} ions. We just noticed that the total glutathione content, which is a major metabolite resulting from S assimilation, was not higher in Na_2SO_4-treated than in control or NaCl-treated plants.

Under saline conditions, oxygen is more frequently activated into reactive oxygen species (ROS) during metabolism. ROS possess a strong oxidative ability and cause cytoplasmic membrane damage, irreversible metabolic dysfunction, and cell death (Liang et al. 2018). To cope with the ROS produced, plants possess complex antioxidative defense system comprising of hydrophilic (AOAM) and lipophilic (AOAD) antioxidants to scavenge ROS (Choudhury et al. 2017). In the present study, we deduced that Na_2SO_4-treated plants more efficiently triggered antioxidant response than plants exposed to NaCl toxicity, as evidenced by a higher accumulation of lipophilic antioxidants in the former case than in the latter one. One may therefore consider that α-tocopherol is an interesting compound that should be analyzed in the future since it is acting as the major lipophilic antioxidant in plants (Bose et al. 2014). Once again, this suggest that, although the hydrophilic fraction which contains glutathione was initially expected to increase, the rice plants displayed a different behavior through increase of antioxidant directly associated with membrane structure.

We confirm that the anionic component of salt stress has a specific impact on rice behavior for a same Na^+ external concentrations. Therefore, chloride salinity was shown to be more detrimental to rice plants than sulfate salinity for most studied parameters. This should not be regarded as a general rule in the plant kingdom, and an opposite trend has been observed in other plant species (Curtin et al. 1993; Datta and Sharma 1990; Gao et al. 2012; Han et al. 2014). The deleterious effect of salinity on rice plants would have been due to the additive effect of cation and anion. The over-accumulation of salt ions in the NaCl-treated plants might alter its system of signal perception and transmission, and at the end, should highly disrupts its defense mechanisms. Beside the level of ion accumulation, the

distribution of ions between tissues, between cells within a given tissue, and even between compartments in a given cell are important parameters to analyze. The precise localization of elements in plant tissue is a crucial aspect to be able to rely toxic ion accumulation and plant symptoms; it however requires very sophisticated and expensive technics to be determined in a reliable way. Another reason that could justify the difference between these two types of salt stress is that the concentration and activity rates of salt ions are far higher in NaCl- than in Na_2SO_4-treated solutions; the concentration and activity rates of the sulfate anion being extremely low. Speciation also needs to be considered: Na^+ and Cl^- free ions represent more than 92% of respective elements according to our speciation program, while SO_4^{2-} represent only 80% or even less of sulphur, the remaining species being $MgSO_4$ and $NaSO_4^-$: SULTR transporter involved in sulfate absorption are rather specific and there is no guarantee that they are able to absorb these ionic species.

Overall, the discrepancy observed in NaCl and Na_2SO_4 effects on rice plant confirms our results that revealed a higher toxicity of NaCl over Na_2SO_4 for most studied physiological parameters such as biomass production, water and nutrients uptake, plant growth, photosynthetic activity and plant yields.

2. Are the differences between the NaCl and Na_2SO_4 toxicities on rice similar at the vegetative and reproductive phase of development from both a quantitative and a qualitative point of view?

According to Itoch et al. (2005), the rice plant developmental course is divisible into three phases, namely embryogenesis, vegetative and reproductive phases. Embryogenesis phase begins with the first zygotic division and ends by seed dormancy breakage. The interval from seed germination to inflorescence development corresponds to vegetative phase while the reproductive one begins from inflorescence development until grain maturation. Throughout our thesis project, we have always considered either the vegetative phase only (without salt stress application at seed germination stage) or the vegetative and reproductive phases

depending on the aim of the experiment. It has to be recall that, according to our experimental procedure, plants that were exposed to salinity during flowering were also exposed to salinity during the vegetative stage. It is therefore difficult to determine if the impact of salt recorded on reproductive and yield-related parameters was only the consequence of the presence of salt during flowering itself and subsequent grain maturation, or could be, at least to some extent, the consequence of a previous exposure during the vegetative period which could compromise plant fitness on a long term basis. Studying another abiotic stress (high temperatures), Shimono and Ishii (2012) demonstrated that an exposure to stress applied only during the vegetative may have strong deleterious impact on the grain behavior, even if the stress was removed during flowering. An alternative would have been to apply salinity starting at the heading stage but we did not choose this option because it does not appear relevant from field conditions where plants are exposed to salinity during the whole cycle.

The scientific community confirms that the impact of abiotic stresses, including salinity, differs according to the plant development phase and that the underlying physiological processes involved in response to salinity are not the same at all phenological stages. From our point of view, no study has been performed with the aim of quantitatively and qualitatively assessing the effects of NaCl and Na_2SO_4 toxicities on the vegetative and reproductive phases of rice plants. Therefore, one of the goals of our research project was to check whether the differences between NaCl and Na_2SO_4 salinities at the vegetative stage of rice plant (as evidenced by our results in different chapters) are quantitatively and qualitatively preserved at the reproductive phase.

For some studied parameters in chapter II of our thesis, the first measurements were performed 14 days after the application of salt stress (and therefore 29 days from the beginning of grain germination): this first phase corresponds to seedling stage. The second set of measurements were taken 30 days after the application of the salt stress (and therefore 45 days from the beginning of grain germination): this second phase corresponds to tillering stage. The second stage theoretically corresponds to the panicle initiation phase but it is difficult to distinguish the tillering phase and panicle initiation phase at this period since the last phase is not visible to

the naked eye. Indeed, we consider that the first phase (seedling) is vegetative and that the second one (tillering) is reproductive.

Considering the quantitative results of chapter II on gaseous exchanges, we deduced that the effects of NaCl and Na_2SO_4 toxicities on rice plant were slightly different at the vegetative and reproductive phases. The differences between these two salt treatments for net photosynthetic rate and instantaneous transpiration rate were respectively 12.3% and 5.6% at vegetative phase while the differences at reproductive phase were respectively 9.6% and 7.5%. Unlike these results on gaseous exchanges, the differences between NaCl and Na_2SO_4 were very high regarding the plant content in photosynthetic pigments. Indeed, the differences between chloride and sulfate salinities for Chla, Chlb and carotenoid contents were respectively 53.7%, 67.9% and 57.2% at vegetative phase while these differences were respectively 29.4%, 38.9% and 26% at reproductive phase .

It is considered that the flag leaf plays a key role in grain filling for all cereals, including rice. In this case, the salinity impact on photosynthetic activity of the flag leaf might be proportional to salinity impact on grains weight. However, we noticed that the difference between NaCl and Na_2SO_4 salinities for the net photosynthetic activity in flag leaf was 17.7% while difference for global 1000-grains weight was 2.3% only. This difference could be explained by the involvement of other leaves or from contribution from other parameters which intervene in grain filling mechanism such as the photosynthetic activity performed at the grain hulls level (lemma and palea) as well as the efficiency in carbohydrates translocation. Mohapatra et al. (2004) indeed demonstrated that various rice cultivars display different behavior in this respect: in lower-yielding traditional rice (such as Pokkali), photosynthesis in the flag leaf mainly contributes to carbon supply to developing grains while in super-yielding rice, not only the flag leaf but also the two leaves below it, equally contribute to grain filling.

Hull are also major (and often neglected) contributors to grain filling in cereals. The size of hull strongly influences grain development and this could be directly related to cell division (Fan et al., 2019) and to phytohormonal status (Tong et al., 2018), both parameters being directly influenced by salinity. Deng et al. (2019) recently performed a proteomic

analysis of glumes and awn in barley under water deficit and demonstrated that an important proportion of differentially accumulated proteins are involved in photosynthesis. Glumes have higher water use efficiency than leaves (Araus et al., 2005) and display a C3-C4 intermediate type of photosynthesis which may contribute for more than 44% to grain filling. In our study, we demonstrated (Chap II) that hull accumulate very high concentration of Na^+, Cl^- and S^{6+} and we suspect that those elements could interfere with hull photosynthesis and thus negatively impact grain weight. In an old study, Seo and Ota (1982) even postulate that sterile spikelets may contribute to grain filling of fertile one although they did not provide full evidences of the translocation of carbohydrates. The impact of salinity on grain weight may result in a decrease of carbohydrates translocation and starch accumulation in relation to an impact on sugar-metabolizing enzyme activities (Fan et al., 2019) but the proportion of protein in the grain increased in the salt-treated rice, and to a similar extent in response to NaCl and Na_2SO_4. According to Saleethong et al. (2016), such an accumulation could be realted to accumulation of stress proteins with protective roles rather than accumulation of classical reserve proteins.

Considering the high level (10 dS m^{-1}) of salt stress applied on rice plants in chapter V, we found that the differences between these two types of salinities for tillers production were 35.08% and 52% for Pokkali and V14 cultivars respectively while the differences for grain weight per plant were 20.25% and 17.5% for Pokkali and V14 respectively. Such difference could explain that the rice plant was able to compensate for the reduction in tillers production and that therefore, this compensation process reduced the difference between the two types of salinity in terms of final yield.

Yokas et al. (2008) analyzed the responses of the tomato (*Lycopersicon esculentum* Mill.) plants exposed at 40 mM NaCl and 20 mM Na_2SO_4 at vegetative and reproductive phases. The results showed that the differences between chloride and sulfate salinities were almost similar at vegetative and reproductive phases for most of studied parameters. As example, the differences between the two salt stresses for seed germination and pollen germination were respectively 5.6% and 3.84% while the differences for seedling height and pollen tube length were 15.4% and 10% respectively. The differences between chloride and sulfate salinities at vegetative phase

could then be kept in reproductive phase depending on plant species, and especially the type of fruits since tomato produced a climacteric fresh fruit while rice is producing a dry kernel.

From a qualitative point of view, it was proved that NaCl was more toxic than Na_2SO_4 for most of the studied parameters in both chapters II and V. This difference was noticed for vegetative as for reproductive phase. At vegetative phase, chloride was more toxic than sulfate salinity for the parameters such as stomatal conductance, photosynthetic pigments concentration, biomass production and tillers production. At reproductive phase, NaCl was more toxic than Na_2SO_4 for the parameters such photosynthetic pigments concentration, spikelets number per panicle, floret fertility, pollen viability, stomatal density and index, grain number per panicle and grains weight per plant. Throughout this thesis project and considering all studied parameters, Na_2SO_4 has never been more toxic than NaCl. We therefore confirm that the differences between these two types of salt stress on rice are qualitatively similar at the vegetative and reproductive phase of development. Overall, we conclude that the differences between NaCl and Na_2SO_4 salinities on rice are not quantitatively similar at the vegetative and reproductive phase of development.

3. Could the "NaCl-resistant" rice cultivars be considered as "Na_2SO_4-resistant" as well?

Throughout this thesis project, three rice cultivars with contrasting tolerance levels were used. The tall indica landrace Pokkali and I Kong Pao (IKP) cultivars are respectively resistant and sensitive to saline stress. These two cultivars were obtained from IRRI (International Rice Research Institute; Philippines) and are often used in research devoted to salt stress in our laboratory. The salt-sensitive cultivar V14 was obtained from the National Institute of Agronomic Research of Burundi. V14 cultivar was used during experiment conducted in field conditions in Burundi and was chosen because it is more suited to the agro-ecological conditions of Burundi than the cultivar IKP. In addition, being the most cultivated cultivar in the Rusizi plain due to its high productivity and organoleptic

qualities, the IRRI researchers (with whom we collaborated in this study), wanted to confirm its sensitivity once again.

For most studied parameters, these three cultivars often displayed different levels of salt-resistance at both vegetative and reproductive phases. At vegetative phase where Pokkali and IKP cultivars were exposed to iso-strength Na^+ nutrient solutions (50mM NaCl or 25mM Na_2SO_4; EC: 5.31 dS m^{-1}) , we took four examples among studied parameters in order to check whether a rice cultivar can be considered as resistant to both NaCl and Na_2SO_4. Regarding biomass production, chloride salinity decreased the shoot dry weight of Pokkali and IKP cultivars by 24% and 52.7% respectively while sulfate salinity decreased the shoot dry weight of Pokkali and IKP by 7.12% and 36.3% respectively. This clearly confirms that, even if the two types of salt act differently on plant, the resistance mechanisms adopted by the plant to these two constraints may rely on similar properties.

The shoot biomass reductions have harmful consequences on rice plant via the reduction in photosynthetic activity as well as the decrease in storage space for carbohydrates synthesized and ultimately lead to plant yield reduction (Negrão et al. 2017). To cope with osmotic stress generated by salinity stress, a plant decrease its osmotic potential in order to maintain sufficient water inside the cells (Sheldon et al. 2017). Therefore, the NaCl-treated plant roots of Pokkali and IKP cultivars decreased the osmotic potential by 83% and 27% respectively while the Na_2SO_4-treated plant roots of Pokkali and IKP cultivars reduced the osmotic potential by 76% and 26.7% respectively. Hence, not only management of the ionic constraint, but also management of the osmotic component of salt stress were more efficient in Pokkali in response to the two types of stress.

As expected results, the plant water content was not significantly affected by salinity in shoots and roots of Pokkali cultivar while the salt stress significantly reduced the plant water content in shoots and roots of IKP cultivar. The reactive oxygen species (ROS) produced in salt-treated plants generate lipids peroxidation process and malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in plant cells (Slama et al. 2017). Therefore, MDA level is known as a marker of

oxidative stress. Our results showed that NaCl salinity increased the MDA contents in shoots of Pokkali and IKP cultivars by 64.17% and 138.27% respectively while the increase in Na_2SO_4-treated shoots were 19.8% and 31.3% respectively. Indeed, it is obvious that Pokkali had always been more resistant than IKP for all these parameters at the vegetative phase. In addition, Pokkali maintained its resistance level to both NaCl and Na_2SO_4 toxicities while IKP remained sensitive to these two salt treatments.

It is tempting to speculate that a dilution effect occurs in tall indica landraces such as Pokkali. This type of varieties produces high shoot biomass (and have thus low harvest index) and may be suspected to absorb toxic ions and distribute them in a higher shoot volume, leading to a decrease in ion concentration. This hypothesis has been frequently mentioned in the literature (and partly validated in some works) (Saleethong et al., 2013; Krishnamurthy et al., 2016; Kaur and Pati, 2017; Mukherjee et al., 2019). If it is valid, it implies that ion concentration expressed on a dry weight basis should be lower in Pokkali than in the two salt sensitive cultivars but this was not the case in our study. Indeed, in Chapter I, Pokkali exhibited similar shoot Na^+ concentration and more Cl^- than IKP while in Chapter V, it even accumulated more Na^+ than V14.

Two additional hypothesis may then be considered. The first one considers that, for an identical amount of toxic ions, distribution of toxic ions was not similar in Pokkali and in salt-sensitive cultivar. The salt resistant cultivar could be able to preferentially accumulate toxic ions in the oldest leaves, which are less important for photosynthesis, thus preserving the young leaves from deleterious accumulation of toxic ions, or in the sheaths rather than in lamina of the leaves. We did not check this hypothesis in our work, but it has been previously demonstrated in the literature that such "organ compartmentation" indeed occur in salt-resistant cultivars (including Pokkali) (Ganie et al., 2019; Reddy et al., 2017; Hoang et al., 2016; Zhu et al., 2000, 2001, 2004). The second hypothesis (which is not incompatible with the first one) considers that Pokkali displayed a typical tolerance strategy and that it is thus able to trigger some gene expression and/or activate specific metabolic pathways affording a more efficient protection against endogenous accumulated ions. Once again, we were not able to test this hypothesis since, for economic reasons, we performed the

transcriptomic approach (Chapter III) on the salt-sensitive IKP only. Nevertheless, the capacity of Pokkali to efficiently activate glutathione cycle, as mentioned in Chapter I, support this hypothesis. Different transcriptional profile were evidenced for contrasting rice genotypes exposed to NaCl (Walia et al., 2005) but this kind of approach was never performed for Na_2SO_4 to the best of our knowledge.

The reproductive phase is crucial for rice crops because it of course widely contributes to the final plant yield. Unfortunately, the reproductive phase is commonly considered as one of the most salt-sensitive development stage in *Oryza sativa* (Lutts et al. 1995). Based on the results of chapter V where we evaluated the effects of NaCl and Na_2SO_4 salinities on some yield-related parameters of Pokkali and V14 cultivars, we showed that these two rice cultivars displayed different levels of salt-resistance. Considering the high level of salt stress (EC of c.a. 10 dS m^{-1}), NaCl decreased the grains weight per plant by 53.4% and 74.5% in Pokkali and V14 cultivars respectively while Na_2SO_4 reduced this yield-related parameter by 33.5% and 56.7% in Pokkali and V14 cultivars respectively. The salt stress applied also hampered the floret fertility. Choride salinity reduced the floret fertility by 49.4% and 68.7% in Pokkali and V14 cultivars respectively while sulfate salinity decreased the floret fertility by 26.3% and 39.6% in Pokkali and V14 cultivars respectively.
Based on these previous results, which prove that Pokkali cultivar remained more resistant to both NaCl and Na_2SO_4 salinities than IKP and V14 salt-sensitive cultivars, we confirm that the NaCl-resistant rice cultivars could be considered as Na_2SO_4-resistant as well. However, our confirmation is only valid within the framework of our thesis project since the number of cultivars used did not correspond to a statistically representative sample.

4. Do the differential responses to chloride and sulfate salinities partly rely on specific gene expression?

As a reminder, the main objective of our thesis project was to discriminate the effects of anionic components of NaCl and Na_2SO_4 in rice (*oryza sativa* L.), knowing that rice responses to Na_2SO_4 salinity only received minor attention. One of the key points of a comparative study like this is to

perform a molecular analysis in order to identify the genes involved in the plant's response to applied stress. Numerous studies on molecular mechanisms of rice tolerance to salt stress have been conducted by mainly focusing on NaCl tolerance (Ganie et al. 2019; Kumar et al. 2013; Shinozaki et al. 2007; Walia et al. 2005; Zhou et al. 2007), but none has investigated the difference between NaCl and Na_2SO_4 tolerance. Throughout our thesis project, two comparative studies on molecular mechanisms of rice tolerance to chloride and sulfate salinities were performed. During the first study (chapter III), a comparative transcriptomic approach offered the first comprehensive picture of genome expression modulation in response to chloride and sulfate salinities in IKP rice cultivar. The second study (chapter IV) aimed to determine the differential effects of NaCl and Na_2SO_4 salinities on Na^+-transport regulatory mechanisms in IKP rice cultivar.

One of the most interesting results of the comparative study of rice transcriptome was the quantitative and qualitative importance in the difference of gene expression under NaCl and Na_2SO_4 toxicities. Most of the genes were specifically expressed in response to NaCl or Na_2SO_4 as evidenced by the fig. 5 in chapter III. Few genes overlapped between these two salt treatments whatever the plant organ considered. Among 1745 common genes selected for further consideration in this study, 1374 genes were specifically expressed in response to NaCl or Na_2SO_4 salinity. As examples, regarding the genes involved in response to abiotic stimulus, 94 and 120 genes were specifically induced in NaCl and Na_2SO_4-treated shoots respectively with an overlap of 52 genes only. Concerning the genes involved in signal transduction, 41 and 57 genes were specifically down-regulated in NaCl- and Na_2SO_4-treated roots with an overlap of 4 genes. For the case of the genes encoding the transport of different elements (osmoprotectants, ions and water) in plant, 49 and 64 genes were specifically induced in NaCl and Na_2SO_4-treated shoots respectively with an overlap of 3 genes only. This constitutes unexpected observation since Na^+ accumulated at high concentration in response to NaCl and Na_2SO_4 and so many differences between differentially expressed genes suggest that this Na^+ accumulation by itself has low impact on gene regulation. In the same view, this would suggest that the anion rather than Na^+ assume a major role in triggering gene expression.

We noticed that among the genes expressed specifically in response to NaCl or Na_2SO_4 toxicity, most of them played a key role in tolerance to salt stress. As examples, the genes Os01g0859300, Os03g0390200 and Os12g0114100 involved in signal sensing, perception and transduction were specifically induced in response to sulfate salinity in shoot. The genes Os01g0187900, Os04g0398000, Os07g0688200 and Os09g0106700 encoding the transcription control during osmotic stress were specifically induced in NaCl-treated shoots. The gene Os07g0666900 involved in vacuolar compartmentalization of excess Na^+ was only induced in Na_2SO_4-treated shoots and roots. The common genes between NaCl and Na_2SO_4 displayed the different expression profiles in term of expression levels. Most of common genes were more induced in Na_2SO_4- than NaCl-treated plants. The gene expression profiles in response to these two salt stresses also differed depending on plant organs, namely shoot and root.

An alternative regarding such a low overlap in gene regulation between NaCl- and Na_2SO_4-treated plants despite similar global Na^+ accumulation in the shoot could be that distribution of this element between cell compartments differ in the two cases. Since the physiological impact of any toxic ion strongly depends on its subcellular distribution, one may assume that contrasting distribution of Na^+ in NaCl- and Na_2SO_4-treated plants may be responsible for the recorded transcriptomic differences. There is therefore an interest to analyze the expression of genes involved in Na^+ transport. Regarding the study on Na^+-transport regulatory mechanisms in IKP rice cultivar, only *OsHKT3* gene was not expressed in both salt-treated plants. This gene was expressed in shoots and roots of NaCl-treated plants but no gene amplification was detected in either shoots or roots of Na_2SO_4-treated plants. Other genes such as *OsSOS1*, *OsNHX1*, *OsNHX2, OsHKT2*, *OsHKT4* and *OsHKT6* showed specific expression in response to NaCl or Na_2SO_4 depending on plant organs (shoots or roots) and stress duration (3h, 24h and 1week).

Although some genes were only expressed in response to sulfate stress in this study, other previous studies have shown that these same genes were expressed in response to NaCl salinity. Studying the phenotypic, metabolic, and transcriptome responses of two related rice genotypes, IR64

(moderately sensitive to salt-stress) and PL177 (salt-tolerant) exposed to 140 mM, Wang et al. (2015) proved that the gene Os07g0666900 was up-regulated in both cultivars. Xu et al. (2018) also showed that Os01g0859300 was induced in Nipponbare rice cultivar (salt-tolerant) exposed to 150 mM NaCl.

As part of our transcriptomic study and only under the experimental conditions considered for this study, we can confirm that the differential responses to chloride and sulfate salinities partly relied on specific gene expression. However, our observation should not be taken as a general rule for several reasons. Firstly, we used a single cultivar when there are more than 100,000 rice cultivars already known in the word and therefore, our sample was not at all representative. Secondly, the gene expression profile varies over time depending on the type of salt stress as revealed by our results in Chapter IV. As we have only considered one time point of stress exposure (48hours), it would be difficult and less careful to confirm that a gene was not expressed throughout its entire life cycle. As proof, the previous paragraph show that some genes not induced under NaCl in this study were induced in other studies using rice cultivars and experimental conditions different from ours. Concerning the genes involved in Na^+-transport regulatory mechanisms (Chapter IV), it is clear that these genes were specifically expressed in response to chloride and sulfate salinities depending on plant organs and stress duration. However, the results of this chapter should be considered with more caution since some genes were induced in control plants without excess Na^+ to exclude or to compartmentalize.

Finally, establishing a direct relationship between gene activation or repression on the one hand, and plant behavior on the other hand may be regarded as a bit short. Kubala et al. (2015) demonstrated that transcriptomic and proteomic analysis in salt-treated *Brassica napus* provided quite different results: even if a gene is induced or overexpressed, the corresponding protein remains unmodified or even absent. It is always tempting to find a “reason” for modification of gene expression and to assume that if this occurs, it must have a finality. Is it so clear? Can we imagine that some modification in gene expression constitute a symptom of injury or that they result from a useless modification in signal

transduction with no precise aim in terms of plant adaptation? If this occurs, it should have a real metabolic cost for the plant and it poses the "old question" of relative efficiency of constitutive versus inducible response to the exogenous constraint.

Overall, the results of these two studies showed that differential responses to chloride and sulfate salinities partly relied on specific gene expression. Indeed, further studies are necessary to confirm our observations and to identify other specific rice genes involved in response to NaCl or Na_2SO_4 salinity. As part of these studies, a representative sample of rice cultivars, the different levels of salt stress as well as different plant development stages should be considered.

5. Are the data obtained for the two types of salinity in controlled conditions and hydroponic culture comparable to those obtained in field conditions on a soil substrate in Burundi?

Burundi's economy is rural and more than 90% of the Burundian population lives from agricultural activity. Among the most consumed foodstuffs, rice ranks second among cereals after maize and more than 50% of national rice production is provided by the Rusizi plain (Gahiro 2011). Unfortunately, more than 15% of the Rusizi plain area has been severely affected by salinity and 182 rice fields of about 0.5 ha each have been completely abandoned in 2010 (Nijimbere 2014); the result being a drastic reduction in rice production at the national level as well as the deterioration in household well-being.

During the study on physico-chemical properties of the saline soil sampled in Rusizi plain, Nijimbere (2014) found that the widely dominant salt ions were Na^+ among cations and SO_4^{2-} among anions unlike many salinized part in the world where the dominant anion remains Cl^-. Therefore, many studies on plant responses to salt stress have been performed using NaCl while Na_2SO_4 effects on plant received minor attention despite its importance. Indeed, one of the major motivation of the present thesis project was to contribute to afford solution for the management of the Na_2SO_4-affected area in Rusizi plain since many soil-restoring techniques

had failed (Kabwa 2010). We would have liked to perform all our experimental studies in field conditions in the Rusizi plain but the economic and security situation in Burundi could not allow it. As an alternative, most of our studies were performed in Belgium (UCLouvain) and the experimental methodology consisted in assessing the effects of NaCl and Na_2SO_4 on physiological, biochemical and molecular parameters of different contrasted rice cultivars using the hydroponic culture. However, the interactions between root-zone environments and plant responses to increased osmotic pressure or specific ion concentrations in the field are complicated by many soil processes such as soil water dynamics, soil structural stability, solubility of compounds in relation to pH and pE (electron concentration related to redox potential), nutrient and water movement in soil (Rengasamy 2010). Knowing this scientific evidence, the last experimental study was conducted in soil conditions in Burundi in order to compare the similarity levels between the results from these two different experimental conditions.

Comparison of these two sets of results is difficult since the stress intensities and rice cultivars used were different. However, the combination of a few parameters measured throughout our thesis project can give us some guidance. Knowing that the pH values of the control nutrient solution and control soil were respectively 4.73 and 6.9, the application of saline stress equivalent to 50 mM Na_2SO_4 and 100 mM NaCl (chapters IV and V) raised the pH values to different levels depending on the type of culture substrate. In nutrient solution, NaCl and Na_2SO_4 salinities increased pH by 1.15 and 1.59 respectively (the final pH values becoming 5.88 and 6.32 respectively) while in soil conditions, NaCl and Na_2SO_4 salinities increased pH by 0.6 and 1.3 respectively (the final pH values becoming 7.5 and 8.2 respectively). We deduce that the increase in pH values was higher in nutrient solution than in soil and between the two types of salinity, the strongest increase has always been recorded for sulfate. In nutrient solution, the pH rose from the acid to almost neutral level (pH threshold favorable for crop growth) while in the soil, the pH rose from neutral to alkaline levels (pH threshold harmful to crop growth). It has been proven that the high soil pH values negatively affect the plant through the ion imbalance of soil solution due to low solubility and high adsorption of

certain nutrients like N, P, K, Ca, Cu, Zn, Fe and Mn (Marlet and Job 2006; Qadir et al. 2006).

Another difference between hydroponics and soil has been recorded in terms of electrical conductivity (EC). Considering the soil experiment performed in Burundi, the soil solution EC values at the end of the experiment (so after harvesting mature rice grains) were almost 10 times lower than the EC values of saline solution initially applied. In contrast to the reduction in EC values as has been observed in soil conditions, the EC values were kept stable throughout the experiment conducted in hydroponic conditions. In soil conditions, the strong decrease in EC values could mainly be explained by the dilution of the soil solution following the plant watering throughout the experiment while the stress level was not regularly renewed and controlled in order to be kept stable. The permanent exchange of ions between the liquid and solid phases of the soil could also partly explain this decrease in EC. Mizanur and Roxana (2005) applied in the soil a saline solution of 13 dS m^{-1} NaCl and recorded only an EC of 2 dS m^{-1} at the end of the full life cycle of rice crop.

The other outstanding fact between these two types of experimental conditions concerns the accumulation of salt ions in the plant. Considering the moderate stress level in chapter V and the experimental conditions in chapter I where we applied a salt stress of 25 mM Na_2SO_4 and 50 mM NaCl, we observed that plants accumulated salt ions differently depending on the type of substrate. Although the stress duration under hydroponic conditions was only two weeks while the stress duration under soil conditions was four months, the accumulation of Na^+ and Cl^- in plant shoots was respectively 5 and 7.5 times higher in the first case than in the latter one. The low accumulation of salt ions recorded under soil conditions could be explained by the dilution effect of the soil solution as well as the migration of some of these ions to the exchange complex.

Even if we are unable to compare the rest of the results obtained in the first four chapters to those obtained in chapter V (since we have used different stress intensities and cultivars), it is obvious that NaCl remained more toxic than Na_2SO_4 and that Pokkali remained the most tolerant cultivar compared to IKP and V14 whatever the culture substrate considered.

Tavakkoli et al. (2010) studied the barley response to NaCl salinity using the hydroponic and soils systems. The authors concluded that the effects of salinity differed between these two experimental conditions. Differences between barley cultivars in growth, tissue water content and ionic composition were not apparent in hydroponics, whereas significant differences occurred in soil. Growth reductions were greater under hydroponics than in soil at similar electrical conductivity values, and the uptake of Na^+ and Cl^- was also greater. These authors concluded that cation exchange capacity of the soil reduces salt stress by facilitating the adsorption of Na^+ into the surface of soil colloids while the buffering capacity provides enough time for the plants to acclimate to soil stress.

It is also important to recall that our experiments in Burundi were performed in pots on an artificially-contaminated substrate and that the pots were maintained in greenhouses. This implies that fluctuations of environmental conditions susceptible to influence plant response to salinity were avoided, or at least strongly reduced. Another difference is also related to the fact that soil salinity is greatly heterogeneous under field conditions, both horizontally and vertically (Bazihizina et al., 2009; Nguyen et al., 2014). Thapa et al. (2017) were performing experiments with spring wheat and observed that the plasticity of the root system allowed it to explore the less saline zone of the profile, providing greater salinity resistance in crops comparatively to plants growing in pots in a limited volume of homogeneous saline substrate.

Our results do not allow us to specify an experimental condition more harmful to the plant. For some parameters such as pH, EC and salt ions accumulation in plant, we confirm that the data obtained for the two types of salinity in controlled conditions and hydroponic culture are different from those obtained in field conditions on a soil substrate. For more detail, we suggest another comparative study on the rice response to NaCl and Na_2SO_4 salinities in soil conditions and in hydroponics. This study should therefore consider the same cultivars and the same stress intensities.

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General conclusion

The results of this research work allowed us to contribute to a better knowledge of the effects of the anionic component of salt stress on physiological, biochemical and molecular parameters of rice plant. This thesis project also provided us the information regarding the tolerance mechanisms adopted by rice in response to NaCl or Na_2SO_4 salinity.

The accumulation of toxic ions in plant depended on salt stress intensities, stress duration and plant organs. The rice plants were exposed to iso-strength Na^+ nutrient solutions or soil solution in all experimental studies and we found that Na^+ ions accumulated to higher concentration in NaCl- than in Na_2SO_4-treated plants. The results demonstrated that even a short-term exposure (3h) to NaCl or Na_2SO_4 was sufficient to induce sodium accumulation not only in roots but also in shoots of salt-treated plants. This suggests that an ionic component of salt stress, and not only the osmotic component as commonly claimed by the biphasic model, may also result from short-term exposure to high salt level. The increase in Na^+ accumulation often resulted in significant reduction in K^+ content but we demonstrated that the two processes dot not necessarily appear concomitantly. Based on the initial concentration of the culture substrates in Cl^- and SO_4^{2-} anions, we observed that rice plant accumulated more chlorine than sulfur. Overall, Pokkali (salt-tolerant rice cultivar) accumulated more toxic ions than IKP or V14 (salt-sensitive rice cultivars).

Salt stress affected the biomass production, the plant growth, the water and nutrient mineral absorption and the photosynthetic activity. The photosynthetic parameters such photosynthetic activity rate, instantaneous transpiration rate and stomatal conductance were significantly reduced by salinity. The salt stress also decreased the synthesis of photosynthetic pigments, namely chlorophyll a, chlorophyll b and carotenoids. It was shown that NaCl was more detrimental than Na_2SO_4 for most of those physiological and photosynthetic parameters. As expected, the impact of salt stress on physiological and photosynthetic activity resulted in deterioration of yield-related parameters. The deleterious impact of chloride salinity was higher than the impact of sulfate salinity for most of

yield-related parameters such as the production of mean number tillers and fertile tillers per plant, the spikelets number per panicle and floret fertility, the straw fresh weight and grains weight per plant. The salt stress also affected the soil chemical properties. The increase in soil pH was only observed under sulfate salinity and this could be related to the adsorption of SO_4^{2-} anions onto soil particles and the subsequent release of OH^- desorbed in the soil. Salinity also increased soil EC as well as Na^+ accumulation on exchange complex and in soil solution.

Our thesis project also revealed some tolerance mechanisms adopted by the rice plant in response to salt stress and these mechanisms may, to some extent, differ depending on the anionic components of salt stress. The salt-induced proline accumulation was higher in NaCl- than in Na_2SO_4-treated plants. Despite the fact that proline is known to play a role in osmotic adjustment, our results revealed that the real absolute contribution of proline in osmotic adjustment considering a cytosolic accumulation was between 0.55 and 1.5%; a notoriously low contribution. In our case, proline could play a key role in signal sensing, perception and transduction as well as in antioxidative defense mechanisms. Accumulation of toxic ions may also contribute to lowering osmotic potential and our results confirmed this hypothesis. The contribution rate of Na^+, Cl^- and S^{6+} in osmotic adjustment were found to be between 48-80.1%, 58.6-97% and 6.1-8.8% respectively, considering a vacuolar accumulation. It should be noticed that the total contribution rates of salt ions in osmotic adjustment were higher for NaCl than for Na_2SO_4 salinity. In this study, we found that rice triggered tolerance mechanisms to saline stress by synthesizing antioxidants. Overall, the plants exposed to Na_2SO_4 managed the salt stress more efficiently than the NaCl-treated plants. Salt-induced increase was more marked for hydrophilic (AOAM) than for liphophilic (AOAD) antioxidants fraction, and for sulfate than for chloride salinity. As tolerance strategy to salt stress, rice plant increased the total protein contents in grains and reduced the stomatal density and index.

The comparative transcriptomic approach showed that the genes were specifically expressed in response to NaCl or Na_2SO_4. It is unexpected observation since Na^+ accumulated at high concentration in response to NaCl and Na_2SO_4 and so many differences between differentially

expressed genes suggest that this Na^+ accumulation by itself has low impact on gene regulation. This would therefore suggest that the anions rather than Na^+ assume a major role in triggering gene expression. The genes involved in Na^+-regulatory mechanisms were differentially expressed depending on plant organs and stress duration. Surprisingly, most of *HKT* genes family did not show amplification and we suggest a confirmatory study which will use several pairs of primers for each gene.

Based on our results, it is clear that NaCl salinity was more toxic than Na_2SO_4 salinity. This could be partly explained by the low concentration and activity rates recorded for the SO_4^{2-} anion as evidenced by the results from the chemical speciation of culture solutions. Another reason would be the low mobility of SO_4^{2-} anion inside the plant. Pokkali was shown to be the most rice tolerant cultivar comparatively to IKP and V14 rice cultivars.

Overall, the results of this study could be useful in rice crop improvement programs aimed at creating new resistant cultivars to Na_2SO_4 salinity. Beside soil management practices, these new cultivars will contribute to increase rice yield on soils salinized by sulfate salinity and to recover the lost areas including the Rusizi lowlands in Burundi.

Limitations and future research directions

As in most research projects, the constraints of time and financial resources have limited the progress of our thesis project. The limitations with their suggested solutions and future research directions are listed below but the list is not exhaustive:

1. In chapter IV, we observed that some genes involved in Na^+-regulatory mechanisms were induced in control plants which, however, had not accumulated excess Na^+. Furthermore, many *HKT* genes family did not show any amplification in salt-treated plants. Those findings were not expected and widely differed with most results of previous studies.
   We suggest a confirmatory study which will use several pairs of primers and whose experimental set-up will be biologically repeated at least three times.

2. Our experimental approach focused on Na^+ transporters: nevertheless, regulations of Cl^- and SO_4^{2-} transporters are also extremely important to consider in order to explain modalities of anion accumulation. Since both Cl and S are essential elements for plant metabolism, specific transporters were selected for their absorption and it may be hypothesized that strategies of down-regulation may occur in response to overaccumulation of these elements within plant tissues but, to the best of our knowledge, no studies until now considered this aspect.

3. Despite its high accumulation in salt-treated plants, proline showed a low contribution in osmotic adjustment. Further studies are needed to check the role of proline accumulated in rice plants exposed to NaCl or Na_2SO_4 salinity. Considering the enzyme activities involved in proline metabolism may help to provide additional information regarding the exact function of the salt-induced increase.

4. Since we confirmed that, for some studied parameters, the data obtained for NaCl and Na_2SO_4 salinities in hydroponic culture were

differed from those obtained in soil conditions in Burundi, it would be better to perform additional studies in field conditions. In addition to soil chemical properties, we suggest to compare the effects of NaCl and Na_2SO_4 on soil physical properties.

5. Throughout this thesis project, we used only three rice cultivars and this sample was not statistically representative since there are more than 100,000 rice cultivars worldwide. In the future, using a larger panel of rice cultivars with diverse genetic backgrounds could provide more details related to rice response to NaCl and Na_2SO_4 toxicities.

6. More physiological traits such as hormonal regulation and accumulation of salt ions in different parts of rice floret could be deeply studied. Furthermore, the determination of salt ions distribution inside the plant cell (precisely the amount of salt ions accumulated in vacuole and cytosol), will allow to further understand whether rice plants are performing toxic ions exclusion or ions balance strategy to tolerate excess salts.

7. As transcriptomic approach did not provide information either on entire set of proteins or metabolites produced by rice plant in response to NaCl or Na_2SO_4 salinity, a combined transcriptomic-proteomic-metabolomic study on a statistically representative sample of rice cultivars and considering several development phases of rice could provide more detailed and useful information. This study should use low or medium doses of salt stress similar to the stress levels usually existing in field conditions and, if possible, consider a kinetic approach with increasing duration of stress in order to have a more complete view of major changes occurring during plant development.

Appendices

Appendix 1. Composition of Yoshida nutrient solution

Source	**Concentration (mmol/L)**
Macronutrients	
NH_4NO_3	1.43
$NaH_2PO_4.2H_2O$	0.323
K_2SO_4	0.512
$CaCl_2.2H_2O$	0.750
$MgSO_4.7H_2O$	1.64
Micronutrients	
$MnSO_4.H_2O$	0.0114
$Na_2MoO_4.2H_2O$	0.00014
H_3BO_3	0.0578
$ZnSO_4.7H_2O$	0.00096
$CuSO_4.5H_2O$	0.0004
Fer-EDTA	0.0427

Appendix 2. Chemical speciation of the Yoshida nutrient solution

Appendix 2.1. Species distribution in nutrient solution salinized with NaCl

Component	Species name
Fe^{3+}	$FeOH^{2+}$
	$Fe(OH)_2^+$
	$Fe(OH)_3$
	$FeHPO_4^+$
NH_4^+	NH_4^+
	$NH_4SO_4^-$
	NH_3
NO_3^-	NO_3^-
	$CaNO_3^+$
	KNO_3
	$NaNO_3$
Na^+	Na^+
	NaCl
	$NaSO_4^-$
	$NaNO_3$
	$NaHPO_4^-$
	NaH_2PO_4
PO_4^{3-}	HPO_4^{2-}
	$H_2PO_4^-$
	H_3PO_4
	$FeHPO_4^+$
	$MgHPO_4$
	$CaHPO_4$
	$CaH_2PO_4^+$
	$NaHPO_4^-$
	$KHPO_4^-$

Component	Species name
PO_4^{3-}	KH_2PO_4
	Na_2HPO_4
	NaH_2PO_4
	$MnHPO_4$
K^+	K^+
	KCl
	KSO_4^-
	KNO_3
	KH_2PO_4
SO_4^{2-}	SO_4^{2-}
	$MnSO_4$
	$MgSO_4$
	$CaSO_4$
	$NaSO_4^-$
	KSO_4^-
	$NH_4SO_4^-$
Ca^{2+}	Ca^{2+}
	$CaCl^+$
	$CaSO_4$
	$CaNO_3^+$
	$CaHPO_4$
	$CaH_2PO_4^+$
Cl^-	Cl^-
	$CaCl^+$
	$MgCl^+$
	KCl
	NaCl
Mg^{2+}	Mg^{2+}
	$MgCl^+$
	$MgSO_4$
	$MgHPO_4$

Component	Species name
Mn^{2+}	Mn^{2+}
	$MnCl_2$
	$MnCl^+$
	$MnSO_4$
	$MnNO_3^+$
	$MnHPO_4$
MoO_4^{2-}	MoO_4^{2-}
	$MgMoO_4$
	$CaMoO_4$
	$HMoO_4^-$
H_3BO_3	H_3BO_3
	$H_2BO_3^-$
Zn^{2+}	Zn^{2+}
	$ZnOH^+$
	$ZnCl^+$
	$ZnCl_3^-$
	$ZnCl_2$
	$ZnSO_4$
	$Zn(SO_4)_2^{2-}$
	$ZnNO_3^+$
	$ZnHPO_4$
Cu^{2+}	Cu^{2+}
	$CuOH^+$
	$CuCl^+$
	$CuCl_2$
	$CuSO_4$
	$CuNH_3^{2+}$
	$CuNO_3^+$
	$CuHPO_4$

Appendix 2.2. Species distribution in nutrient solution salinized with Na_2SO_4

Component	Species name
Fe^{3+}	$FeOH^{+2}$
	$Fe(OH)_2^+$
	$Fe(OH)_3$
	$FeHPO_4^+$
NH_4^+	NH_4^+
	$NH_4SO_4^-$
	NH_3
NO_3^-	NO_3^-
	$CaNO_3^+$
	KNO_3
	$NaNO_3$
Na^+	Na^+
	NaCl
	$NaSO_4^-$
	$NaNO_3$
	$NaHPO_4^-$
	NaH_2PO_4
PO_4^{3-}	HPO_4^{2-}
	$H2PO_4^-$
	$FeHPO_4^+$
	$MgHPO_4$
	$CaHPO_4$
	$CaH_2PO_4^+$
	$NaHPO_4^-$
	$KHPO_4^-$
	$MnHPO_4$
	KH_2PO_4

Component	Species name
PO_4^{3-}	Na_2HPO_4
	NaH_2PO_4
K^+	K^+
	KCl
	KSO_4^-
	KNO3
	$KHPO_4^-$
	KH_2PO_4
SO_4^{2-}	SO_4^{2-}
	$MgSO_4$
	$CaSO_4$
	$NaSO_4^-$
	KSO_4^-
	$NH_4SO_4^-$
Ca^{2+}	Ca^{2+}
	$CaCl^+$
	$CaSO_4$
	$CaNO_3^+$
	$CaHPO_4$
	$CaH_2PO_4^+$
Cl^-	Cl^-
	$CaCl^+$
	$MgCl^+$
	KCl
	NaCl
Mg^{2+}	Mg^{2+}
	$MgCl^+$
	$MgSO_4$
	$MgHPO_4$
Mn^{2+}	Mn^{2+}
	$MnCl^+$

Component	Species name
	$MnSO_4$
	$MnNO_3^+$
	$MnHPO_4$
MoO_4^{2-}	MoO_4^{2-}
	$MgMoO_4$
	$CaMoO_4$
	$HMoO_4^-$
H_3BO_3	H_3BO_3
	$H_2BO_3^-$
	NaH_2BO_3
Zn^{2+}	Zn^{2+}
	$ZnOH^+$
	$ZnCl^+$
	$ZnSO_4$
	$Zn(SO4)_2^{2-}$
	$ZnNO_3^+$
	$ZnHPO_4$
Cu^{2+}	Cu^{2+}
	$CuOH^+$
	$CuCl^+$
	$CuSO_4$
	$CuNH_3^{2+}$
	$CuNO_3^+$
	$CuHPO_4$
	$CuH2BO_3^+$