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SALT ACCLIMATION AND IONIC HOMEOSTASIS ARE ASSOCIATED WITH PRIMING AND MEMORY IN RICE PLANTS

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Thesis presented to the Post-Graduate Program in Soil Science of the Federal University of Ceará, as a partial requirement to obtain Doctor's degree in Soil Science. Area of concentration: Soil Chemistry, Fertility and Biology.

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To God.
To my parents, Joseane and Edson.
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“Believe you can and you're halfway there.” (Theodore Roosevelt)

ABSTRACT

This thesis aimed to test the hypotheses that the pre-exposure of rice plants to eustressors in the early stages of life can generate stress memory. This memory would be responsible for promoting more efficient metabolic responses under salt conditions and to achieve an acclimation state faster. Thus, the first part of the thesis consists of a contextualization on the importance of studies on soil salinity and rice production for food security. Then, a brief review of concepts of great relevance in the context of research, such as acclimation and stress of plants, as well as the concepts of eustress and priming and their relevance in the scientific environment. Subsequently, we started the experimental part, seeking to identify salinity dose capable of promoting eustressor effects in rice plants. For this, we conducted an experiment with different salinity doses (low - 16 mM NaCl; moderate - 25 mM; and high - 50 mM) applied in exposure-recovery cycles to identify possible differences in the responses due to stress repetition, as well as the spatial effect of ionic accumulation on tissues. The results indicate that saline doses from low to moderate, when applied in the vegetative phases, present potential for stress memory formation. So, the exposed plants have morphological alterations in relation to the control plants, yet without severely compromising crop productivity. From the results of this first experiment, a second experiment was carried out using moderate salinity (25 mM NaCl for 7 days) as priming agent at different stages of plant development to identify the effect of application time on metabolic and morphological responses. The tested treatments were priming during germination and during traditional transplanting moment (V6) followed by saline stress (50 mM NaCl), imitating the actual condition of exposure of seedlings after transplanting. As a result, we obtained that the seed priming (SPS) presented high beneficial potential for the crop. Despite reduction in leaf length, there was an increase in dry biomass production and tillering compared to the control treatment. On the other hand, the priming treatment applied at the time of transplanting (VPS) showed a strong distressor effect, with lower parameters than those obtained by the salt stress treatment alone (S). In addition, it was possible to visually identify the premature necrosis of the flag leaf tissues, although they did not present toxic ions' accumulation. Together, the results of these studies show the great phenotypic plasticity of rice, presenting differential responses in each tissue and adapting according to the moment of exposure to the stressful factor, stress intensity, acclimation state. More studies are needed related to the epigenetic part to unravel the mechanisms behind this plasticity, considering the space-time scale of these organisms with such complex and dynamic behavior.

Keywords: Eustress; *Oryza sativa* L.; phenotypic plasticity; priming.

RESUMO

Esta tese teve como objetivo testar as hipóteses de que a pré-exposição de plantas de arroz a fatores euestressores nas fases iniciais da vida conseguem gerar memória do estresse. Essa memória seria responsável por promover respostas metabólicas mais eficientes para alcançar um estado de aclimatação mais rapidamente. Desse modo, a primeira parte da tese consiste em uma contextualização sobre a importância dos estudos sobre salinidade do solo e produção de arroz para a segurança alimentar. Em seguida, é feita uma breve revisão de conceitos de grande relevância no contexto da pesquisa, tais como aclimatação e estresse de plantas, bem como os conceitos de euestresse e *priming* e sua relevância no meio científico. Na sequência, iniciamos a parte experimental buscando identificar uma dose de salinidade capaz de promover efeitos euestressores em plantas de arroz. Para isso, conduzimos um experimento com diferentes doses de salinidade (baixa - 16 mM de NaCl; média - 25 mM; e alta - 50 mM) aplicadas em ciclos de exposição-recuperação para identificar possíveis diferenças nas respostas devido à repetição do estresse, bem como seu efeito espacial nos tecidos. Os resultados indicam que doses salinas de leve a moderadas, ao serem aplicadas nas fases vegetativas, apresentam potencial de formação de memória do estresse, de modo que as plantas expostas apresentam alterações morfológicas em relação às plantas controle, contudo sem comprometer severamente a produtividade da cultura. De posse dos resultados desse primeiro experimento, um segundo foi conduzido utilizando salinidade moderada (25 mM de NaCl por 7 dias) como agente de priming em diferentes fases do desenvolvimento das plantas para identificar o efeito do tempo de aplicação nas respostas metabólicas e morfológicas. As etapas testadas foram *priming* durante a germinação e durante o momento de tradicional transplantio (V6) seguidas de estresse salino (50 mM de NaCl), imitando a condição real de exposição das plântulas após o transplantio. Como resultado, obtivemos que o *priming* aplicado durante a fase de semente (SPS) apresentou alto potencial benéfico para a cultura. Apesar da redução no comprimento das folhas, houve um aumento na produção de biomassa seca e perfilhamento em relação ao tratamento controle. Por outro lado, o tratamento de *priming* aplicado no momento do transplantio (VPS) apresentou forte efeito distressor, com parâmetros inferiores aos obtidos pelo tratamento de estresse salino sozinho (S). Além disso, foi possível identificar visualmente o necrosamento prematuro dos tecidos da folha bandeira, apesar de esta não apresentar acúmulo de íons tóxicos. Em conjunto, os resultados desses estudos apresentam a grande plasticidade fenotípica do arroz, apresentando respostas diferenciais em cada tecido e adaptando-se conforme o momento de exposição ao fator estressante, da intensidade do estresse, do estado de aclimatação. Mais estudos são necessários relacionados a parte epigenética para desvendar os mecanismos por trás dessa plasticidade, considerando a escala espaço-tempo desses organismos de comportamento tão complexo e dinâmico.

Palavras-chave: Euestresse; *Oryza sativa* L.; plasticidade fenotípica; *priming.*

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LIST OF ABBREVIATIONS AND ACRONYMS

A	Net CO_2 assimilation rate
ABA	Abscisic acid
APX	Ascorbate peroxidases
AsA	Reduced ascorbate
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CAT	Catalase
Cl^-	Chloride
DHA	Dehydroascorbate
E	Transpiration rate
EC	Electrical conductivity
FAO	Food and Agriculture Organization
gs	Stomatal conductance
GPX	Glutathione peroxidases
GS	Glutamine synthetase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
K^+	Potassium
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate redutase
Na^+	Sodium
NADPH	Nicotinamide adenine dinucleotide phosphate
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RWC	Relative water content
SOD	Superoxide dismutases
TBARS	Thiobarbituric acid reactive substances
WUE	Water use efficiency

CONTENTS

1 INTRODUCTION

Food security is a global challenge that affects millions of people around the world. According to the Food and Agriculture Organization (FAO), about 820 million people suffer from chronic hunger and more than 2 billion people lack access to nutritious food. Thus, food production is fundamental to ensure access to essential nutrients for human well-being. In this context, it is necessary to develop strategies to improve cultures' resilience to the oscillating environment to sustain food production.

There is a forecast from the United Nations (2024) of an increase in the world's population to 9.7 billion people by 2050. In addition, it is estimated that the world's population will reach a peak of about 10.4 billion people from 2080 to 2100. Considering this scenario, rice cultivation plays a crucial role as one of the main sources of food for more than 3.5 billion people, fundamental to global food security, especially in the Asian and African regions.

There are many rice species of economic importance cultivated worldwide. This culture can adapt to many environmental conditions (each species with its own specificity of the ideal environment), however with climate change, rice quality and production are being hindered. Fahad *et al.* (2019) lists several factors that compromise rice productivity in the world and, among them, is soil salinity.

The salt problem in soils is very common in arid and semiarid regions due to the climate conditions that favor salt rise from the lower levels of soil to the surface. It is also common in coastal areas due to the seawater presence. However, human activities enhance soil salinity through the bad management of lands, compromising food production and, once rice is a very salt sensitive culture, the rise of saline areas can undermine food security (Dias *et al.*, 2021; Sentís, 2021).

FAO estimates that 10% of the total global land area is already affected by salinity (Konyushkova, 2024). This report also suggests some strategies in order to overcome this issue and to increase food production, considering the prediction of population rise. Among the solutions is plant breeding and bioremediation to remove harmful elements from soil, but the rational management of land and culture also has potential on inducing a quicker acclimation to the detrimental environment.

Seed priming consists in a management technique identified centuries ago for researchers such as Darwin and Gaius, but its modern concept has been developed since the 60’s aiming a faster acclimation when plant is exposed to a stress factor (WAQAS *et al.*, 2019). For its efficiency in improving plants' responses to external stimuli, this method has been subject of many researches in order to unravel the mechanisms behind the best performance of the culture (BARBOSA *et al.*, 2023; FERREIRA; PEREZ-MARIN, 2022).

Several priming agents have been tested on seeds (chemical, physical or biological agents) aiming to identify the more viable to be used on a large scale. Its functioning is based on the activation of a memory of stress. It begins when the seed is briefly exposed to an external stimulus, leading to the activation of an alert state. Thus, when a plant is exposed again to a stressful condition, it is capable of quickly responding and overcoming the condition through acclimation (FERREIRA; PEREZ-MARIN, 2022; WAQAS *et al.*, 2019; YADAV *et al.*, 2023).

Taking into consideration the increase in salinity all around the world, the high demand for food with the growing population, the strong economic importance of rice culture and its sensibility to salt and the long time needed to develop new cultivars more tolerant to this specific issue, this thesis aimed to better understand rice plants responses to salinity focusing on the spatial scale.

2 LITERATURE REVIEW

2.1 Epigenetics and plant memory

Plants, as sessile organisms, face the persistent challenge of adapting to a constantly fluctuating environment. Within this context, epigenetics emerges as a fundamental mechanism for comprehending their capacity for adaptation.

Epigenetics refers to heritable modifications in gene expression that occur without altering the underlying DNA sequence. These modifications directly influence how DNA is transcribed and subsequently translated into proteins, thereby critically impacting plant development and its response to environmental stimuli. It is related to biochemical modifications of the genome that cause reversible conformational changes in chromatin, allowing plants to adjust their physiology and metabolism to face adverse conditions without having to go through several generations of evolution.

Crucially, unlike permanent genetic mutations, epigenetic alterations are reversible and can be directly influenced by environmental factors, conferring plants with a vital adaptive plasticity (BASHIR *et al.*, 2025; LÄMKE; BÄURLE, 2017).

From the epigenetic marks, the concept of phenotypic plasticity arises, which deals with different expressions of phenotypes within the same genome shaped by environmental fluctuations. The epigenetic marks are responsible for gene expression regulation so that the plant expresses the phenotype more adapted to the particular condition it is subjected to, leading to acclimatization (VOGT, 2023).

When we talk about plant memory, we refer to the retention of information by the plant from past stressful events that will give rise to different responses than those obtained by “naive” plants (which had not passed through stressful events previously). This may turn the plant into a more tolerant state in case of recurrence of the stress event (SIDDIQUE *et al.*, 2024).

Among the mechanisms involved in plant stress memory is the accumulation or post-translational modification of one or more signaling proteins that can be synthesized or modified and remain inactive while favorable conditions (SIDDIQUE *et al.*, 2024). Another mechanism involves transcriptional responses resulting from the first exposure that induce changes in gene expression or modified responses mediated by transcription factors. The phytohormones also act: ABA remains higher in a second stimulus, and can be associated with the H_2O_2 accumulation, reduction of gibberellin, involvement of acetylsalicylic acid and jasmonic acid (GODWIN; FARRONA, 2020). This is associated with epigenetic modifications (chromatin packaging, histone modification, methylation, RNA polymerase inactivation, sRNA wrapping) as a loop (LOCATO; CIMINI; DE GARA, 2018).

The creation of memory depends on the duration of exposure and the intensity of the stressful factor, so that different stress levels can also lead to different levels of stress memory. The duration of this memory will also depend on the stability of the alleles responsible for stress memory. When based on increase in the level of metabolites or transcription factors, memory has a shorter duration (days to weeks), but information can be retained for longer if it involves processes in chromatin (months) (LÄMKE; BÄURLE, 2017; SIDDIQUE *et al.*, 2024; VILLAGÓMEZ-ARANDA *et al.*, 2022).

Memory can extend to descendants, contributing to adaptation, being intergenerational (when it passes to a next generation) - or transgenerational - when it passes to 2 or 3 descendants. However, there is still the possibility of "resetting" the memory and the plant returning to its original phenotype. This is because by removing or modifying stressful conditions, some phenotypes can become deleterious. Thus, the epigenetic mechanism allows the plant to revert to its initial state, that is, the plant stops to respond according to the previously acquired memory (LÄMKE; BÄURLE, 2017; VILLAGÓMEZ-ARANDA *et al.*, 2022).

To effectively utilize the knowledge of plant stress memory in agricultural contexts, priming stands out as a key intervention. It involves exposing plants to a sub-lethal dose of stress (a "primer") that doesn't cause significant damage but is sufficient to activate their defense mechanisms and initiate the acquisition of stress memory. This beneficial low-level stress is often referred to as eustress (LIU, HAIPEI; ABLE; ABLE, 2022; VILLAGÓMEZ-ARANDA *et al.*, 2022).

The underlying principle behind eustress and priming aligns perfectly with the hormesis theory. This theory posits that substances or conditions that are harmful at high doses can be beneficial at low doses, stimulating a positive adaptive response. In plants, this means a mild, carefully managed exposure to stressors like salinity, drought, or extreme temperatures, can trigger a cascade of protective molecular, physiological, and morphological adjustments, preparing the plant to better withstand more severe future challenges (VILLAGÓMEZ-ARANDA *et al.*, 2022).

The priming concept is based on the learning capacity of living beings. In plant physiology, it refers to the process of plants' pre-exposure to a slight or mild stress factor in order to activate defense mechanisms. This activation involves gene regulation, hormonal production and other metabolic changes that allow plants to respond quicker when exposed to a new stressful event. The primed state increases plants' capacity to acclimate, but its effectiveness depends on many factors, such as timing, duration, and intensity of the stress, as well as the plant's developmental stage (FILIPPOU *et al.*, 2013; KAMANGA *et al.*, 2020)

The priming itself consists of a technique that promotes memory development. Thus, there are many agents to be used to induce the priming effect: a warmer day (physical), an insect bite (biological) or even the root contact with a saline soil layer (chemical) are situations in which plants are susceptible to on a daily basis that can contribute to its strengthening. The paths traveled to overcome the stressful factor can be “stored” to be “used” again when plants face a similar situation (SIDDIQUE *et al.*, 2024).

This technique has proven to be very effective, however its effect is dependent on the interval between the priming and the stress, as well as the intensity and duration of the priming factor. There are numerous researches on this topic, increasingly considering the space-time scale in research, seeking for the best priming agent and the development window that allows each culture to express its maximum productive potential (MUNNÉ-BOSCH, 2022; SWIFT *et al.*, 2022).

The application of this technique on seeds presents numerous benefits: more uniform germination, increased seedlings vigor, tolerance development, etc. Besides, it is simple, low-cost and its effects can last the entire culture cycle, improving crops' performance. Another benefit is the generated cross tolerance, that refers to the ability of a plant already acclimated to a type of stress also tolerate other types of adversities. It depends on the plant's ability to rearrange its metabolism and gene expression in response to stimulus (LÄMKE; BÄURLE, 2017; LOCATO; CIMINI; DE GARA, 2018).

In an experiment conducted by Zhou *et al.* (2014), pre-treated tomato plants with moderate cold, paraquat and water deficit presented better responses to subsequent cold, water deficit and photo-oxidative stress. Yi *et al.*, (2015), who also worked with pre-treatment of tomato plants with high doses of CO_2, identified that these plants showed increased photosynthetic efficiency, reduction in transpiration and lower accumulation of Na^+ in shoots when subjected to saline stress. Zhang, Wang, *et al.*, (2016) & Zhang, Zhou, *et al.* (2016) identified that wheat plants pre-treated with heat and drought conditions increased tolerance to high temperatures in the post-anthesis.

An eustressor can be used as a "priming" agent, changing the regulation of genes, promoting faster and stronger responses, increasing the resistance of the plant due to previous exposure to this stimulant. According to Korres *et al.* (2022), rice seed hydropriming can improve germination processes as well as crop growth and establishment. Sriskantharajah *et al.* (2022) also shows how pre exposure to a stress factor can lead to a faster acclimation.

In brief, understanding epigenetic mechanisms can be useful to develop more resistant plants to environmental stress, especially in the current context of climate change, where agricultural crops will need to adapt quickly to extreme environmental conditions. Studying it can help to understand how plants can evolve without relying exclusively on genetic mutations.

2.2 Acclimation x Plant stress

Acclimation is a natural process that consists of physiological, morphological and biochemical changes aiming to adapt to a change in environmental conditions. In other words, acclimation is the mechanism that allows plants to overcome difficult situations and complete their life cycle (KAMANGA *et al.*, 2020). On the other hand, the concept of plant stress is related to the inability to deal with the shifting condition to which the plant is exposed. The new environment may include biotic, such as pathogens, or abiotic factors, such as drought, heat and salinity (YORDANOV; VELIKOVA; TSONEV, 2000).

It is important to differ between those concepts because physiological answers vary similarly in both cases. In summary, acclimation is a gradual process of constant change that is happening due to internal (circadian cycle) or external factors (oscillating environment); plant stress begins when plant metabolism is not capable of responding to maintain homeostasis, and then the organism as a whole start to collapse. The duration and intensity of the external stimulus are the main determining factors to define which of the two will take place (KORRES *et al.*, 2022), but also plant age, development stage, water availability, etc.

Bringing those concepts to practical terms, let’s use as an example the exposure of a rice plant to the condition of saline soil: salinity causes immediate plant growth stop. This leads to a reduction in leaf length, visually identified (LIU, CUIYU; JIANG; YUAN, 2024; SILVEIRA *et al.*, 2016). This isolated effect is often improperly used as an indicator of stressed plant. We must consider the ability that plant has of resuming its growth and development, overcoming the external condition and completing its life cycle, even if the stressful factor culminates with reduction in the crop's productive potential.

This is resulting from plants’ plasticity, in which plants reduce their growth rate to reduce water loss and increase water use efficiency. Other parameters must be taken into consideration along with the growth reduction. Photosynthetic rate, for instance, is another important parameter. Tissue might present lower photosynthetic area, but still have the same or even higher yield, meaning the plant is well acclimated. Or photosynthesis may fall drastically, necrotic areas appear, life cycle being shortened, meaning the plant is collapsing (SILVEIRA *et al.*, 2016).

Another practical example is excessive heat or light. It affects the electron chain transport and photosynthesis. When stress intensity is too high or exposition to the stressful condition is too long, or even when it is combined with other kinds of stress factors, it can aggravate the photosynthetic apparatus impairment to irreversible levels (BALFAGÓN *et al.*, 2020).

It is significant to mention that stating a plant is acclimated doesn't mean it will produce the same as if it was on the ideal environmental conditions. The acclimation can be partial, with levels where a plant can express various phenotypes. It is possible to acclimate very well and keep life going with high quality yield. Or maybe it doesn't acclimate very well and presents a reduced productivity, inferior quality of grains (KIMM *et al.*, 2021; MANZI *et al.*, 2025)

In short, to understand and recognize plant's physiological responses has fundamental importance in order to recognize an acclimation event or a stress episode. For this, many parameters, such as photosynthetic rate, membrane integrity and production quality must be considered together to have a precise diagnosis. Understanding these concepts is fundamental to develop effective and sustainable plant management and cultivation strategies.

2.3 Rice as a staple food

Rice is a staple food for millions of people around the world. It is grown in a wide variety of climatic conditions, from tropical to temperate regions. With the increase in the global population and the need to feed more people, rice cultivation becomes even more important, and it is necessary to seek new methodologies for increasing crop productivity.

Knowledge about phenology is fundamental for crop management, to obtain a good harvest and is essential in the development of new technologies to maximize production. From the understanding of phenology it is possible to predict responses to certain stresses. In rice, the phenology is divided into stages and each stage has distinct characteristics as shown in table 1.

Table 1: Development stages of the rice plant.

Development stages	Description
S	Seed ready to germinate, being able to start the process of germination from the coleoptyl or radicle.
V	Designation of the vegetative stage, with numbering according to the number of leaves that the plant has.
R	Designation of the reproductive stage, with numbering according to the progress in the development of the panicle (reproductive organ) of the main plant.
V3 - V8	Sheet emission and profiling phase.
V9 - V13	Transition to the reproductive stage, when the differentiation of the panicle occurs with the elongation of the stalk that will support it.
R0	Initiation of the emission of panicle.
R1	The panicle is already formed within the branches.
R2	Formation of the collar of the flag leaf, place from which will begin the emission of the panicle, microsporogenesis and rubbing (indicates that flowering will begin).
R3	The tip of the panicle is exposed above the collar of the flag leaf.
R4	Emergence of florets (tiny flowers) able to be pollinated.
R5	Elongation of the panicle (growth of grains).
R6	At least one cariopse finished growing inside the bark.
R7	Beginning of yellowing of the peels indicating reduction in moisture content of grains.
R8	Presence of ripe grains, with brown color peel.
R9	The whole panicle has brown shells, indicating that it has reached maturity.

Source: Adapted from Counce *et al.* (2000).

For being a crop that can develop well under waterlogged conditions, rice plants have a very permeable root system, which makes it a very sensitive crop to salinity, with its threshold about 3 dS m^{-1} (30 mM NaCl) (HAQUE *et al.*, 2021; MUNNS; TESTER, 2008). However, studies show that the effect of temporary salinity on rice plants can increase their resistance to other stressors. In an experiment by Rossatto *et al.* (2023), a short-term pre-treatment of rice plants with high salinity during the vegetative stage activated mechanisms that mitigated water stress during the reproductive stage. These responses were observed at both physiological and hormonal levels. The pre-treated, or primed, plants exhibited greater root growth, which allowed for the recovery of relative water content in their leaves.

2.4 Salinity effects in plants

To understand salinity mechanisms on plants, we first need to know the concepts of water potential. The water movement is determined by potential differences among two points, so water migrates from areas with higher to lower potential, aiming to equilibrate forces. On soil, two are the main potential forces present: matric, referring to the one that holds water on a particle's surface, and osmotic, related to the minerals solved on soil solution. Salt excess dissolved in soil solution reduces water potential due to the reduction of the osmotic factor and this is how salinity effect begins on plants (DE SWAEF *et al.*, 2022; SILVEIRA *et al.*, 2016).

A soil is defined as saline when it presents electrical conductivity superior to 4 dS m^{-1} (HAILU; MEHARI, 2021; COCA *et al.*, 2023; SANTANOO *et al.*, 2023). To be able to absorb water and nutrients from soil, root cells need to present lower water potential than soil solution. When the root meets a saline area, where water potential is lower in soil than in cells, it is not capable of absorbing water and nutrients and a cascade of signaling begins (DE SWAEF *et al.*, 2022; SILVEIRA *et al.*, 2016).

2.4.1 Osmotic effect

The first salinity effect is the osmotic. The low water potential in soil leads to an immediate growth stop due to the temporary inability to absorb water and nutrients. The root, in turn, produces the abscisic acid hormone (ABA) that will promote the elongation of the root system seeking for areas with greater water potential. This hormone is also transported via xylem to the aerial part and will promote stomatal closure, delaying water loss by evapotranspiration and tissue wilting (ARIF *et al.*, 2020; SILVEIRA *et al.*, 2016).

Stomata are leaf structures responsible for regulating gas exchange between the plant and the environment. Its main function is the capture of atmospheric CO_2 for fixation through the photosynthetic process. They are also fundamental in the maintenance of raw sap rise through transpiration and O_2 release produced by photochemical phase of photosynthesis (ZHANG, YUQI *et al.*, 2022).

The stomatal movement is a dynamic process, dependent on several factors, such as light and water availability. Wavelengths close to 440 nm (blue) stimulate the activity of H^+-ATPases present in the plasma membrane of the guard cells, generating an electrochemical gradient between the internal and external environment. With the decrease of pH in the apoplast, K^+ channels are activated, allowing their inflow into the cell, reducing its water potential and favoring the entry of water. Then, turgidity leads to stomatal pore opening (INOUE; KINOSHITA, 2017).

Under salinity conditions, ABA production induces stomatal closure. This hormone acts on the inactivation of H^+-ATPases. This causes the inactivation of the K^+ channels present in the plasma membrane and induces the influx of Ca^{2+} by channels that neutralize polarization of the membrane, inducing the K^+ exit from the guard cell for the companion cells. With the water potential increase in the guard cells, water is also lost, the cells become flaccid and the stomata pore is closed (PACHECO; LAZZARINI; ALVARENGA, 2021; COCA *et al.*, 2023).

With the stomatal closure, gas exchanges are interrupted, but the plant continues to receive sunlight, and the photochemical stage of photosynthesis continues to occur. During the photochemical stage of photosynthesis, O_2 is produced in chloroplasts from water photolysis while the photon energy is captured by the complex antenna. This energy is used to excite electrons in the photosystems, and it generates excessive reducing power in the stroma. Excess energy, if not dissipated, can be used on molecular oxygen reduction, generating reactive oxygen species (ROS), highly reactive molecules capable of damaging many cell components. Some enzymatic and non-enzymatic mechanisms used to neutralize the excess of ROS by plants are going to be described in the next topic (HAO *et al.*, 2021; SILVEIRA *et al.*, 2016).

The plant also presents mechanisms for dissipation of excess reducing power generated. An example is photorespiration, resulting from the oxygenase activity of the rubisco. With the stomata closure, there is a reduction in CO_2 concentration along with the accumulation of O_2 in chloroplasts from water photolysis (MILLER *et al.*, 2010). Thus, rubisco assumes oxygenase activity, being an alternative way to dissipate excessive reducing power and to protect against oxidative damage.

2.4.2 Ionic effect

To overcome the osmotic effect of salinity, it is necessary for plants to osmotically adjust to be able to resume growth (SHAHZAD *et al.*, 2022). With this, the plant begins to suffer from the ionic effects, resulting from the accumulation of salt ions in its tissues (PARIHAR *et al.*, 2015). A very common salt in saline areas is sodium chloride (NaCl). Both sodium (Na^+) and chloride (Cl^-) are toxic to plant cells at high concentrations, interfering with metabolic and structural processes and impairing the ability to photosynthesize efficiently (COCA *et al.*, 2023).

Under salinity conditions, salts can compete with other elements for absorption by the roots (COCA *et al.*, 2023). Cl^-, being a micronutrient, presents passive transport from channels (MUNNS; TESTER, 2008). The Na^+ normally enters the plant by the same transporters of potassium (K^+) and calcium (Ca^{2+}) (WILLADINO; CAMARA, 2010). Thus, a higher absorption of Na^+ results in a reduction in the absorption of other elements, leading to a break in the ionic homeostasis and destabilizing the protein complexes, including those involved in photosynthesis (COCA *et al.*, 2023).

K^+ is an element that is not part of any organic compound, remaining in the cells in its ionic form. Its main functions are as enzymatic activator and osmotic regulator, being important in several stages of photosynthesis, from the regulation of the stomatal opening to the activation of the rubisco enzyme. As it has a low affinity for organic compounds, K^+ must be present in high concentrations to ensure optimal enzymatic activity (COCA *et al.*, 2023; WILLADINO; CAMARA, 2010).

With K^+ absorption compromised by the excess of Na^+ under saline conditions, there is a reduction in the K^+/Na^+ ratio, breaking the ionic homeostasis. This ionic imbalance negatively affects several metabolic processes, once the activity of many enzymes is dependent on K^+ as a cofactor. Furthermore, Na^+ toxicity directly damages cellular structures. It compromises the integrity of membranes and the function of organelles, thereby impairing essential metabolic processes crucial for plant survival (SILVEIRA *et al.*, 2016).

The absorption of Ca^{2+} is also impaired by the presence of excess Na^+ (COCA *et al.*, 2023). As a crucial element for structural integrity, Ca^{2+} is an essential component of the cell wall and acts as a cementing agent in the middle lamella. Additionally, it is vital for conferring stability to proteins and maintaining membrane integrity. Consequently, a deficiency of Ca^{2+}, resulting from its compromised absorption, can lead to a reduction in plant growth and development. In severe or prolonged cases, this deficiency can result in tissue necrosis due to the loss of membrane integrity (ACIELY *et al.*, 2024).

2.4.3 Plant mechanisms to prevent salt stress

Plants have mechanisms to prevent the salt ions from being distributed through the plant. The first mechanism is the exclusion in the root, rejecting the absorption of the unnecessary element (SHAHZAD *et al.*, 2022). With the increase of salinity, this mechanism becomes insufficient. The plant begins to compartmentalize the excessive ions in vacuoles to prevent them from accumulating at toxic levels in the cytosol, as well as in less sensitive tissues, such as older leaves, to preserve younger and growing tissues. In addition, plants can also synthesize organic compounds to bind to toxic ions and prevent them from damaging the cell (INJA *et al.*, 2017; COCA *et al.*, 2023).

To prevent the Na^+ reaching toxic levels in the cytosol, mechanisms of its compartmentalization in the vacuoles and extrusion to the apoplasm are activated through Na^+/H^+ countertransport systems present in the plasmalemma (SOS1) and tonoplast (NHX). For this, H^+ATPases present in the plasmalemma and tonoplast pump H^+ to the apoplast or vacuole. The H^+ ions return to the cytosol following the concentration gradient by means of the counter-transporters proteins, while the Na^+ is transported from the cytosol to the vacuoles or apoplast (BASU *et al.*, 2021; LI *et al.*, 2020; LIU, CUIYU; JIANG; YUAN, 2024).

The SOS1 itself also acts as a signal in the presence of excess Na^+ in the external medium causing an increase in the free Ca^{2+} content in the cytosol. High levels of Ca^{2+} in the cytoplasm activate a series of cellular signaling pathways that regulate gene expression, enzymatic activity and other metabolic processes related to salt tolerance. It can also induce the formation of insoluble salts, obstructing plasmodesmata and regulating the inflow of Na^+.

In short, under saline conditions, salt accumulation occurs from the bottom. The plant activates salt avoidance mechanisms to prevent salt absorption, but as stress persists, those mechanisms become insufficient and salt begins to reach the lower tissues. Thus, to prevent the spread of ions, toxic ions are compartmentalized in older tissues, which are sacrificed to the detriment of younger and reproductive tissues, ensuring the propagation of the species (BASU *et al.*, 2021; LIU, CUIYU; JIANG; YUAN, 2024).

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2.4.4 Antioxidant salinity responses

ROS are highly reactive molecules normally produced in organelles such as chloroplasts, peroxisomes and mitochondria and have signaling function as part of the metabolism. Its synthesis and neutralization are natural processes of the organism. However, in response to environmental stresses, such as salinity, its production on a larger scale than the neutralization capacity generates oxidative stress, since these molecules can easily bind to proteins, lipids, nucleic acids, dissociating, inactivating or even denaturing them, damaging several cellular components (MAIA *et al.*, 2021).

The main ROS produced by plant cells are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen (O_2*) and hydroxyl radical (•OH). To neutralize them, plants have antioxidant mechanisms, enzymatic and non-enzymatic. Among the enzymatic mechanisms are the enzymes SOD, CAT and APX, and the main non enzymatic involve ascorbate and glutathione (LIU, CUIYU; JIANG; YUAN, 2024; SILVEIRA *et al.*, 2016).

Superoxide dismutase (SOD) is considered the first line of cell defense. It catalyzes the conversion of the O_2^- into H_2O_2 and O_2. There are several isoforms of SOD, ranging from the coupling element and the site of synthesis/action. For example, Fe-SOD is present in chloroplasts, while Mn-SOD can be found in peroxisomes and mitochondria (HAO *et al.*, 2021; SILVEIRA *et al.*, 2016).

$$2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$$

Catalase (CAT) is addressed in peroxisomes and is responsible for catalyzing the reaction that converts H_2O_2 into H_2O and O_2 (HAO *et al.*, 2021).

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Ascorbate is a molecule synthesized in virtually every part of the plant. It can interact directly with the ROS, donating electrons to the neutralization of •OH and O_2*.

$$\text{Ascorbate} + \bullet\text{OH} \rightarrow \text{Dehydroascorbate} + H_2O$$
$$\text{Ascorbate} + O_2{}^* \rightarrow \text{Dehydroascorbic acid}$$

In its reduced state (AsA), ascorbate acts as an electron donor to the reduction of H_2O_2 in H_2O and O_2 in a reaction catalyzed by the APX enzyme. This reaction generates monodehydroascorbate (MDHA) which can be reduced to AsA by the action of the enzyme monodehydroascorbate reductase (MDHAR) using NADPH as electron donor. However, MDHA can also be converted to AsA and dehydroascorbate (DHA) in a spontaneous reaction and the regeneration of DHA to AsA uses glutathione in reduced form (GSH) as an electron donor. Ascorbate also plays an important role in maintaining the reduced state of tocopherol (vitamin E), another antioxidant molecule (HAO *et al.*, 2021).

Glutathione is a molecule that relates to the regulation of the absorption of sulfur in the roots, also having strong antioxidant action. It acts on redox buffering, so a high GSH (glutathione in its reduced form) pool is necessary to prevent the formation of disulfide bridges between proteins (enzymatic inactivation) and between proteins and ROS. It is also able to neutralize H_2O_2 in a reaction catalyzed by the GPX enzyme, generating glutathione in the oxidized form (GSSG) and H_2O. GSSG is regenerated to GSH in a reaction catalyzed by the glutamate reductase (GR) enzyme using NADPH as electron donor (HAO *et al.*, 2021).

Under environmental stress, such as saline conditions, the photoreduction of O_2 retained in the intercellular spaces generates superoxide anion (O_2^-), which is converted to H_2O_2 by the action of the SOD enzyme and then reduced to H_2O and O_2 by the chloroplastic APX enzyme. Photorespiration is also stimulated, producing 2-phosphoglycolate (2PG) by the oxygenase activity of rubisco. For the regeneration of 2PG to 3-phosphoglycerate, 2PG is dephosphorylated to glycolate and sent to peroxisome, where it will be oxidized to glyoxylate producing H_2O_2 which will be neutralized by CAT enzyme.

The plant’s response to salinity can vary according to intensity and duration of stress, as well as age, genotype and several other factors. The temporal dynamics of the processes helps to understand how plants can cope with extremely variable environments. The environment has a lot of variability and this caused plants to evolve into chaotic dynamics. The greater the irregularity of a temporal phenomenon, the greater the ability to respond in a more appropriate way to oscillations and to have greater capacity to recover from an external event (SOUZA; POSSO; DE CARVALHO OLIVEIRA, 2024).

Spatial variation is another component of complexity, since there may be differences in local responses at different times. For example, what happens on a leaf on one side of the canopy affects leaves on the other side. The plant is a modular organism and each module receives a set of different signals. The spatial heterogeneity does not depend only on the perception and local response of the modules, but also on a signal integration of other distant modules (ROS, calcium, hormones, etc.) (LÜTTGE, 2021).

One of the main ways priming effect acts is on improving antioxidant mechanisms. According to Kamanga *et al.* (2020), tomato plants pre exposed to low salinity presented improved antioxidant activity, avoiding oxidative stress when exposed to a stressful saline condition. Thus, plant memory has an important role on the acclimation process, acting mainly on the avoiding the oxidative damage on cells.

In general, salinity promotes reduction in the photosynthetic rate, which leads to a strong reduction in growth and biomass production, affecting grain production. Considering rice plants, some morpho-physiological parameters modified by salinity stress are a general reduction in biomass production and yield. The biomass reduction is due to reduction in plant tillering, as well as reduction in leaf area. As a consequence, with fewer tillers, fewer panicles are produced, some sterile spikelets may occur and final yield is compromised (HAQUE *et al.*, 2021).

Drawing from the principles of complex dynamic systems and the spatio-temporal heterogeneity inherent to plant organisms, we hypothesize that the pre-exposure of rice plants to moderate salinity at early life stages activates a "memory" that leads to a more efficient and rapid acclimation to subsequent stressors. This study posits that the timing, intensity, and repetition of the stress event act as "initial conditions" that influence the plant's unique, chaotic dynamic trajectory. The resulting phenotypic plasticity is therefore a manifestation of the plant's ability to navigate and find new, more stable orbits in response to environmental disturbances, with differential responses occurring across various tissues and developmental stages.

3 OBJECTIVES

3.1 General

The general objective of this thesis is to obtain a better understanding of rice plant responses to salinity focusing on the spatial scale, and how salt priming can induce modification of stress responses, leading to better and faster acclimatization.

3.2 Specifics

1. To identify whether salt can induce plant stress memory when used as a priming agent, and to determine the most effective dose for eliciting eustress-related effects.
2. To assess how toxic ions accumulate in plant tissues at a spatial scale and how this accumulation differentially affects specific tissues.
3. To evaluate the responses of flag leaves from both control and acclimated plants under saline stress conditions.
4. To investigate the optimal timing for pre-exposing rice plants to the priming agent and to analyze spatially physiological and structural modifications it induces.
5. To establish the leaf response pattern according to age on a spatial scale, considering ion accumulation and stress indicators.

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4 SPATIAL IONIC ACCUMULATION IN LEAVES AND SALT STRESS UNDER CYCLES OF NaCl-EXPOSURE/RECOVERY IN RICE (*Oryza sativa* L.) PLANTS

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Abstract

Under field conditions, plants commonly cope with fluctuating environmental conditions such as soil salinity. Recent research has demonstrated that inducing responses in the form of stress/recovery cycles enables plants to acquire resistance through priming-memory-stress mechanisms. To this end, studies involving physiological responses to stress-recovery cycles are essential. In this study, we tested the hypothesis that the application of salinity/recovery cycles induces responses that are dependent on salt dose and on the leaf spatial position. Rice (*Oryza sativa* L.) plants were exposed to different doses of salinity (control – 0 mM NaCl, low – 16 mM, moderate – 25 mM, and high – 50 mM) supplied in three cycles of 7 days of salt exposure and 3 days of recovery, performing 21 days of salinity exposure. The experiment was conducted under hydroponic conditions and the first cycle started with plant on V6 stage (15 days after germination). Plant growth and development presented a dose-dependent reduction. However, stress indicators, electrolyte leakage, lipid peroxidation, and glutathione redox state were slightly altered by stress/recovery cycles in L12 leaves (fully expanded). These results were similar to those presented by photosynthetic CO_2 assimilation and stomatal conductance. In contrast, salt ions (Na^+ and Cl^-) presented a dose-dependent progressive accumulation in the oldest leaves whereas the K^+ content was less affected. Altogether, these results suggest that salinity supplied as stress/recovery cycles induces dose-dependent effects, but the stress intensity is dependent on the spatial position of rice leaves.

Keywords: *Oryza sativa* L., Ionic Homeostasis, Priming, Salinity, Stress.

4.1 Introduction

The process of soils salinization occurs naturally in regions with reduced rainfalls and high temperatures, such as arid and semi-arid climate areas, as well as on coastal zones due to seawater intrusion. However, human activities are contributing to accelerate this process, especially in agricultural areas due to irrational crop management. Soil salinity contributes to the reduction of crop productivity and new management strategies need to be developed in order to weaken salt effects on food production and to avoid food insecurity.

The first salinity effect on plants is a growth arrest due to the osmotic effect, making it difficult to absorb water and nutrients due to soil’s water potential reduction. To overcome the osmotic effect of salinity, it is necessary for plants to adjust osmotically. As a result, the plant begins to suffer from ionic effects, resulting from the accumulation of saline ions in its tissues (Dias *et al.*, 2021; Korres *et al.*, 2022; Silveira *et al.*, 2016; Zhao *et al.*, 2020).

A very commonly identified salt in saline areas is sodium chloride (NaCl). Both ions are toxic to plant cells in high concentrations, interfering with plant metabolic and structural processes. The main issue is caused because the excess of salts in soil compete for the absorption with other nutrients. Na^+, for instance, competes especially with K^+, which is required in large quantities, leading to a breakdown of ionic homeostasis in the cellular environment (COCA *et al.*, 2023), reducing the activity of some K^+ dependent enzymes and harming the normal functioning of cells (PAVLOVIĆ *et al.*, 2019; SILVEIRA *et al.*, 2016).

The plants species present different levels of salt resistance, which relates to its ability to exclude or compartmentalize excess toxic ions in the vacuoles (CHEN *et al.*, 2021; SILVEIRA *et al.*, 2016). Among plants salt avoidance mechanisms are root exclusion, rejecting the absorption of the unneeded element, and control of xylem ion loading, to prevent cell damage due to excess of toxic ions (SHAHZAD *et al.*, 2022). With increasing salinity, this mechanism becomes insufficient and the concentration of saline ions begins to rise in the cytosol until ionic homeostasis is broken.

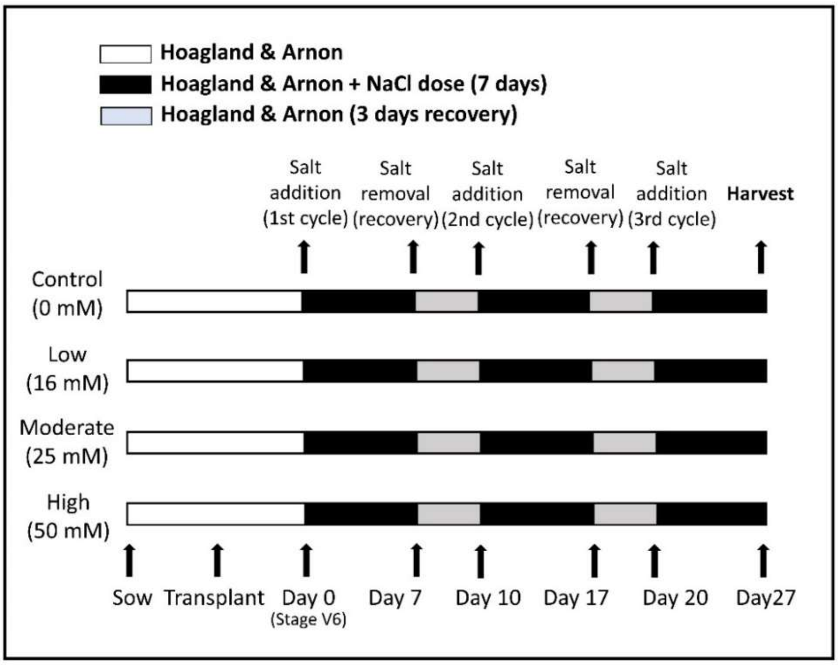
The aim of this paper is to identify a pattern of salt ion's compartmentalization and redox responses in rice plants in a spatial scale according to salinity severity as well as to elaborate a method of seed priming production through cycles of exposure/recovery. Once salinity is an increasingly common phenomenon in agricultural soil, we hypothesized that there is a salinity dose that, when applied in cycles, develops plant memory reducing salinity’s effects on plant development.

4.2 Material and methods

4.2.1 Plant material, growth conditions and treatments

Rice seeds (*Oryza sativa* spp. *Japonica;* cv. Nipponbare) were germinated in Germitest® paper under 240 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR), 27 ± 2 °C, 80% relative humidity and 12-h photoperiod. Eight days after sown, rice seedlings were transferred to 3-L plastic pots filled with ¼ strength Hoagland-Arnon's nutritive solution (HOAGLAND; ARNON, 1950) during 7 days. Then, nutritive solution was completely changed to ½ strength for 7 more days and then to full strength. The seedlings were grown in a greenhouse (average maximum PAR of 800 µmol m^{-2} s^{1}; 29 ± 2 °C; 12-h photoperiod; and 68% relative humidity). When plants achieved stage V6, treatments were applied: 0 mM (control), 16 mM (low salinity), 25 mM (moderate salinity) and 50 mM (high salinity) NaCl dissolved in nutrient solution. After 7 days of treatment, nutrient solution was completely changed and plants recovered for 3 days. After recovery, another cycle was carried out and plants were collected at the end of the third salinity exposure as shown in figure 1. At the end of the experimental period, leaf segments were harvested to determine electrolyte leakage. Main culm and leaves (L10 – senescent; L11 – mature; L12 – full expanded; L13 – expanding) were frozen in liquid N_2 and stored at -80 °C for later determinations of biochemical analysis.

Figure 1 – Experimental design.



4.2.2 Biomass, root and shoot morphology

For salinity tolerance assay, morphological measurements (root and shoot length, number of leaves and tillers) were carried out at the end of each saline and recovery cycles. After the experimental period (43 days), roots and shoot fresh matter were determined. Shoot main culm, divided into basal (0-15 cm), middle (15-30 cm) and apical (>30 cm), main culm leaves and panicles, were collected separately, frozen in liquid nitrogen (N_2) and kept under -80 °C.

4.2.3 Photosynthetic parameters

CO_2 assimilation (A), stomatal conductance (g_s) and transpiration (E) were measured on L12 in the morning (8 to 11 a.m.) of the day before harvest using a portable infrared gas analyser – IRGA (LI-6400-40, LI-COR, Lincoln, NE, USA). The conditions inside the IRGA chamber during measurements were: PPFD 1000 µmol photons m^{-2} s^{-1}, 28 °C, air vapor pressure deficit of 1.0 ± 0.2 kPa and air CO_2 partial pressure of 40 Pa. The amount of blue light was set to be 10% of PPFD to maximize stomatal aperture (FLEXAS *et al.*, 2008).

4.2.4 Ionic determinations

Na^+ and K^+ contents were determined as described by (CAVALCANTI *et al.*, 2004). Dry leaves were finely grinded, and 50 mg samples were extracted with 20 mL of deionized water at 95 °C for 60 min in hermetically closed tubes. After cooling, the extract was filtered, and the ion content was determined using a flame photometer (Micronal mod. B462). Na^+ and K^+ concentrations were estimated using standard curves of NaCl and KCl, respectively, with concentrations ranging from 0 to 50 mmol L^{-1}.

Chloride content was determined by Mohr method, described in SILVA *et al.* (2021). 150 mg of finely grinded dried leaves were added to 125 mL Erlenmeyer containing 15 mL of calcium nitrate (0,085 M), shaken for 15 min and filtered. 1ml of indicator solution of potassium chromate (0,258 M) was added to 10 mL of extract and it was used to titration with silver nitrate (0,0282 M) until red color (V1). The blank was prepared in parallel and subtracted from the sample (V0). Chloride content was expressed as umol g^{-1} DM.

4.2.5 Oxidative stress indicators

Membrane damage (MD) was measured by electrolyte leakage as described previously by Blum & Ebercon (1981). Segments of a mature rice leaf (10 segments of 3 cm from the middle part of leaf 11 – L11) were placed in tubes containing 10 mL of deionized water. The flasks were slightly agitated for 24h and solution electrical conductivity was measured (C1). Solution with segments were then boiled (98 °C) for 1 h and the electrical conductivity was measured again (C2). The relative membrane damage was estimated by MD (%) = C1/C2 x 100.

Lipid peroxidation was measured based on the formation of thiobarbituric acid-reactive substances (TBARS) in accordance with Heath & Packer (1968). 200 mg of fresh mass were added to 1.5 mL of TCA 5%, centrifuged at 12,000 g (4 °C) for 20 min and the supernatant was collected. 500 µL of the supernatant was added to tubes containing 2 mL of TBA solution, left on 98°C water bath for 60 min and rapidly cooled in ice bath. After centrifuged at 10,000 g for 5 min, absorbance was followed at 532 and 660 nm in the spectrophotometer TBARS concentration was calculated using absorption coefficient (155 mM^{-1} cm^{-1}) and results were expressed as ηmol MDA-TBA g^{-1} FM.

Glutathione content was assayed as described by Griffith (1980). Fresh leaf samples (100 ug) were homogenized in HCl 0.2 N (1 mL), centrifuged at 16,000 *g* (4 °C) for 10 min and the supernatant was then used. The neutralized extract was prepared by adding 400 uL of extract, 40 uL of 200 mM phosphate potassium buffer pH 5.6 and 320 uL of 0.2 M NaOH. To determine total glutathione (GSH+GSSG), 20 uL of neutralized extract was added to a cocktail with 200 uL of 200 mM phosphate potassium buffer pH 5.6, 20 uL of 5mM NADPH, 40 uL of 6 mM DTNB (5,5-dithio-bis-(2-nitrobenzoic acid)) and 100 uL of distillated water. The mixture was stabilized at 30 °C for 10 min. 20 uL of 20U/mL glutathione redutase was added to the mixture and absorbance was followed at 412 nm for 180 s in the spectrophotometer. The GSSG analysis used a derivatized extract. 2-VP (2-vinylpyridine), an alkylating agent, was used to avoid GSH from reacting. GSH content was determined by difference of total glutathione and oxidized glutathione and redox state was determined by the ratio of reduced glutathione and total glutathione. All contents were expressed as µmol g FW^{-1}.

4.2.6 Statistical analysis

The experiment was arranged in a completely randomized design, with five independent replicates, each consisting of one pot containing two plants. Sigmaplot 14.5 program was used for statistical analysis of ANOVA and Tukey test with significance level set at 5% ($p<0.05$).

4.3 Results

4.3.1 Growth and development

In the present study, rice plants were exposed to three salinity cycles of 7 days exposition and 3 days recovery with different salt levels added through nutritive solution. At the end of the first cycle, salinity effects were silent and differences started to appear at the recovery period. It was observed that moderate and high salinity plants did not recover completely, showing a reduction on tiller number (data not shown) and, as a consequence, reduction on number of leaves (data not shown). At the second saline cycle, low salinity plants also showed a reduction of tiller and leaves number (Figures 2B and 2C), with no recovery after. At the end of the third saline cycle, plant height was also affected (Figure 2A), implicating a strong reduction on shoot biomass (Figure 3A). Root biomass was also harmed (Figure 3B) due to reduction in new roots emission.

The end of the experiment coincided with the beginning of the reproductive stage (R3-R4). There was a late emission of panicles with salinity, with a dose-dependent reduction on its number (Figure 4A) and weight (Figure 4B). The panicles weight reduction was due to reduction in seed weight (data not shown).

Damage in flag leaves (L13) was also observed. All saline treatments presented damaged flag leaves, as well as faster senescence of mature leaves (L11). However, the leaf right above flag leaf (L12) was healthy and vigorous.

The photosynthetic parameters were measured on L12 (Figure 5). They presented a slight reduction due to salt exposure. CO_2 assimilation differed from control only from moderate to high salinity, but those presented no significant difference from low salinity (Figure 5A). Stomatal conductance and transpiration in high salinity-treated plants also reduced compared to control, but presented no difference from plants treated with low and moderate salinity (Figures 5B and 5C).

Figure 2 – Effects of multiple salt doses (0, 16, 25 and 50 mM NaCl) on plant height (A), leaf number (B) and tiller number (C) of rice plants at the end of each saline cycle (S1 – end of 1st saline cycle; S2 – end of 2nd saline cycle; S3 – end of 3rd saline cycle). Data represents averages from four replicates ± SD and different letters represent significant differences among different NaCl concentrations at p<0.05 indicated by the Tukey's test.

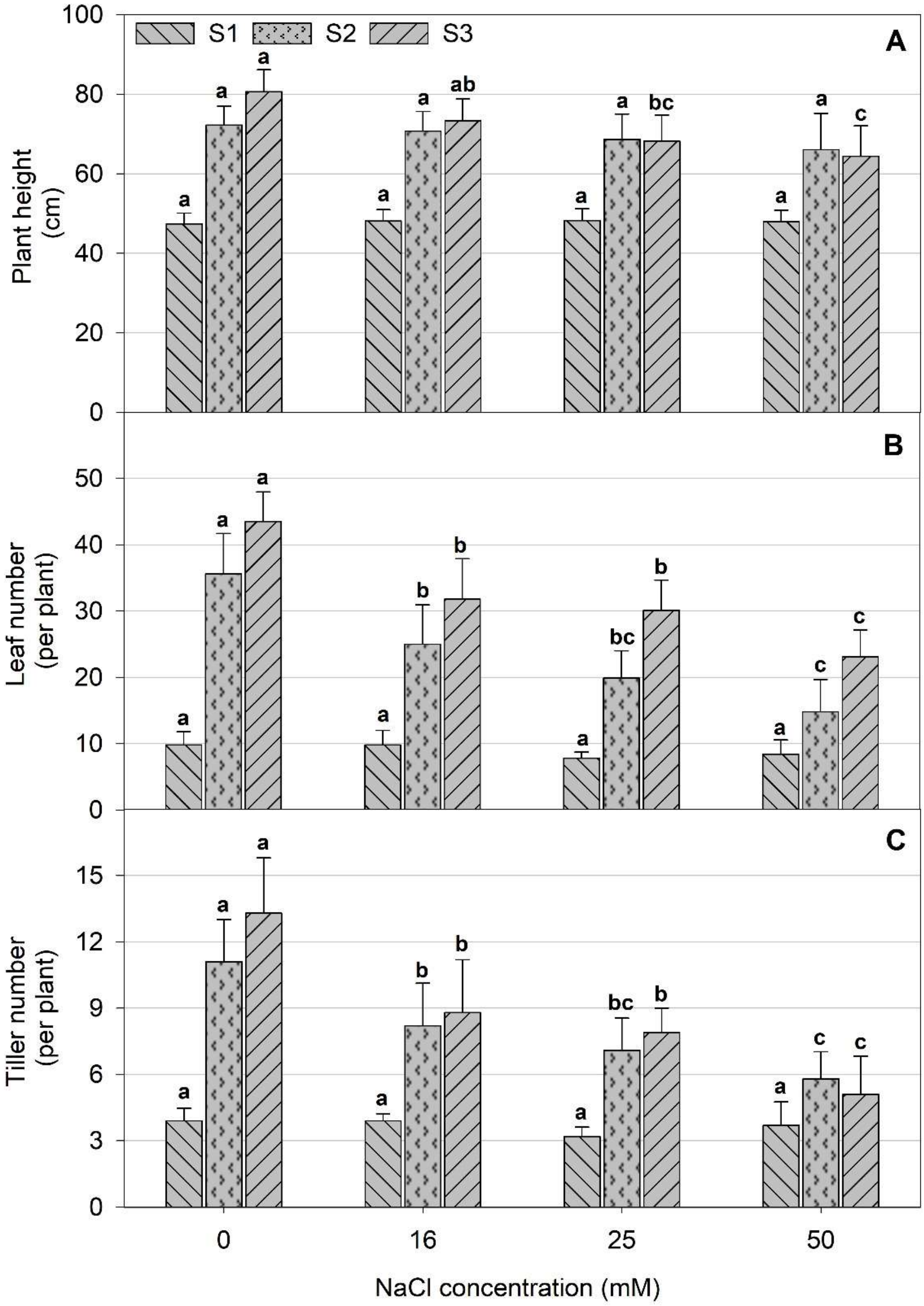


Figure 3 – Effects of multiple salt doses (0, 16, 25 and 50 mM NaCl) on shoot (A) and root (B) dry matter of 50-day-old rice plants at the end of the 3rd saline cycle. Data represents averages from four replicates ± SD and different letters represent significant differences among treatments at p<0.05 indicated by the Tukey's test.

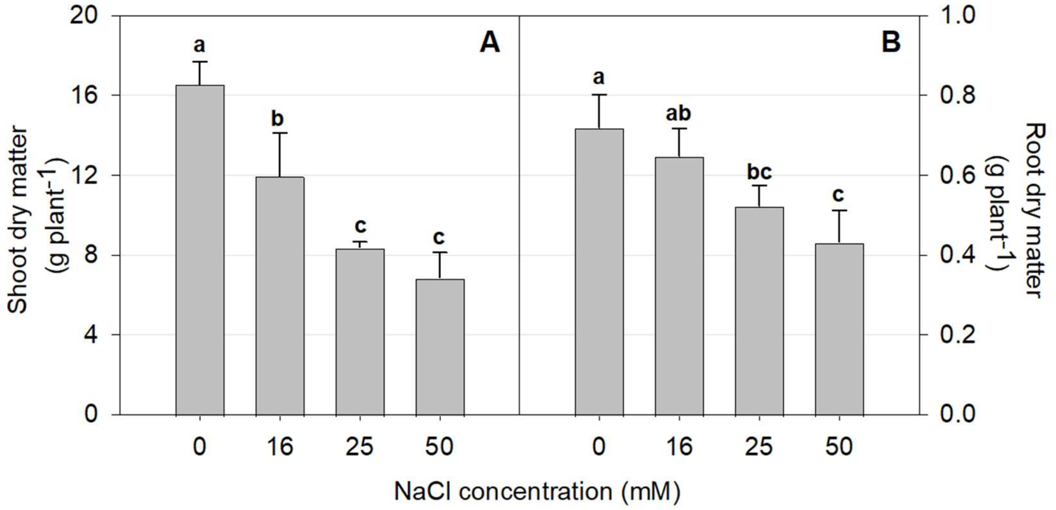


Figure 4 – Effects of multiple salt doses (0, 16, 25 and 50 mM NaCl) on panicle number (A) and weight (B) of 50-day-old rice plants at the end of the 3rd saline cycle. Data represents averages from four replicates ± SD and different letters represent significant differences among treatments at p<0.05 indicated by the Tukey's test.

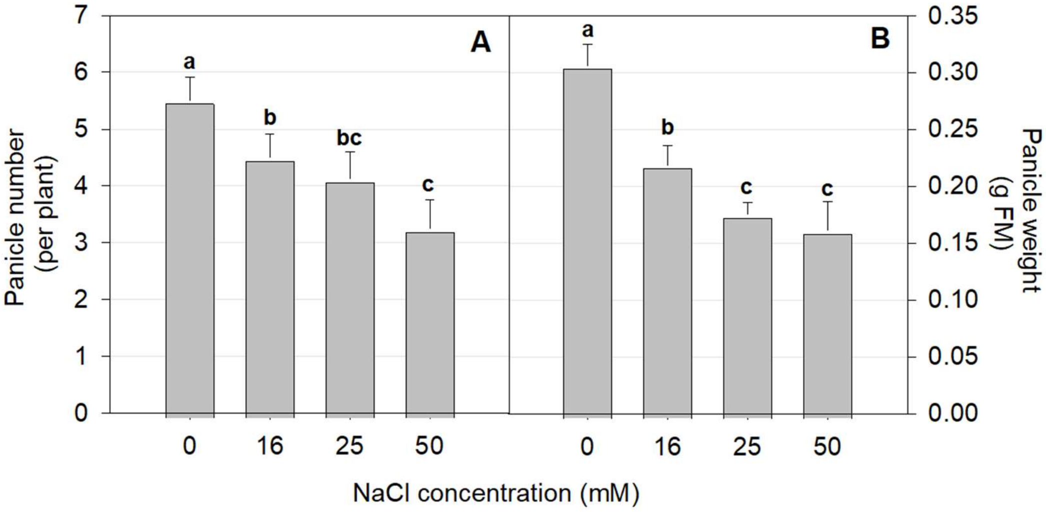
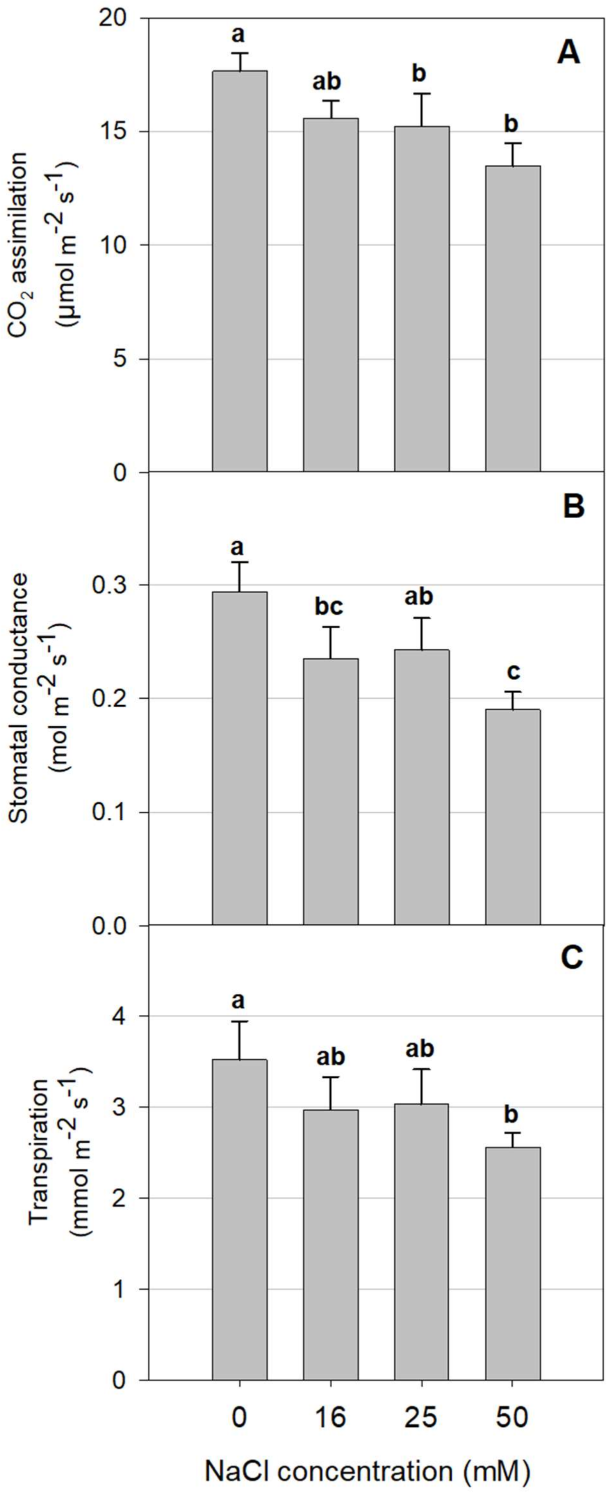


Figure 5 – Effects of multiple salt doses (0, 16, 25 and 50 mM NaCl) on CO_2 assimilation (A), stomatal conductance (B) and transpiration (C) of 50-day old rice plants at the end of the 3rd saline cycle. Data represents averages from four replicates ± SD and different letters represent significant differences among treatments at $p<0.05$ indicated by the Tukey's test.

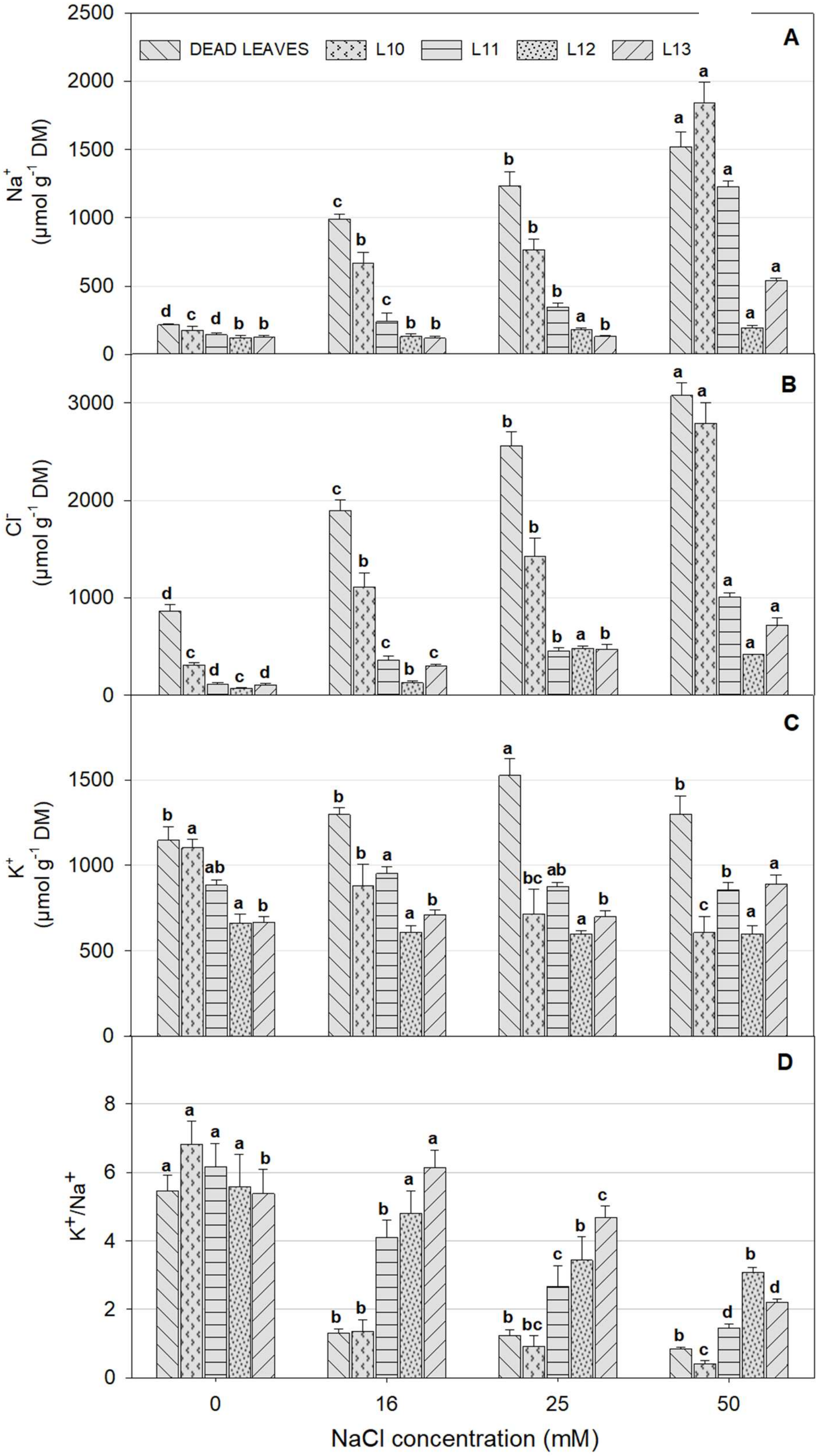


4.3.2 Ionic spatial distribution

Salinity resulted in ionic storage at the plant basal region after the three saline cycles (Figure 6A and 6B). Elements accumulated in dead and senescent leaves (L10), which worked as deposits of toxic ions, avoiding accumulation on more efficient tissues. Under low to moderate salinity, the plant was able to compartmentalize ions in older leaves, preventing salt them from reaching toxic levels in younger tissues. However, under high salinity, salts reached higher levels on flag leaves (L13) than on the one right above it (L12).

The K^+ content was reduced in basal leaves (L10), but it was kept on other tissues (Figure 6C). With the increasing Na^+ content, K^+/Na^+ ratio was affected (Figure 6D). Senescent leaves were the most affected due to reduction on K^+ and increase in Na^+ accumulation. On the other hand, L12 was the leaf that presented the lowest levels of toxic ions and was capable of maintaining K^+ levels, being the most effective tissue on keeping ionic homeostasis.

Figure 6 – Effects of multiple salt doses (0, 16, 25 and 50 mM NaCl) on spatial distribution/accumulation of sodium – Na+ (A), chloride – Cl- (B), potassium – K+ (C) and K+ / Na+ ratio (D) on 50-day-old rice leaves at the end of the 3rd saline cycle. Data represents averages from four replicates ± SD. Different uppercase letters compare in each NaCl treatment and lowercase letters compare different leaves.



4.3.3 Stress indicators

Electrolyte leakage analysis were done using L11. It was observed high percentage of membrane damage under high salinity, while the other treatments presented no significant difference from control (Figure 7).

TBARS content (Figure 8) was measured on the four leaves present on the main culm. All saline treatments presented a raise on TBARS content considering L10 (senescing) compared to control, however the input presented no significant difference among them. L12 presented the lower content of TBARS among the leaves analyzed. It presented an increase on TBARS content on the more severe treatment, while the other remained similar to control. On L11 and L13, TBARS content was not affected by salinity.

Glutathione redox state was also measured in the four leaves of the main culm (Figure 9). The glutathione redox state tends to be similar in all the analyzed tissues under controlled conditions. When exposed to salinity, redox state presented a tendency of being higher on older leaves and reduce with leaf spatial position. Despite no significant difference on GSH content on the different leaves, the GSSG content was spatially affected by salinity. Higher levels of GSSG were identified on L13 under high and moderate salinity compared to control; a slight increase on L12 GSSG content in all saline treatments; and no significant difference considering L11; and a strong reduction on L10 GSSG content exposed to salinity compared to control, especially on 25 mM. The GSH content was only affected on L10, which increased with salinity.

Figure 7 – Effects of multiple salt doses (0, 16, 25 and 50 mM NaCl) on electrolyte leakage in mature 50-day-old rice leaves (L11) at the end of the 3rd saline cycle. Data represents averages from four replicates ± SD and different letters represent significant differences among treatments at $p<0.05$ indicated by the Tukey's test.

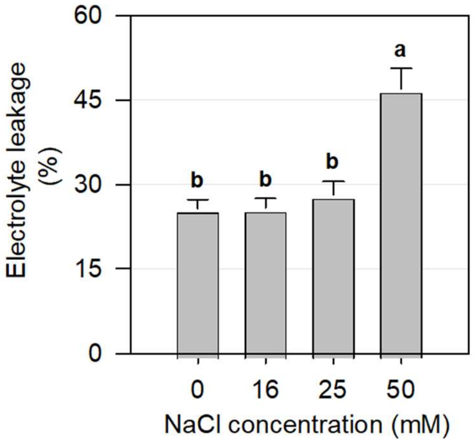
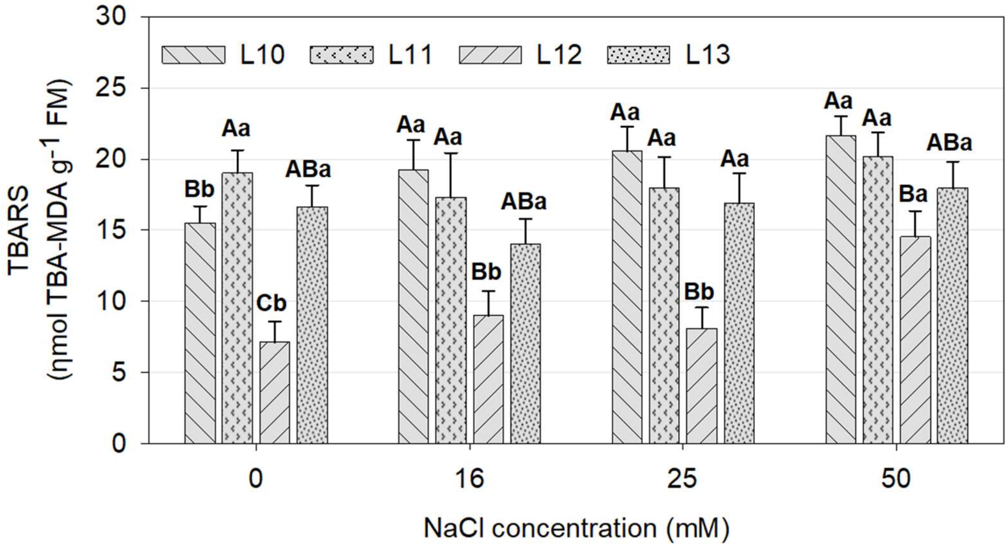
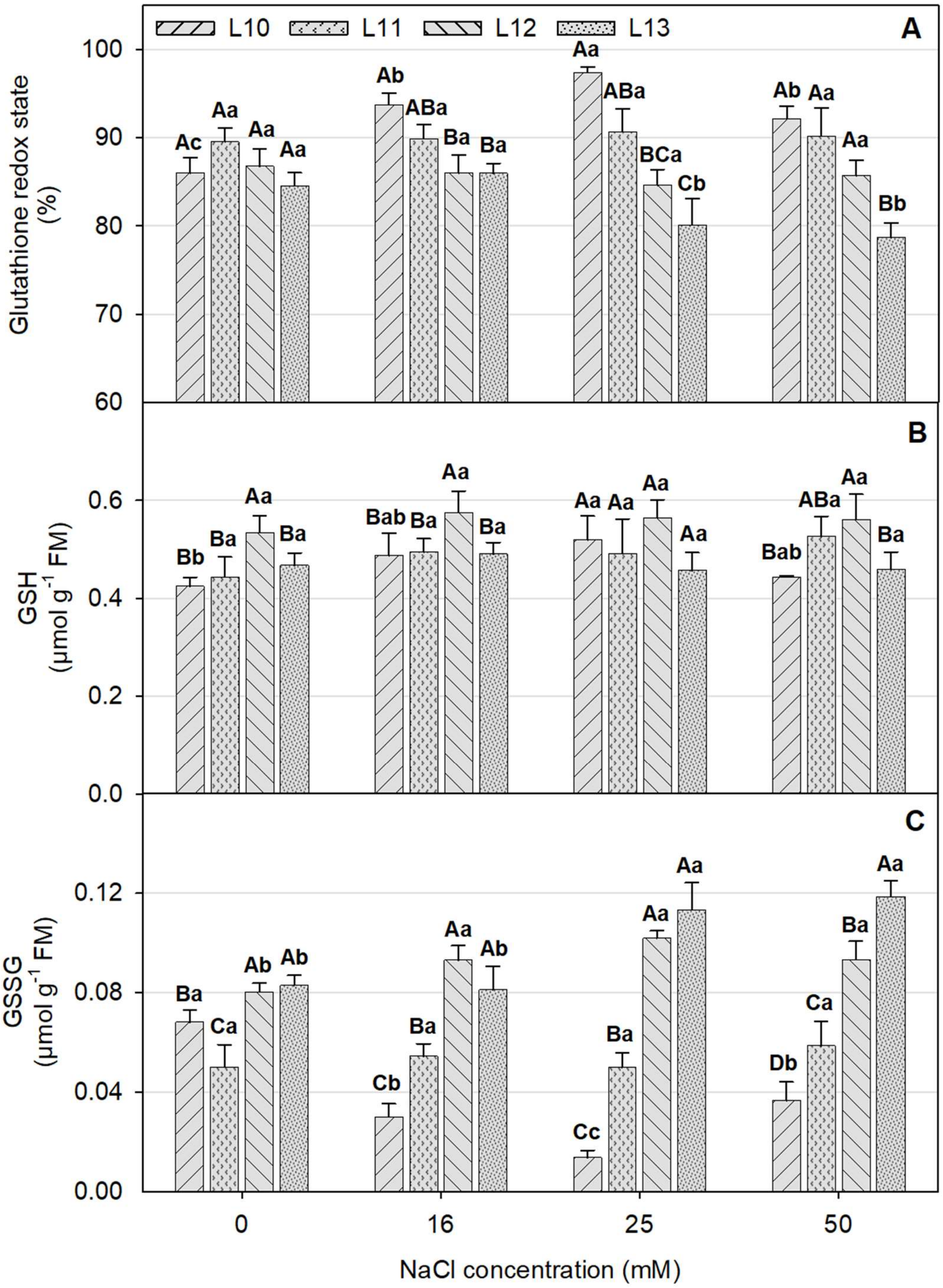


Figure 8 – Effects of multiple salt doses (0, 16, 25 and 50 mM NaCl) on content of thiobarbituric acid reactive substances – TBARS in 50-day-old rice leaves at the end of the 3rd saline cycle. Data represents averages from four replicates ± SD. Different uppercase letters compare in each NaCl treatment and lowercase letters compare different leaves.



Harvest was carried out in plants on reproductive stage with panicles emitted (R3-R4).

Figure 9 – Effects of multiple salt doses (0, 16, 25 and 50 mM NaCl) on glutathione redox state (A), reduced – GSH (B) and oxidized – GSSG (C) in 50-day-old rice leaves at the end of the 3rd saline cycle. Data represents averages from three replicates ± SD. Different uppercase letters compare in each NaCl treatment and lowercase letters compare different leaves.



4.4 Discussion

4.4.1 Saline cycles effect is dose dependent, suggesting biomass reduction is an acclimation strategy

Considering the first two salinity cycles, being the first one a priming and the second a stress agent, during recovery the memory should have stabilized and the plant would have respond better, acclimating quicker (VILLAGÓMEZ-ARANDA *et al.*, 2022; WILLADINO; CAMARA, 2010). However, results suggest that low salinity would be enough to hinder rice yield in plants primed in V6 for 7 days.

Once the priming concept considers yield (ADHIKARI; OLORUNWA; BARICKMAN, 2022; FERREIRA; PEREZ-MARIN, 2022; YADAV *et al.*, 2023), the impairment of production resulting from the reduction in tillering would be a very strong argument to consider that none of the doses were adequate to be applied as priming agent for rice at V6 stage. It is possible that, despite mother plant not respond well and not being capable of recovering from a stress episode, its offspring may be able to acquire some resistance (WANG *et al.*, 2018).

It is known that salinity may hinder shoot growth while stimulates roots to grow in order to reach a salt free region and reestablish shoot development (SILVEIRA *et al.*, 2016; ZHANG, YUQI *et al.*, 2022). Considering this, an increase in root length, not necessarily followed by the dry matter content, would be a common response to salinity. However, there weren't differences among root length, but dry matter was reduced in the same proportion as shoot. Root length may have not been affected by salinity due to pot limitations, once the experiment was conducted on hydroponic conditions.

The plant struggled under all saline levels, but different tissues presented different damage levels. The visual effect of the early salinity stages in rice plants is a faster senescence of mature leaves. Due to the osmotic effect, new tissues stop to expand and growth is hindered (ADHIKARI; OLORUNWA; BARICKMAN, 2022; ZHANG, YUQI *et al.*, 2022). The plant responses to these stressful conditions is complex, only understandable considering a space-time scale.

Considering a spatial distribution, in the beginning of salt exposition, we can observe the plant senescing from the bottom to the top. During osmotic adjustment, salts are up taken and cells compartmentalize them inside vacuoles in order to prevent them from spreading to other tissues (SHAHZAD *et al.*, 2022). Trying to acclimate to saline condition, excessive saline ions are held on less effective tissues to prevent damage on more photosynthetically active parts (HAO *et al.*, 2021).

With the long duration and high intensity of exposition, the older leaves die and the toxic ions begin to reach younger tissues (LIU, CUIYU; JIANG; YUAN, 2024). Due to the high photosynthetic and transpiratory activity of the flag sheet (L13), as well as being in expansion process, this tissue presents itself as a strong drain, carrying much of the sap for this region joining with the saline ions. Once it is a very young tissue with little vacuoles (INJA *et al.*, 2017), small concentrations of saline ions may be able to cause a lot of damage and flag leaf is strongly injured. Thus, the leaf right above flag leaf (L12) may become the most important on species survival, responsible for feeding panicle to promote grain filling.

In terms of temporal distribution, the higher the salinity, the faster the negative effects are manifested on the plants. Once salinities above 2 dS m^{-1} are already harmful for rice plants (GRATTAN *et al.*, 2002), low salinity hinders rice development, but plant cycle can be completed once damage spreads slowly; plants may be able to stand moderate salinity at cost of a lower production; and under high salinity, ions spread too quickly damaging tissues before it can complete its life cycle. Thus, acclimation settles under low salinity and moderate salinity, while high salinity leads to stress due to the damage to spread faster than the plant's capacity to cope (SOUZA; POSSO; DE CARVALHO OLIVEIRA, 2024).

The impairment on growth and development is related to the photosynthetic capacity during salinity. However, considering L12 metabolism, gas exchange presented discreet reduction compared to control. Stomatal conductance reduction occurs due to the osmotic effect and the hormone balance regulation, raising abscisic acid (ABA) levels on tissue to trigger stomatal closure. It reduces transpiration rate and slows down salt uptake. Y. Zhang *et al.* (2022) identified that the saline effect on photosynthesis is stronger in tomato older leaves due to reduction in photosynthetic capacity and stomatal response rate with tissue aging. Bearing this in mind, modifications in L12 responses may be indicative of inability on dealing with salinity.

4.4.2 Rice plants present a mechanism of spread avoidance of toxic ions through plant

Rice plants exposed to salinity exhibit intense accumulation of saline ions in their basal tissues. This ionic toxicity damages cell membranes, increases the production of ROS, and disrupts the homeostasis of other essential ions (HAO *et al.*, 2021). In response, apoptosis mechanisms are activated to isolate the damage and protect the organism as a whole. Therefore, maintaining ionic homeostasis is crucial to avoid toxicity caused by excessive salts.

When exposed to salinity, rice plants demonstrate an efficient mechanism for retaining Na^+ and Cl^- ions primarily in their basal parts. We can affirm this because, while salinity at any dose impedes plant growth, the rate of ion deposition isn't uniform across the entire plant. A striking example of this is seen in flag leaves. Despite exhibiting a considerable reduction in length regardless of the salinity dose (data not shown), these leaves maintained Na^+ content similar to control plants.

The observed high deposition rate of potentially toxic ions in older tissues, coupled with low Na^+ content in young and mature leaves, and the 'self-destruction' strategy, strongly suggest an exclusion mechanism. This mechanism, similar to the ammonium exclusion identified by Sousa *et al.* (2021), effectively avoids ionic toxicity and preserves vital young tissues in rice plants.

Cl^- exhibits more flexible movement within the plant and is important in various physiological functions. Even under low salinity, a slight upward translocation of this element from middle to top leaves was observed. Cl^- is particularly crucial in salinity responses, primarily regulating stomatal function through its osmotic effects. Its accumulation in flag leaves, for instance, may be linked to ABA accumulation, which also plays a key role in stomatal regulation (CHEN *et al.*, 2021; PAVLOVIĆ *et al.*, 2019).

Under conditions of low and moderate salinity, rice plants exhibit a pattern of rapid ion compartmentalization, followed by programed cellular death. This strategy aims to sequester salt within specific tissues. Consequently, essential and excess elements become deposited on them. As the level of salinity increases within these senescent tissues, their capacity for ion remobilization diminishes. This leads to an unfortunate outcome: senescent tissues accumulate a high concentration of ions, effectively wasting essential elements in the process of attempting to remove or isolate toxic ones.

As salinity intensifies, toxic ions accumulate in the flag leaf, driven by its rapid growth rate and high photosynthetic activity. Since the flag leaf is a young tissue, its vacuolar salt accumulation mechanism is inefficient (INJA *et al.*, 2017), leading to ion toxicity and cellular death. The extent of flag leaf injury directly correlated with the salt level, potentially sparing the L12 leaf. The K^+/Na^+ ratio demonstrated the loss of ionic homeostasis among plants' parts. When plant starts collapsing, it increases its attempts on finalizing panicle and for this reason, flag leaf may be so severely damaged before others. Under this circumstance, L12 becomes the main leaf to feed panicles instead of L13.

The salinity effect may be enhanced or mitigated according to other factors. When combined with heat, salinity harmful effects were enhanced inducing higher Na^+ accumulation in photosynthetic tissues and membrane damage, despite stimulating antioxidative mechanisms (MENDES *et al.*, 2024).

4.4.3 Low and moderate salinity promote acclimation, while high salinity leads to stress

One of the main metabolic disturbances induced by ionic toxicity is ROS production. Since they are highly reactive, ROS accumulation can induce oxidative stress when reacting with lipids, harming membrane integrity; oxidizing amino acid residues of proteins, modifying its functions; interacting with DNA nitrogenous bases leading to mutation or cellular death, resulting in widespread damage, etc. (CHEN *et al.*, 2021; MILLER *et al.*, 2010; SILVEIRA *et al.*, 2016; ZHAO *et al.*, 2020).

Stress threshold from electrolyte leakage in rice plants can vary based on factors such as the specific experimental conditions, the rice variety, and the severity and type of stress. In general, unstressed plants often exhibit low electrolyte leakage, typically below 20%. In this work, harvesting conditions may have contributed to slightly higher values. However, the significant increase in electrolyte leakage in high salinity can suggest stress conditions. To validate those results, TBARS content were determined.

The selective retention of salts in basal or older leaves helped to protect younger and more metabolically active tissues from the toxic effects of excessive salt accumulation. So, young leaves might be more effective in neutralizing ROS and preventing lipid peroxidation compared to older leaves. The flag leaf, due to its unique behavior, must be studied separately from others.

TBARS content of flag leaves more closely resembles that of mature and senescent leaves than younger leaves of similar age. Since flag leaves' main objective is to promote the grain filling, they may present a shorter and more intense life. The observed pattern of lower TBARS levels in young leaves (L12) compared to the other leaves in all treatments may have been influenced by plant mechanisms that contribute to ion regulation and tolerance to salinity. However, under high salinity TBARS content in L12 increased, indicative of lipid peroxidation affecting membranes' integrity.

The discrepancy between high electrolyte leakage and non-significant changes in TBARS suggests that high salinity is affecting cellular membranes, but the specific nature of the damage may involve mechanisms beyond lipid peroxidation or involve the action of other compounds capable of altering membrane permeability.

Intensity of oxidative damage depends on the activity of many antioxidants mechanisms. Glutathione is one of the main non-enzymatic mechanisms of oxidative protection. It acts as electron donor on reduction of H_2O_2 or dehydroascorbate (DHA), oxidized form of ascorbate (MORADBEYGI *et al.*, 2020; SILVEIRA *et al.*, 2016). High pool of GSH is an indicative of efficient antioxidative activity and reduction of its redox state indicates actuation on oxidative protection. Despite the tendency for a reduction in the redox state of glutathione with the reduction in tissue age, GSH pool is unaltered among treatments. L12 did not present significant difference among treatments, however, it can be observed a raise in GSH pool. This may be insufficient to deal ROS neutralization, leading to the raise in lipid peroxidation.

Rice plants responses to salinity are complex and highly dose-dependent, involving a dynamic interplay between physiological, biochemical, and structural mechanisms. While low and moderate salinity levels may trigger acclimation processes, high salinity overwhelms the plant’s capacity to cope, leading to stress, damage, and reduced productivity. Our study shows that none of the tested salinity levels at the V6 stage can be considered effective priming agents on the applied method of exposure/recovery cycles, as even low salinity compromised tillering and yield.

Taken together, these findings underscore the rice plant’s attempt to balance growth, survival, and reproduction under saline stress through ion compartmentalization, tissue-specific sacrifice, and antioxidant activity. However, when stress exceeds the threshold of tolerance, the plant’s protective mechanisms collapse, leading to physiological dysfunction and reduced yield. Understanding these mechanisms is crucial for developing salinity-resilient rice varieties and optimizing agricultural strategies in saline-prone environments.

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SUPPLEMENTARY

Figure S1 – Visual aspects of whole plant of rice plants at the end of the 3rd saline cycle.

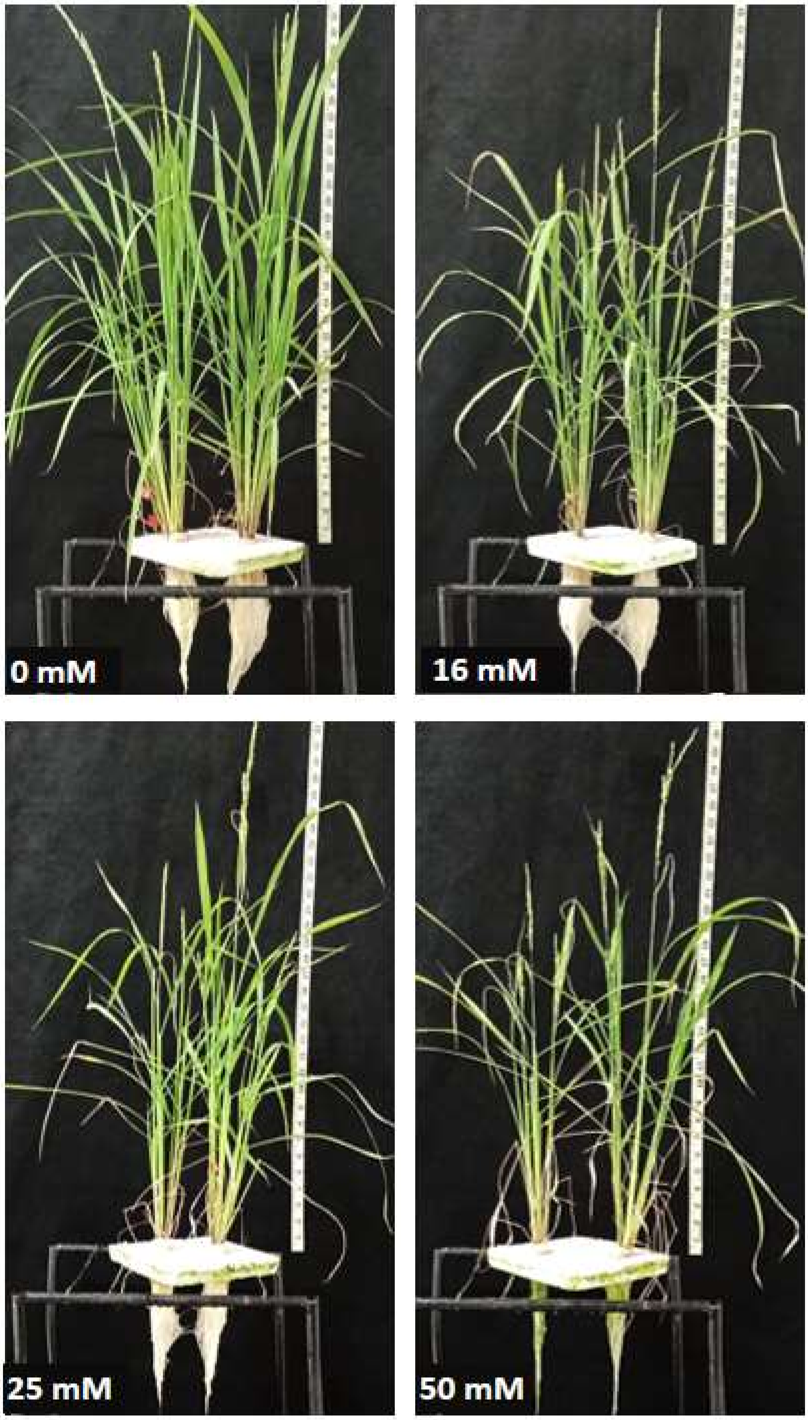


Figure S2 – Visual aspects of panicles and flag leaves of rice plants at the end of the 3rd saline cycle.

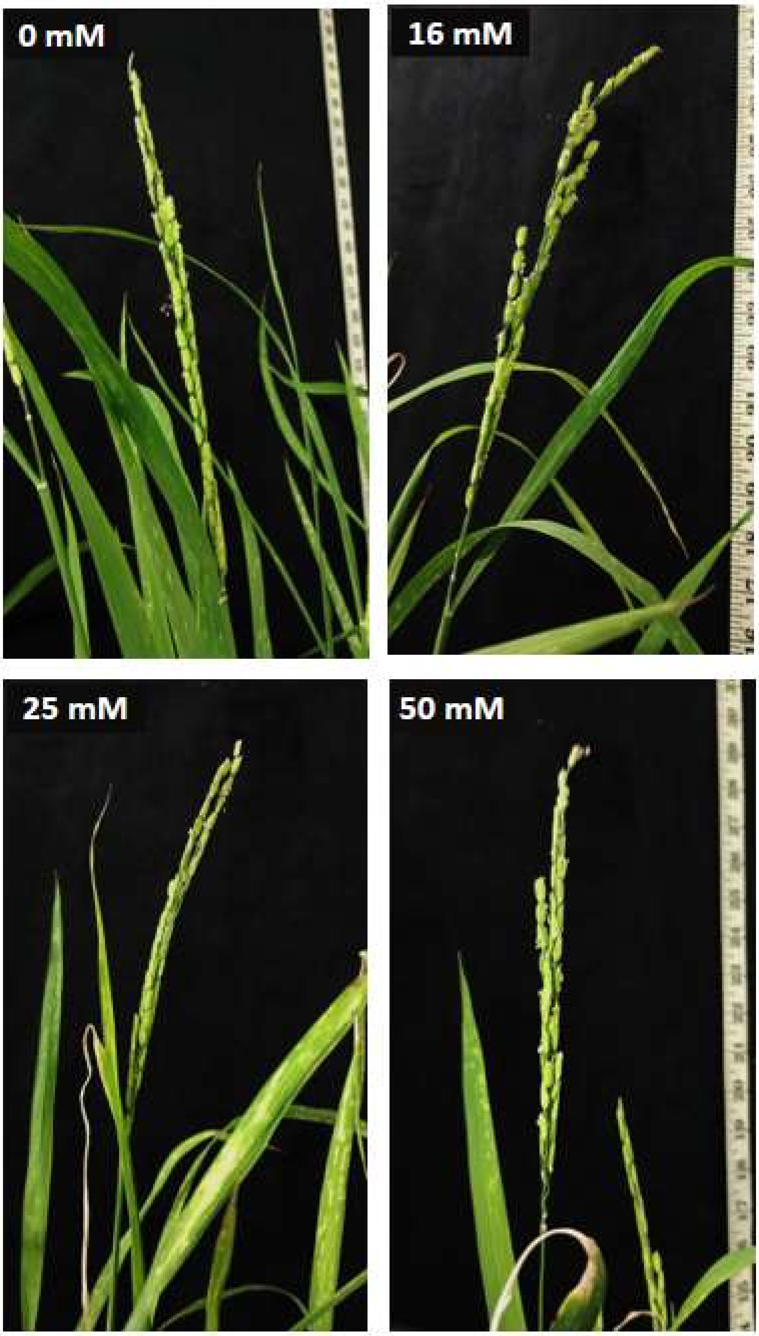


Figure S3 – Visual aspects of tillers of rice plants at the end of the 3rd saline cycle.

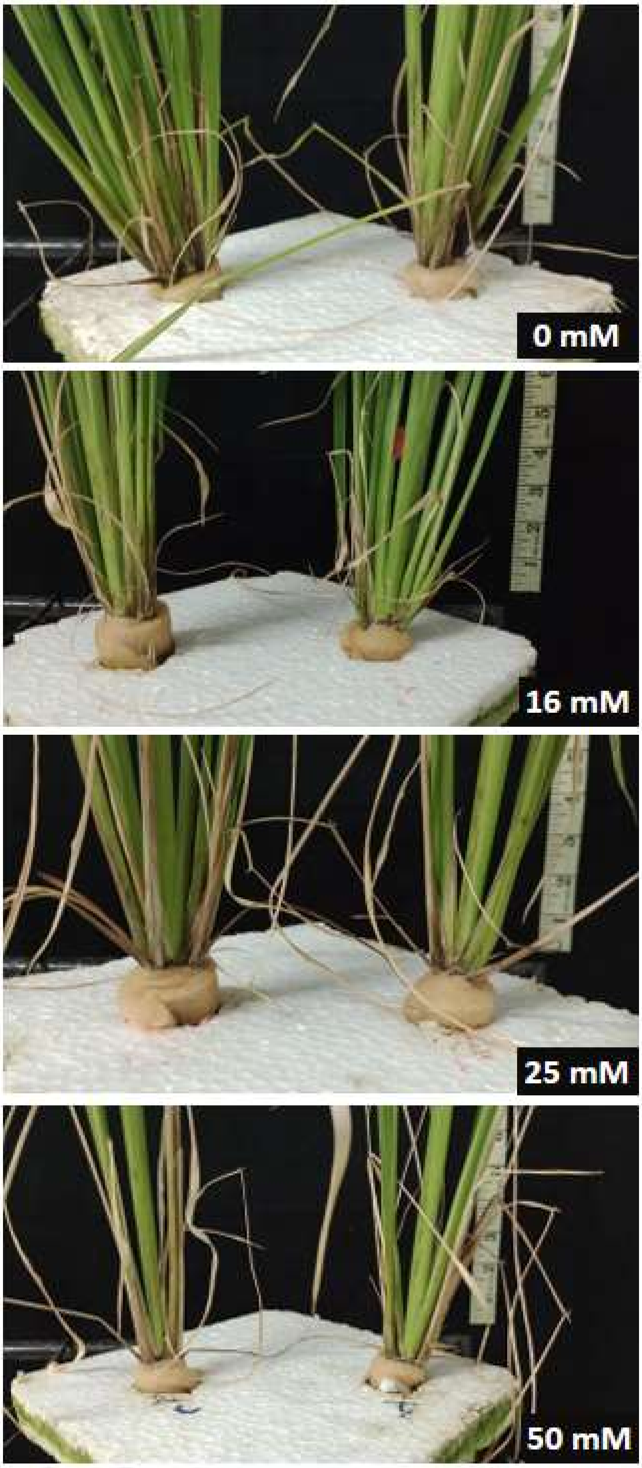
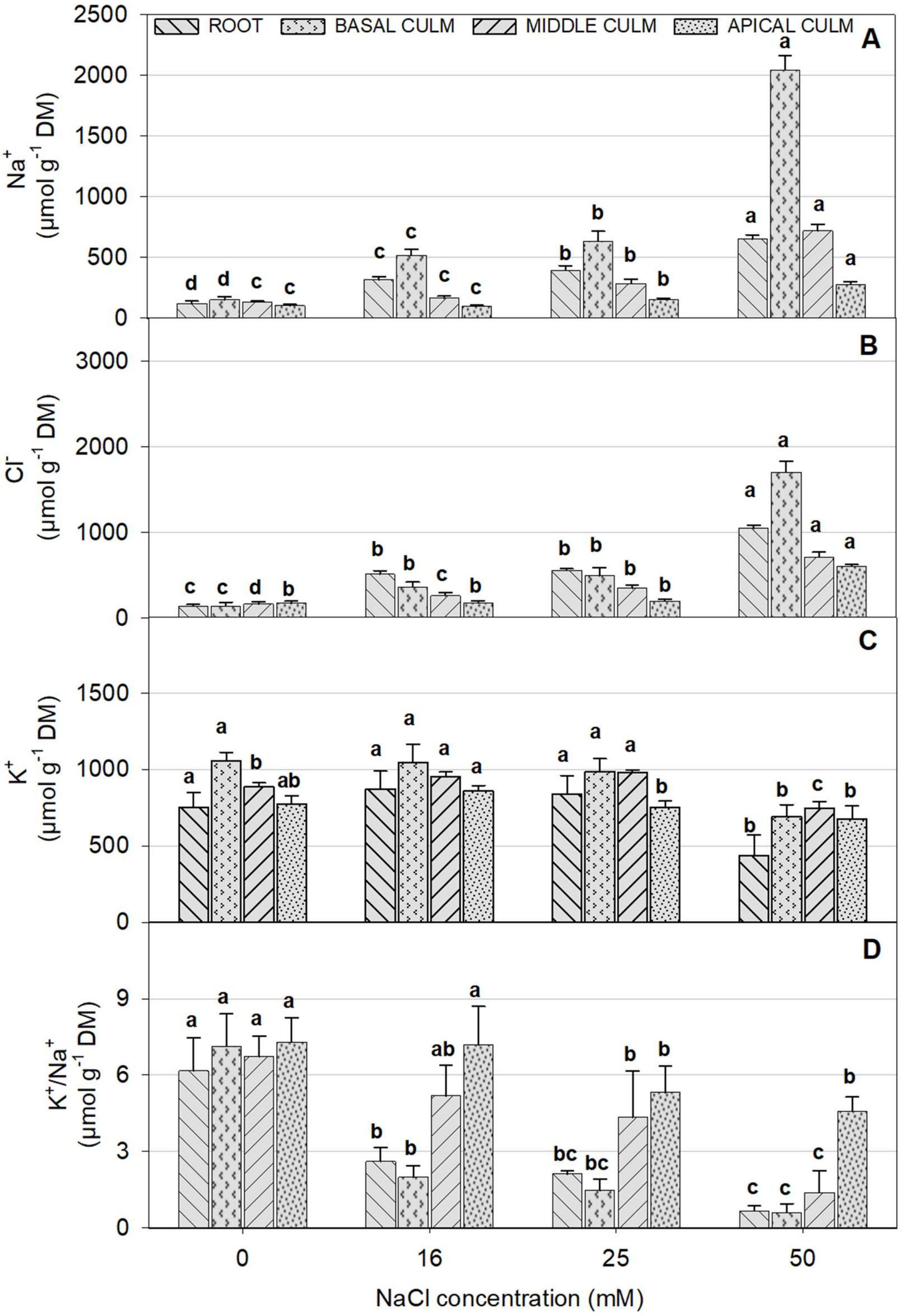


Figure S4 – Spatial distribution/accumulation of sodium – Na^+ (A), chloride – Cl^- (B), potassium – K^+ (C) and K^+ / Na^+ ratio (D) on rice culm at the end of the 3rd saline cycle. Data represents averages from four replicates ± SD and different letters represent significant difference among treatments by Tukey's test ($p<0.05$).



5 NEGATIVE OR POSITIVE MEMORY TRIGGERED BY NaCl PRE-EXPOSURE IS DEPENDENT ON THE DEVELOPMENT PHASE IN RICE (*Oryza sativa* L.) EXPOSED TO SALINITY

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Abstract

In order to overcome the oscillating environmental condition in which plants are inserted, new techniques are being developed to improve food production and to guarantee food security. Thus, this experiment aimed to study different moments of priming exposure of rice plants in order to enhance plants' performance under stressful conditions. For this, rice plants were primed with moderate salinity (25 mM NaCl for 7 days) in two different stages: during germination and vegetative development (V6). Later, when achieving V10 stage, plants were exposed to high salinity (50 mM NaCl for 16 days). It was identified positive effect of seed priming through input of dry matter, no difference in photosynthetic parameters, and the development of a different salt removal mechanism based on Ca^{2+} transport, regulating the senescence process. On the other hand, vegetative priming presented negative effects even compared to salt treatment, with lower dry matter content, poor photosynthetic adaptation to the stressful environment, and no changes in ion accumulation pattern compared to salt. While oxidative stress data requires further analysis, our findings highlight the promising potential of seed priming in improving rice resilience to salinity, emphasizing the need for future research to fully elucidate the associated antioxidative mechanisms.

Key-words: Acclimation, Ionic homeostasis, *Oryza sativa* L., Priming, Stress.

5.1 Introduction

Soil salinity is a globally significant phenomenon, with a particularly high incidence in arid and semi-arid regions. In these environments, elevated temperatures and limited rainfall favor salt accumulation in soil superficial layers. Furthermore, inadequate management of agricultural land enhance this natural process by destroying soil’s capacity for agriculture, once many of the commercial cultivars lack tolerance to high salt concentrations (Dias *et al.*, 2021; Haq *et al.*, 2009).

Plant breeding has mitigated the challenges posed by oscillating environmental, resulting in varieties adapted to particular developmental environments. However, the growing frequency of extreme weather events due to climate change is now compromising global food security and new techniques need to be quickly developed in order to maintain food production (BALFAGÓN *et al.*, 2020; LOBELL; GOURDJI, 2012).

Given the urgent need for novel techniques to sustain food production under the increasing environmental pressure, a complementary and highly effective approach to plant breeding involves the rational management of agricultural systems, to rapidly enhance plant responsiveness to environmental stresses. Seed priming stands out as a management technique due to its simplicity and effectiveness. This pre-sowing stimulus triggers epigenetic mechanisms during germination, leading to stress memory development, processes that are under intense investigation for their potential to improve crop resilience in the face of escalating salinity and other environmental threats. (LOUIS; DHANKHER; PUTHUR, 2023).

In our previous studies, we have shown that low and moderate salinity levels may trigger acclimation processes, so it has potential to be used as a priming agent in rice plants. The present study aimed to confirm the positive effect of this priming on V6 stage and to compare with seed priming. We hypothesized that vegetative primed plants would develop as well as plantlets primed during germination when both were challenged with a more intense salt stress at a later developmental stage.

5.2. Material and methods

5.2.1 Plant material and growth conditions and treatments

Rice seeds (*Oryza sativa* spp. Japonica cv. Nipponbare) were sown in Germitest ® paper during 14 days in a growth chamber (150 mmol m^{-2} s^{-1} photosynthetic active radiation – PAR, 25 °C, 60-70% relative humidity and 12 h photoperiod). 14 days after sowing, seedlings were transferred to 3-L pots containing ½ diluted Hoaglang-Arnon nutrient solution (HOAGLAND; ARNON, 1950) and were grown in a greenhouse (average of maximum photosynthetically active radiation – PAR of 800 µmol m^{-2} s^{-1}; average temperature of 30 °C; 12-h photoperiod; and average relative humidity of 75%). During the growth period, the nutrient solution was completely changed every week. A priming treatment was applied at two different sets of plants: one at the germination establishment – seed priming – and the other at the stage V6 – vegetative priming. When reached stage V10, plants were exposed to the following treatments: salt (S), seed priming + salt (SPS) and vegetative priming + salt (VPS). Salt treatments consisted in adding 50 mM NaCl to the nutritive solution for 16 days. For salinity tolerance assay, plants' measurements were carried out once a week (plants' height, leaves and tillers number). At the end of the experiment, photosynthesis, roots and shoot parts fresh and dry mass were determined. Panicles and roots were harvested and oven-dried at 70 °C until constant mass. Shoot was divided into basal (0-15 cm), middle (15-30 cm) and apical (>30 cm) culms and leaves, frozen in liquid N_2 and stored at -80 °C for biochemical determinations.

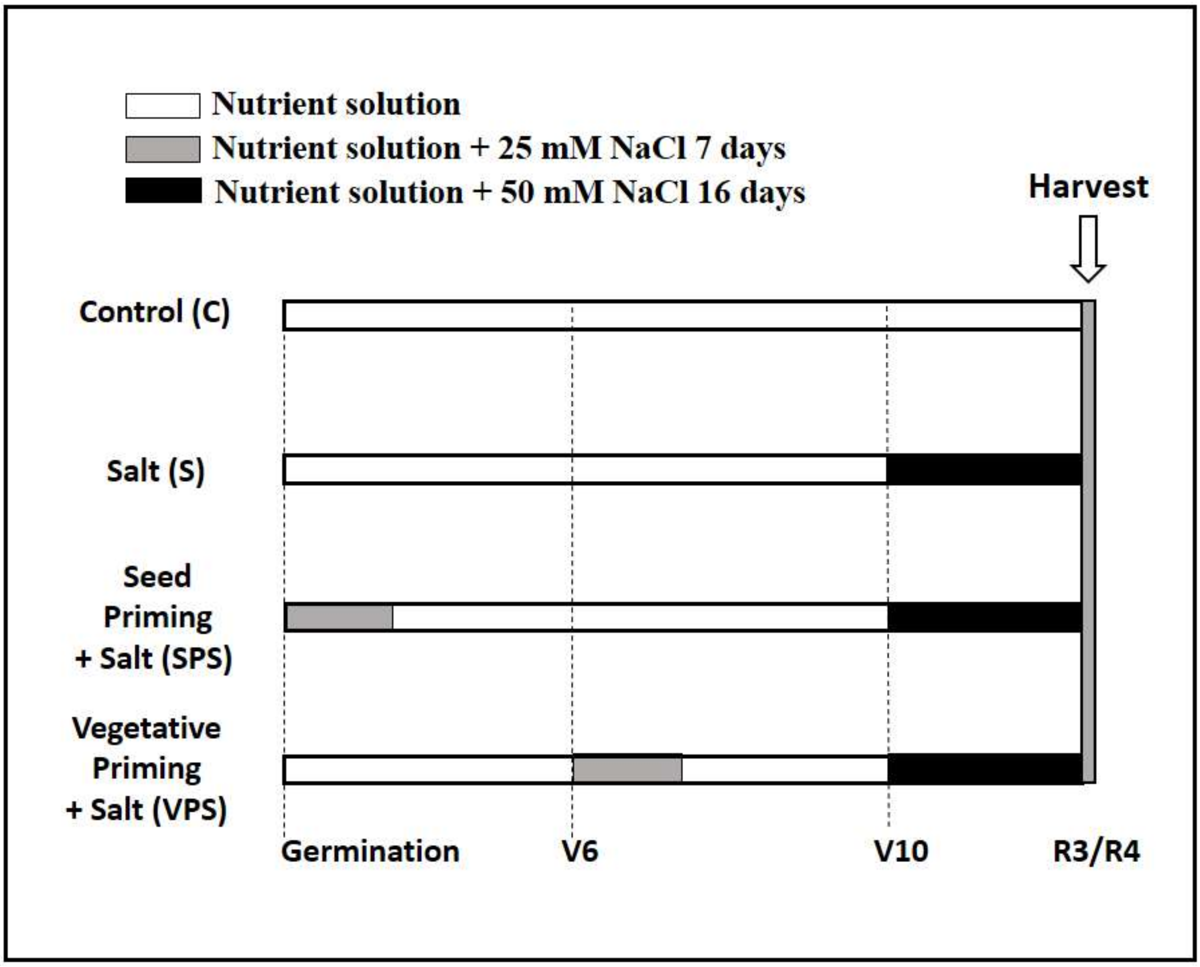
5.2.2 Priming treatment

Priming was performed by exposing plants to a 25 mM NaCl solution for 7 days, applied at two distinct development stages. The seed priming involved sowing seeds in germitest paper moistened in saline solution for 7 days. Following this period, the primed seedlings were transferred to a new germitest paper moistened with distilled water for an additional 7 days. In contrast, control seeds (C) were sowed in germitest paper moistened exclusively in distilled water for the full 14-day period. For the vegetative priming, a 25 mM NaCl solution was added to the nutrient solution when plants reached stage V6 (28 days after sowing) and after 7 days the solution was completely replaced with a fresh nutrient solution.

5.2.3 Salt treatment

Salt stress was performed by exposing plants to a 50 mM NaCl solution for 16 days applied when plants reached V10 stage (42 days after sowing). Three groups of plants were started: salt (S) group, which received no prior priming; seed priming + salt (SPS) group; and vegetative priming + salt (VPS) group. The experimental design is shown in figure 10. After 16 days, all plants were harvested for subsequent analysis.

Figure 10 – Experimental design.



5.2.4 Leaf area and gas exchange measurements

The determination of leaf area was made using a leaf area meter model LI-3100C. Gas exchange parameters were measured by using a portable infrared gas analyzer system (LI-6400XT, Li-COR, Lincoln, NE, USA) in mature middle leaves (L12). PPFD and temperature inside the measurement chamber were kept at 1000 μmol m^{-2} s^{-1} and 28°C, respectively. The amount of blue light was set up to 10% of the PPFD to maximize stomatal aperture (FLEXAS *et al.*, 2008), CO_2 partial pressure was kept at 40 Pa and the leaf-to-air vapor pressure difference was 1.85. Measurements were recorded when the total coefficient of variation was lower than 5% and temporal stability was achieved (about 3 min).

5.2.5 Oxidative stress indicators

Membrane damage (MD) was measured by electrolyte leakage as described previously by Blum & Ebercon (1981). Segments of a mature rice leaf (10 segments of 3 cm from the middle part of leaf 11 – L11) were placed in tubes containing 10 ml of deionized water. The flasks were slightly agitated for 24h and solution electrical conductivity was measured (C1). Solution with segments were then boiled (98 °C) for 1h and the electrical conductivity was measured again (C2). The relative membrane damage was estimated by MD (%) = C1/C2 x 100.

Lipid peroxidation was measured based on the formation of thiobarbituric acid-reactive substances (TBARS) in accordance with Heath & Packer (1968). 200 mg of fresh mass were added to 1.5 mL of TCA 5%, centrifuged at 12,000 g (4 °C) for 20 min and the supernatant was collected. 500 μL of the supernatant was added to tubes containing 2 mL of TBA solution, left on 98°C water bath for 60 min and rapidly cooled in ice bath. After centrifuged at 10,000 g for 5 min, absorbance was followed at 532 and 660 nm in the spectrophotometer TBARS concentration was calculated using absorption coefficient (155 mM^{-1} cm^{-1}) and results were expressed as ηmol MDA-TBA g^{-1} FM.

H_2O_2 content determination was performed using Amplex® red kit in accordance with Zhou *et al.* (1997). 150mg of fresh leaf samples were macerated with liquid N_2 in the presence of 1 ml of 100 mM phosphate buffer (ph 7.5), centrifuged at 12,000g (4 °C), and the supernatant was immediately used for H_2O_2 determination.

Aliquots of 200 µL of the supernatant were added to a reaction medium consisting of 200 µL of the amplex red solution and incubated for 30 min at 25° C protected from the light. The absorbance was determined at 560 ηm. Blank was prepared in parallel and subtracted from the sample. H_2O_2 content was calculated from a standard curve and results were expressed as µmol H_2O_2 g^{-1} FM.

5.2.6 Ions determination

Macerated samples were dried at 70 °C for three days. Ionic extraction was done according to F. C. da (ed.). SILVA, (2009). 25 mg of dried samples were added to erlenmeyers containing 25 ml of HCl 1M. The erlenmeyers were mixed for 1h and filtered after 2h of repose. Sodium and potassium in the extract were determined by flame photometry and Ca^{2+} content was determined by atomic absorption. Na^+ and K^+ concentrations were estimated using standard curves of NaCl and KCl respectively, with concentrations ranging from 0 to 50 mmol L^{-1}.

5.2.7 Experimental design and statistical analysis

The experiment was arranged in a completely randomized design totaling four treatments and four replicates per treatment. Each replicate was represented by one container with 2 plants. Sigmaplot 14.5 program was used for statistical analysis of ANOVA and Tukey test with significance level set at 5% ($p<0.05$).

5.3 Results

5.3.1 Growth and development

In the present study, rice plants were submitted to moderate NaCl doses (25 mM) during 7 days on different ages (seed and V6 priming) before being exposed to a high salinity condition (50 mM NaCl). Figure 11 shows the morphological development during the initial stress period.

Before salt stress begins (D0), it was possible to identify that seed priming plants (SPS) presented superior stature than other treatments and a slightly higher number of tillers and leaves, visually indicating induction of eustressor effect. On the other hand, vegetative priming plants (VPS) presented the opposite effect. A distressor effect was observed in VPS even when compared to salt treatment (S), especially on tillering, which led to a reduction in leaf number.

On the 1st week of salt (D7), vegetative growth was more oriented towards height, considering the little input of dry matter through tillers and leaves on control plants. After 2 weeks (D14), plants were in the reproductive stage (R3/R4) and growth in height was no more the focus. Control plants (C) gained biomass through increasing number of tillers and, as a consequence, increasing on leaf number. S and SPS plants kept the same pattern of growth of raise in tillers and leaves as control plants, while VPS plants did not emit new tillers during the whole salt period. They continued to emit leaves under salt stress, but the senescence process took place at the end of the experiment.

The dry matter content of shoots and roots it’s shown in figures 12A and 12B. Salt treatment presented a reduction of approximately 40% on shoot dry matter content compared to control; SPS plants increased by about 20%; and VPS plants presented a reduction around 70%. Similar pattern was identified for root dry matter, 60% higher for SPS and lower 37% and 83% for S and VPS, respectively.

Shoot dry matter was divided in culms and leaves, as shown in figures 12C and 12D. There was no difference among control and SPS, as well as among salt and VPS on these parameters. However, the average reduction found between control and salt treatment was of about 50% for culms and 60% for leaves. Considering VPS, the reduction was of about 72% for culms and 70% for leaves compared to control.

The growth pattern of the flag leaves was also measured considering length and width (data not shown). Salt stress caused reduction in leaf length in all treatments. However, the reduction in SPS was inferior, about 15%, compared to salt and VPS plants, which presented a reduction around 30%. The main difference lies in leaf width, which presented no significant difference on SPS, and reduction from 30 to 35% on the other treatments compared to control.

Flag leaf area and total leaf area results presented no difference between control and SPS, as well as for salt and VPS for both parameters (data not shown).

The photosynthetic parameters were measured after 13 days under salt stress, as shown in figure 13. No significant difference was identified on the measured parameters between control, SPS and VPS. However, salt plants presented a reduction of around 30% on CO_2 assimilation, 45% on stomatal conductance and 20% on transpiration.

Figure 11 – Plant height (A), leaf number (B) and tiller number (C) of rice plants after 16 days of salt (D0 – before salt; D7 – 7 days of salt; D14 – 14 days of salt). Data represents averages from four replicates ± standard deviation (SD) and different letters represent significant differences among treatments by Tukey's test (p<0.05).

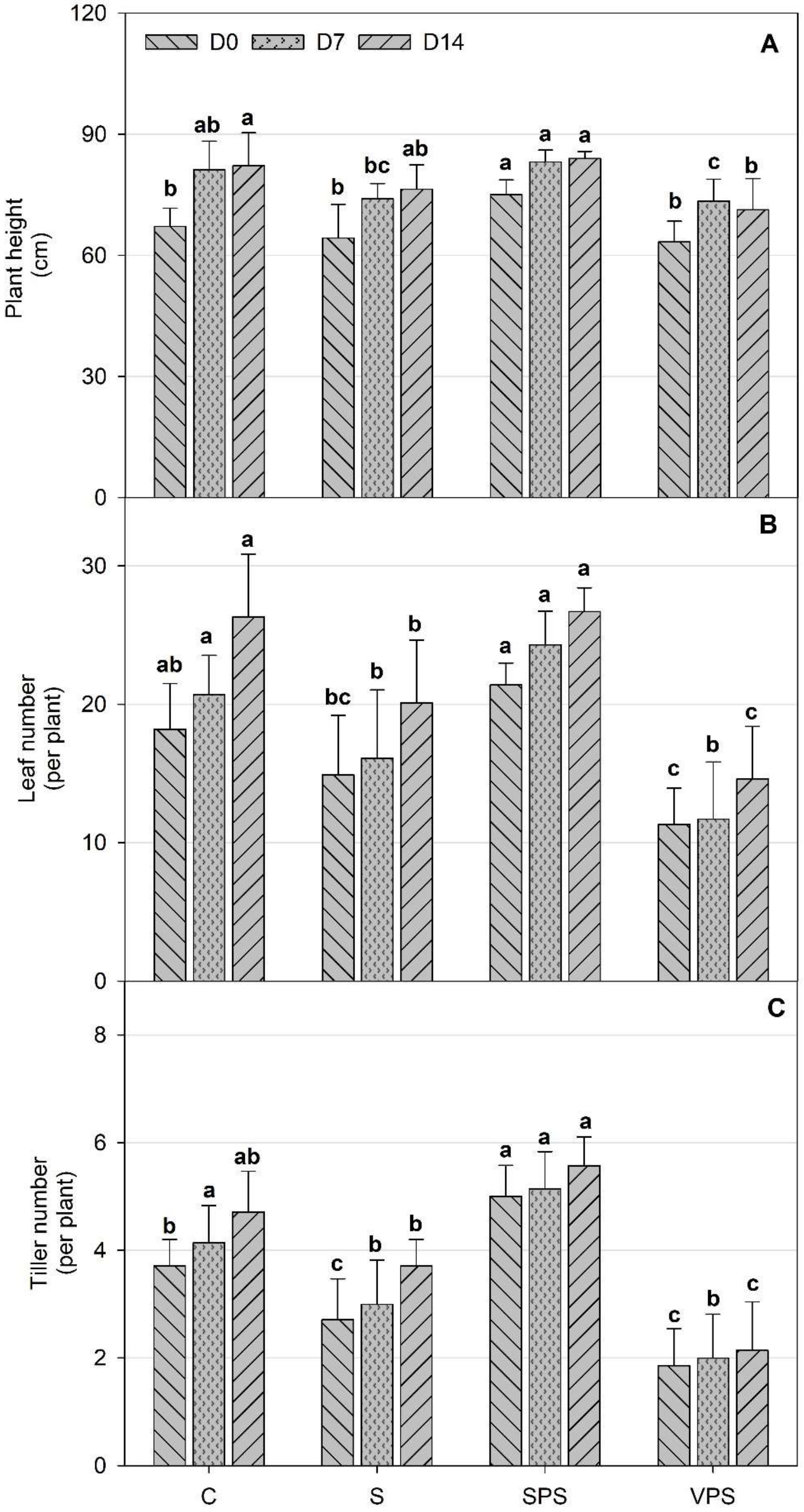


Figure 12 – Shoot (A), root (B), culm (C) and leaves (D) dry matter of rice plants after 16 days of salt. Data represents averages from four replicates ± SD and different letters represent significant difference among treatments by Tukey's test (p<0.05).

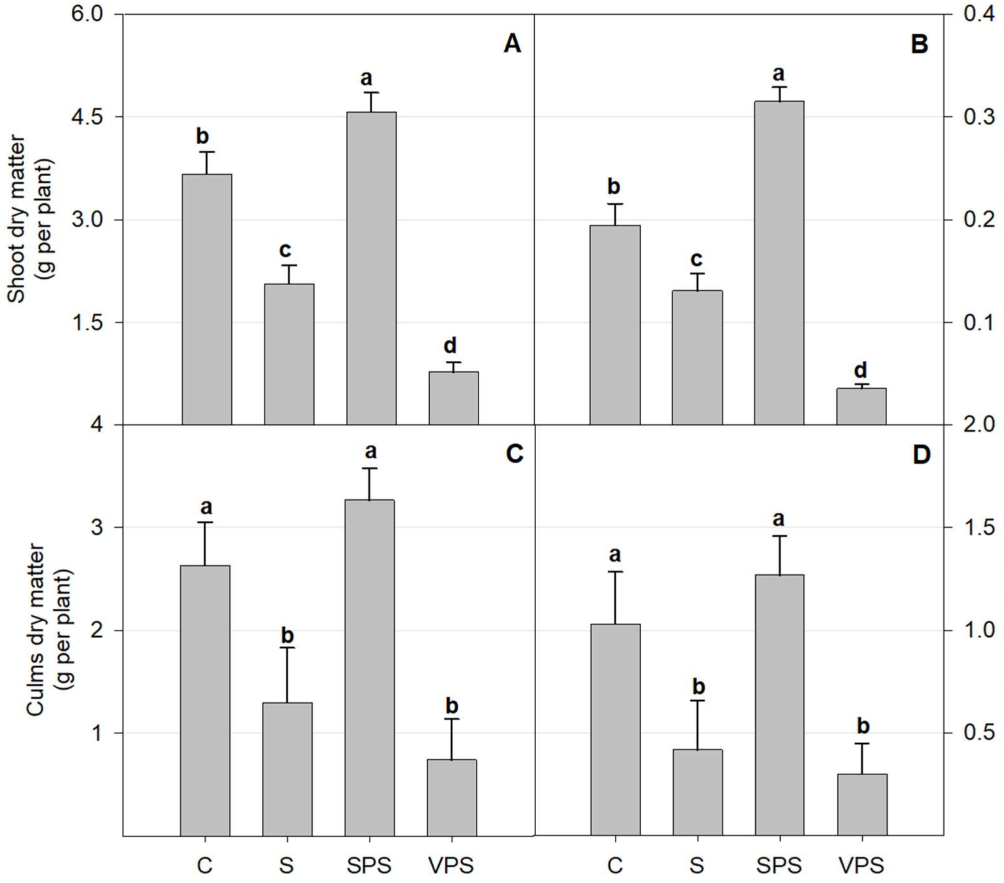
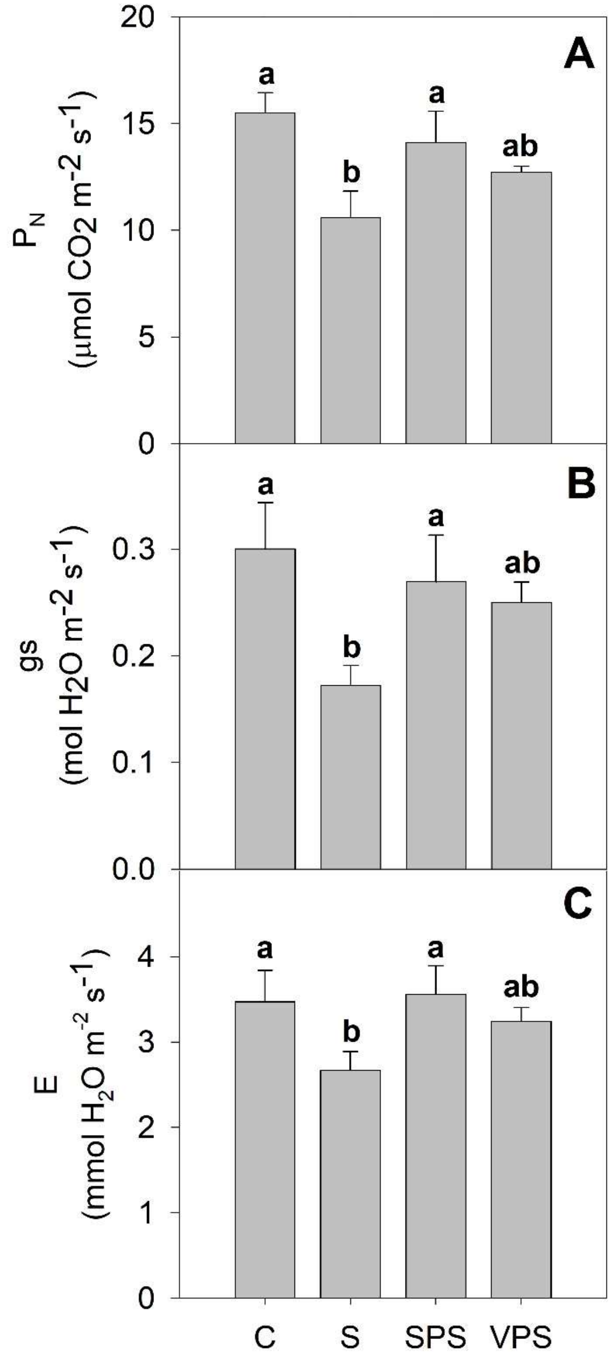


Figure 13 – CO_2 assimilation (A), stomatal conductance (B) and transpiration (C) of rice plants after 13 days of salt. Data represents averages from four replicates ± SD and different letters represent significant differences among treatments by Tukey's test ($p<0.05$).



5.3.2 Ionic spatial distribution

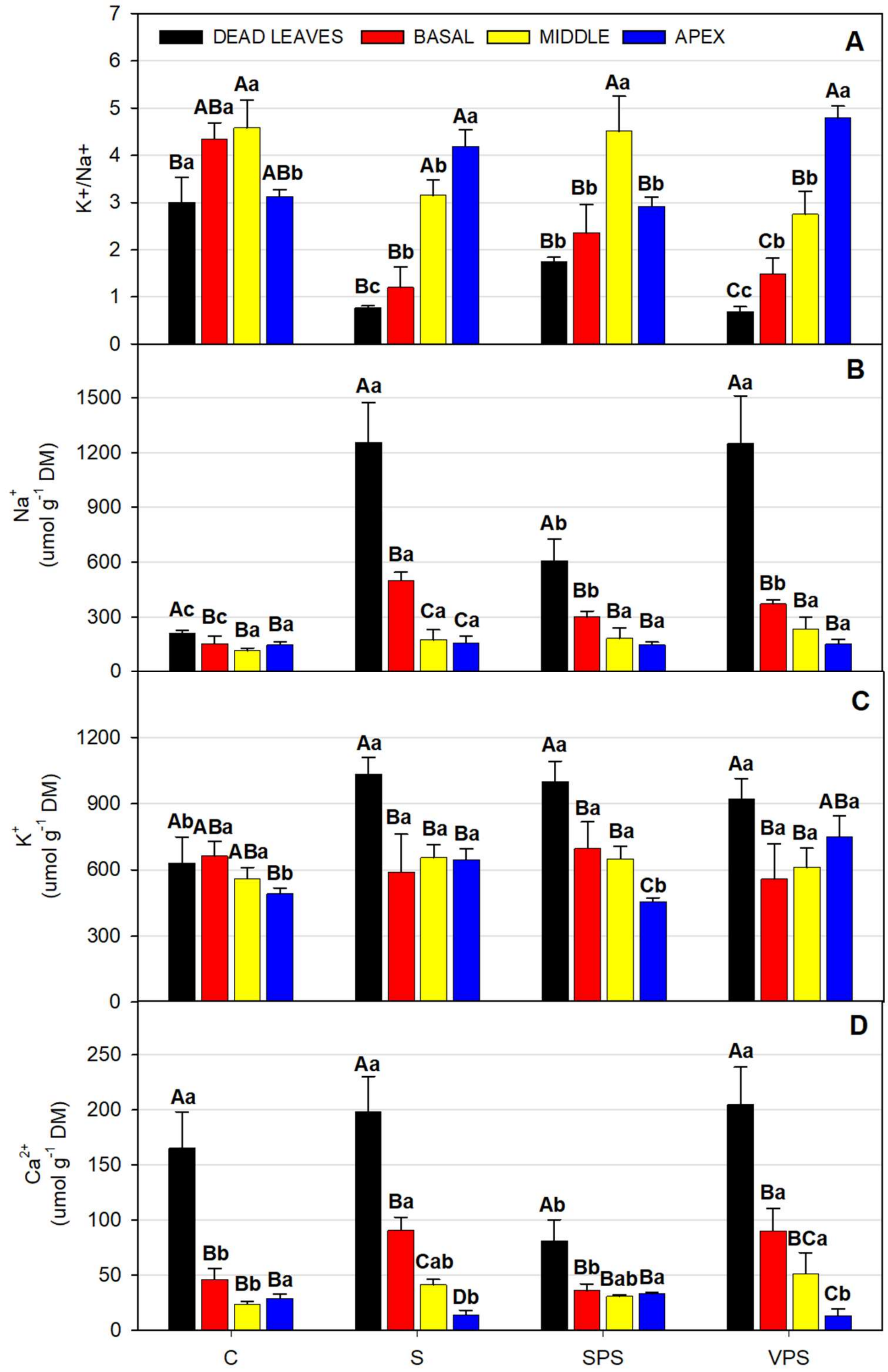
The spatial distribution of ions is displayed on figure 14. The control pattern of ion accumulation consists of no significant difference among green leaving leaves. However, the dead leaves tend to accumulate higher content of the determined elements, especially Ca^{2+}, which increased around 250% compared to basal leaves.

Salt treatment presented a different pattern of ion accumulation, with higher content of ions near the base, and reducing as it approaches the apex. Some of the elements determined presented a marked increase in dead leaves compared to control dead leaves: about 486% for Na^+ and 64% for K^+. Considering the leaf living tissues, Na^+ and Ca^{2+} also differed, with apical leaves presenting reduction of 69% and 84% in the content of Na^+ and Ca^{2+}, respectively, related to basal leaves. Compared to control, basal leaves presented an increase of 223% for Na^+ and 98% for Ca^{2+}, while K^+ differed only in apical leaves, with an increase of 31%. The exact same ion accumulation pattern of salt treatment was identified for VPS plants.

SPS plants presented an ion accumulation pattern similar to control plants, with no significant difference among the living tissues and higher ion content on dead leaves. However, strong reduction on storage patterns of Na^+ and Ca^{2+} was identified on dead leaves (51% and 60%, respectively) and on basal leaves (40% and 60%, respectively) compared to salt treatment. It was also observed reduction on K^+ content on apical leaves to similar levels to control plants.

The pattern of K^+ lies on 3 to 5 folds the Na^+ content in healthy tissues. This pattern was kept on middle and apical leaves of saline treatments, but not for dead and basal leaves due to the excess of Na^+ on those tissues.

Figure 14 – Spatial distribution of K^+/Na^+ ratio (A) and ionic accumulation of sodium – Na^+ (B), potassium – K^+ (C) and calcium – Ca^{2+} (D) on rice leaves after 16 days of salt. Data represents averages from four replicates ± SD and different letters represent significant differences among treatments by Tukey's test ($p<0.05$). Uppercase letters compare within the same treatment, while lowercase letters compare between treatments.



5.3.3 Redox responses

The electrolyte leakage and TBARS and H_2O_2 contents are shown in figures 15 and 16.

The electrolyte leakage analysis was carried out using a mature leaf (L11). There was significant difference among control and VPS treatments with increase of 70% on membrane damage. Salt and SPS presented no significant difference from control.

The TBARS content on basal leaves was superior on salt and VPS treatments on about 45% compared to control, but SPS presented similar levels. Considering middle and apical leaves, no significant difference was found among treatments, but on SPS plants, TBARS content was 27% superior on apical leaves compared to the basal ones, slightly different from control tendency.

The H_2O_2 content also did not present a significant difference among treatments on basal and apical leaves, except on SPS plants, which presented superior levels of H_2O_2 on apical leaves than in the basal ones. The levels of H_2O_2 on middle leaves almost doubled in salt treatment (81%), tripled in SPS (169%) and almost quadrupled in VPS treatment (235%). Compared to salt treatment, H_2O_2 levels increased 48% and 84% for SPS and VPS, respectively.

Figure 15 – Electrolyte leakage in mature rice leaves (L11) after 16 days of salt. Data represents averages from four replicates ± SD and different letters represent significant differences among treatments by Tukey's test ($p<0.05$).

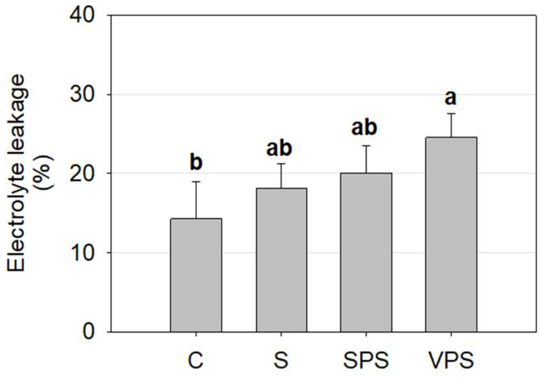
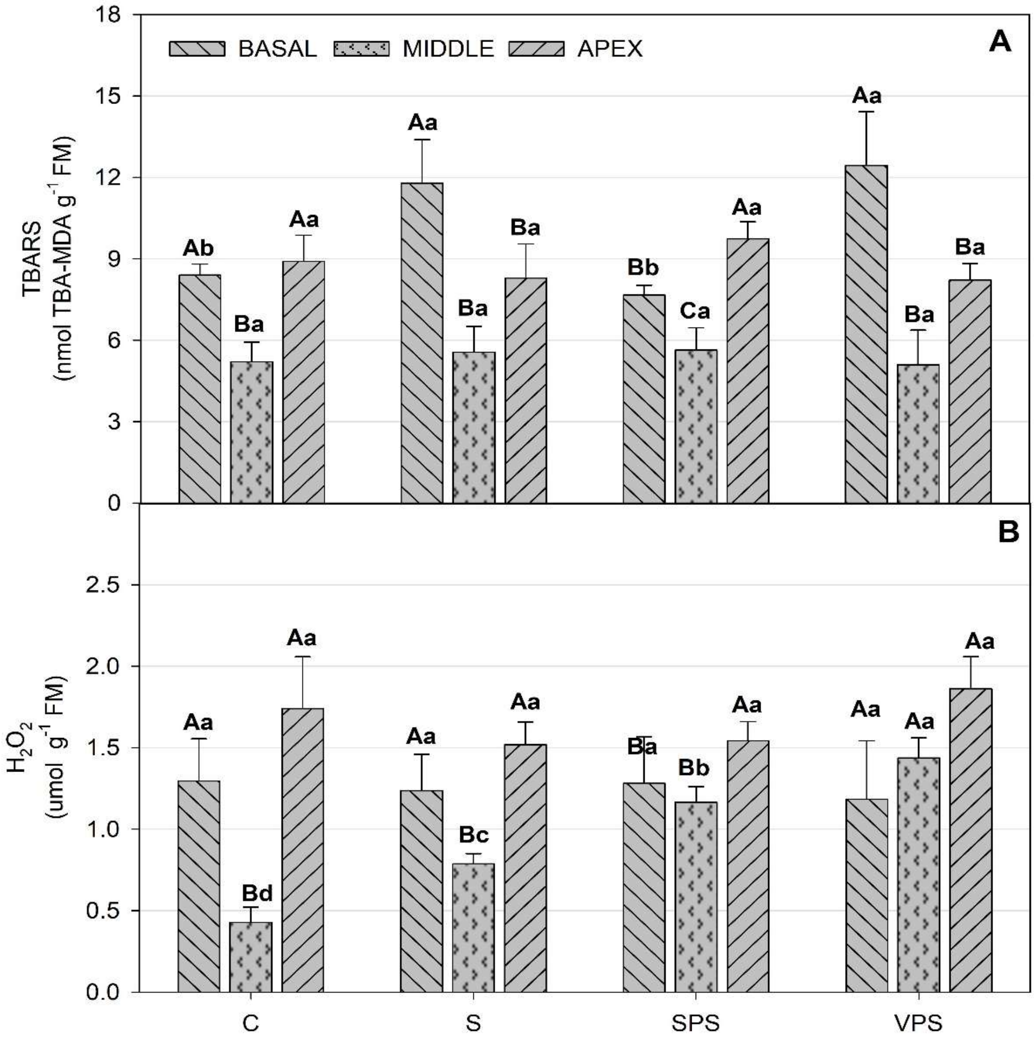


Figure 16 – Contents of thiobarbituric acid reactive substances – TBARS (A) and hydrogen peroxide – H_2O_2 (B) in rice leaves after 16 days of salt. Data represents averages from three replicates ± SD and different letters represent significant differences among treatments by Tukey's test ($p<0.05$). Uppercase letters compare within the same treatment, while lowercase letters compare between treatments.



Harvest was carried out in plants on the reproductive stage with panicles emitted (R3-R4).

5.4. Discussion

5.4.1. Stress or acclimation due to salinity might be identified through leaf width

From the beginning of the salinity period, it was possible to identify the beneficial effect of seed priming on growth, with taller plants. After 16 days of salt, despite no significant difference on number of leaves and tillers, height and leaf area, and also the smaller leaf length on SPS plants compared to control, SPS formed more vigorous plants with higher content of shoot and root dry matter. According to Coca *et al.* (2023) and Sousa *et al.* (2021), salinity is capable of inducing many changes in plant morphology, such as leaf thickness and vessel diameter. Other studies have shown that many primers are able to improve salt tolerance through higher biomass production (EL-SERAFY *et al.*, 2021; SILVA *et al.*, 2022). Thus, it can be said that the seed priming induced an eustressor effect in rice plants due to the plant capacity to maintain plant growth and development like control even under salt stress.

On the other hand, the exposure to moderate salinity on early vegetative stage (V6) acted as a stress factor instead of a priming, hindering plant's growth. VPS plants presented a lower biomass content visually identified through the reduced number of leaves and tillers even before salt stress begins. Beyond this, the reduction on dry matter content was not proportional to the reduction on height, tillering and leaves number, meaning salinity promoted changes in morphology that possibly increased tissue fragility. Many researches show the lower dry matter content as a common effect of salinity (HAQ *et al.*, 2009; HUANHE *et al.*, 2024; KORRES *et al.*, 2022; PIRES *et al.*, 2015; COCA *et al.*, 2023). Despite several experiments show the positive effect of seed priming on maintaining shoot dry matter content (EL-SERAFY *et al.*, 2021; SILVA *et al.*, 2022), Rossatto *et al.* (2023) results corroborate those of this experiment, in which vegetative priming was not able to maintain shoot dry matter content.

Shoots and roots biomass presented the same tendency of development among treatments, maintaining shoot/root ratio constant. From these results, it is possible to infer that salinity did not stimulate differential root development in any treatment, possibly due to the combined effect of hydroponic condition and vase limitation for growth, once many studies shown increase in root development under saline conditions (KORRES *et al.*, 2022; ROSSATTO *et al.*, 2023; SANTANOO *et al.*, 2023).

The more interesting result lies on the not proportional difference among dry matter content and morphological results. Despite visual identification of higher biomass production on SPS plants compared to control and lower biomass production on VPS plants compared to salt (Figure S3), the difference was not due to difference on leaf and/or tiller emission. Tissues were differently built according to timing of priming exposition, but which elements were favored and which were disadvantaged require more in-depth studies. Huanhe *et al.* (2024) found difference in nonstructural carbohydrates content in rice plants stems due to the isolated effect of salinity and drought with stronger effect when combined. On the other hand, El-Serafy *et al.* (2021) identified increase in proline content due to salinity in non-primed rice plants, while seed primed plants presented reduction in proline content and increase in the total phenolic compounds. Yadav *et al.* (2023) attributed the rise in rice fresh and dry matter to a more efficient nutrition promoted by the primers, which were able to induce metabolic changes in order to improve the acclimation state.

Another interesting finding was related to the possibility to quickly identify plant struggle through leaf width. Rice leaves grown under controlled conditions presented an average width of 14 to 15 mm in the intermediate part. Under salt, plants' response may vary. Yao *et al.* (2023) identified that salinity was able to induce raise in *Lycium barbarum* L. leaf area, where low salinity improved leaf's length and width, while high salinity promoted a thicker leaf blade. Considering rice plants, Korres *et al.* (2022) identified that salinity reduces leaf growth rate, width and length. In this experiment, despite reduction in leaf length on plants under salt stress, seed primed plants were capable to maintain leaf blade's width and it did not affect biomass production as in salt and VPS plants. Thus, rice leaf width might have potential to be used as acclimation parameter.

Leaf area reduction due to salinity brings many consequences, beginning with reduction on photosynthetic rate, low biomass production, low final yield or even early senescence (MORADBEYGI *et al.*, 2020; SANTANOO *et al.*, 2023; TALEISNIK *et al.*, 2009). The changes in leaf tissue structure due to priming promoted modification on photosynthetic pattern on SPS plants, allowing them to maintain high photosynthetic rates. New studies considering leaf morphological changes need to be carried out in order to better understand how photosynthetic mechanism was able to be improved through seed priming.

5.4.2 Seed priming plants develop a mechanism of ion exclusion or selective uptake involving Ca^{2+} absorption

Control plants basal leaves are prone to accumulate higher contents of K^+ and Ca^{2+} compared to apical leaves and not harmful contents of Na^+. Considering the dead leaves, Na^+ and K^+ content remain similar to the senescent leaves on the base, however Ca^{2+} presents a very distinct behavior, reaching up to 4 folds the basal leaves amount. This accumulation may be related to a regulatory mechanism for the progression of leaf senescence, acting in signaling and maintaining cell integrity, supported by Huang *et al.* (1990). This author stated that high content of Ca^{2+} were necessary to slow rice leaves senescence. On the other hand, SPS plants presented a strong reduction on Ca^{2+} accumulation on dead leaves. Following Huang *et al.* (1990) reasoning, these plants may have developed a mechanism of fast salt removal through the non-accumulation of Ca^{2+} in order to spare the other tissues and organs, once the other living tissues present no significant difference on Ca^{2+} content from control plants.

Calcium is an element of structural function with little movement on plants. Its main functions are keeping membrane integrity (which is very affected by salinity) and signaling in response to stresses (ACIELY *et al.*, 2024). It seems that calcium is sent to lower tissues under salt stress, and this occurs at the expense of upper leaves, that will present a more fragile structure and more prone to suffer other kinds of stress.

To overcome the upper leaves calcium deficiency on Salt and VPS treatments, those tissues presented an increase on K^+ content. Cruz *et al.* (2006) found on their experiment with passionfruit that salinity reduced K^+ content in all organs analyzed. Pires *et al.* (2015), on the other hand, stated that salinity doesn't affect K^+ content and, for this, K^+/Na^+ ratio is not so important to determine rice plants salt stress. This raise in K^+ accumulation may be due to its effect as osmotic regulator and also to speed up grain filling, leading to a K^+/Na^+ ratio higher than control treatment. The same didn't happen for SPS plants, which remained similar to control plants.

Under salt, rice plants were able to hold Na^+ on basal tissues, maintaining ionic homeostasis similar to control on middle and apical tissues. SPS plants presented a slightly distinct behavior considering the reduction on Na^+ content on dead leaves, improving salt avoidance mechanism. Conversely, VPS presented the exact same pattern of Na^+ accumulation than Salt treatment, indicating this priming moment is not capable of developing memory.

5.4.3 Acclimation parameters

As mentioned by Silveira *et al.* (2016), growth reduction alone cannot be an indicative of plant stress, but rather as the beginning of a very complex acclimatizing response. Plants metabolism presents several mechanisms in order to cope with the oscillating environment in which they are inserted. The stress diagnosis involves the determination of parameters such as membrane damage ratio and redox responses.

Considering electrolyte leakage analysis were carried out using middle leaves, which were not struggling as the basal ones, the levels were low, not indicative of stress. Usually, salinity elevates the level of membrane damage to up 25%, which were the maximum obtained in this experiment. Also, still considering the middle leaves, no difference on TBARS content was identified. However, H_2O_2 content on middle leaves appears to follow the same pattern than membrane damage.

What studies show is that salinity effects on plants are due to reduction on enzymatic antioxidant activity (HUANHE *et al.*, 2024) and priming works on improving the antioxidant mechanism through increasing enzymes activities and also the non-enzymatic antioxidant compounds (EL-SERAFY *et al.*, 2021).

5.5 Conclusion

The results of this study highlight the potential of seed priming as a promising tool to improve the resilience of rice to saline stress, inducing tolerance mechanisms that manifest in better biomass development, maintenance of leaf morphology, and efficient ionic regulation. The absence of similar effects in vegetative priming suggests that the timing of priming application is crucial for the induction of physiological memory in rice plants under saline stress. Future research is needed to elucidate the underlying molecular and physiological mechanisms of these differential responses and to explore the potential for optimizing priming strategies to enhance tolerance to multiple abiotic stresses under field conditions.

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SUPPLEMENTARY

Figure S5 – Seed priming treatment (SP) - seedlings at 7th germination day on germitest paper under controlled (A) and priming (B) conditions.

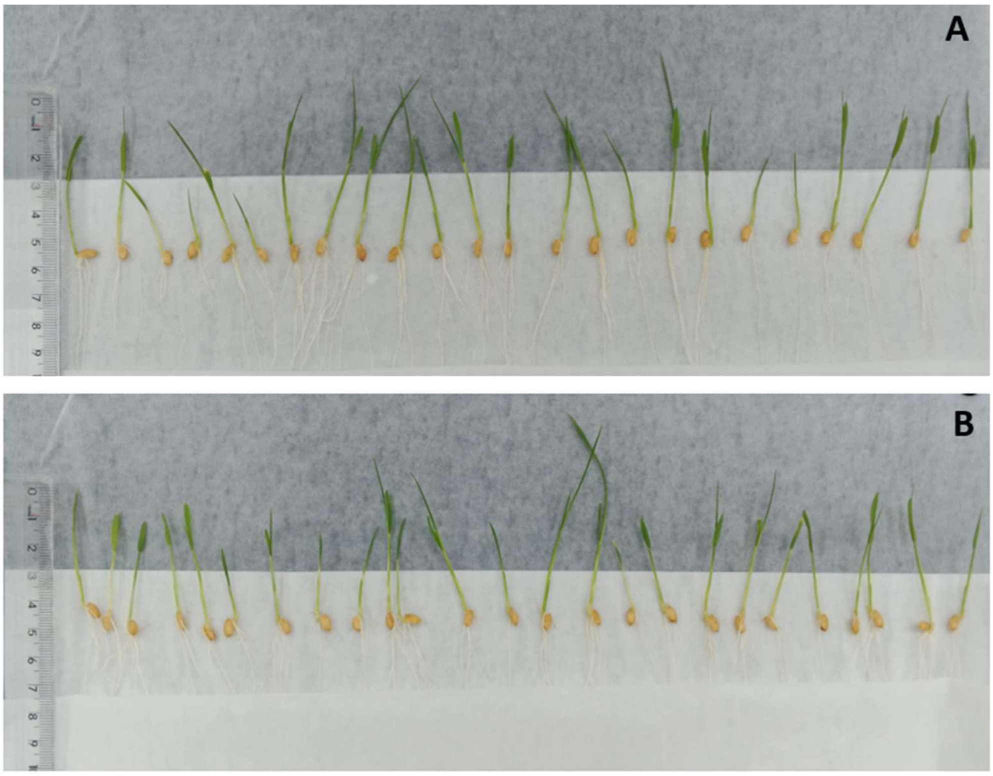


Figure S6 – Vegetative priming treatment (VP) - Rice plants at the end of the 7th day of priming under controlled (A) and primed (B) conditions.

Figure S7 – Visual aspects of whole rice plants after 14 days of salt.

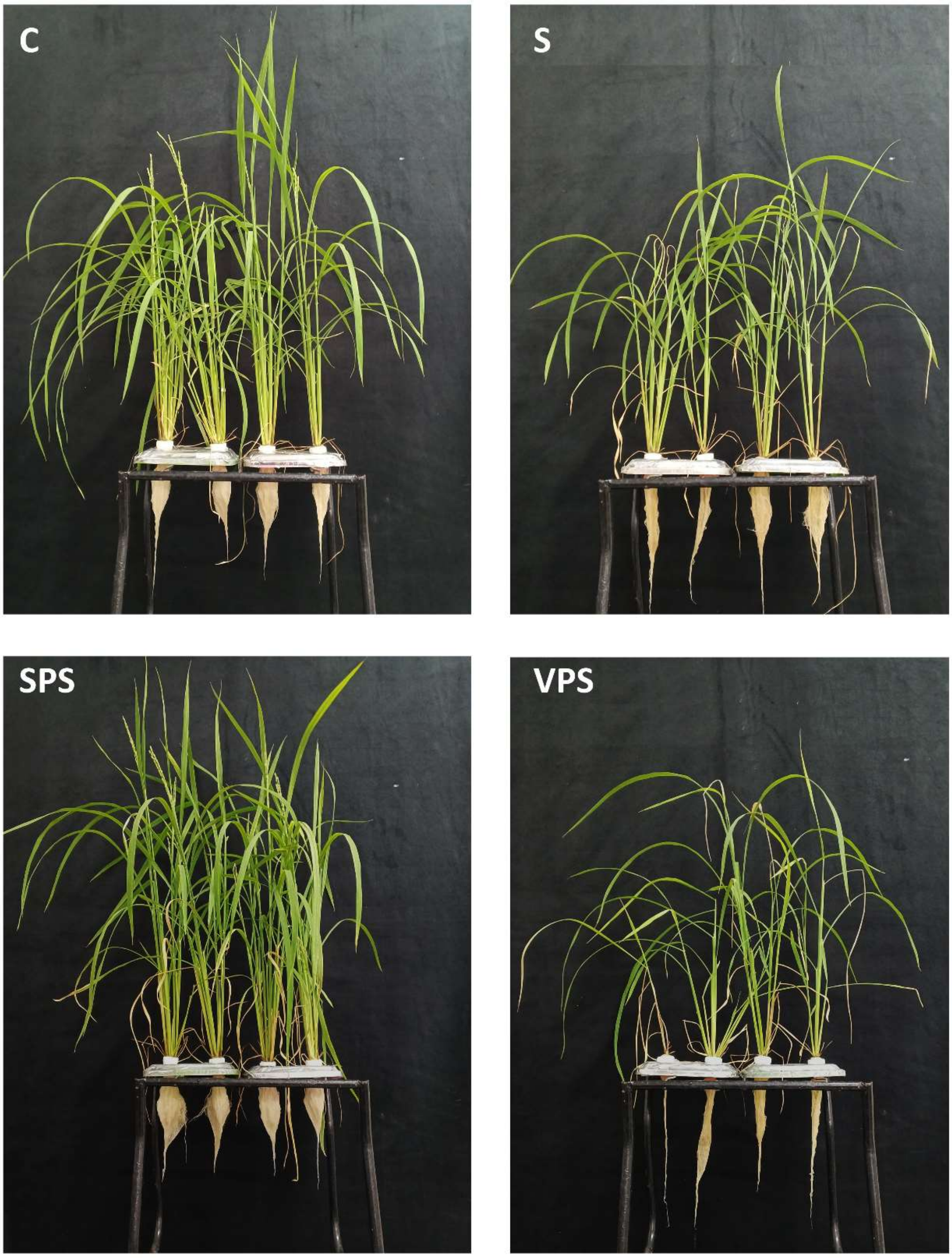


Figure S8 – Visual aspects of panicles of rice plants after 14 days of salt.

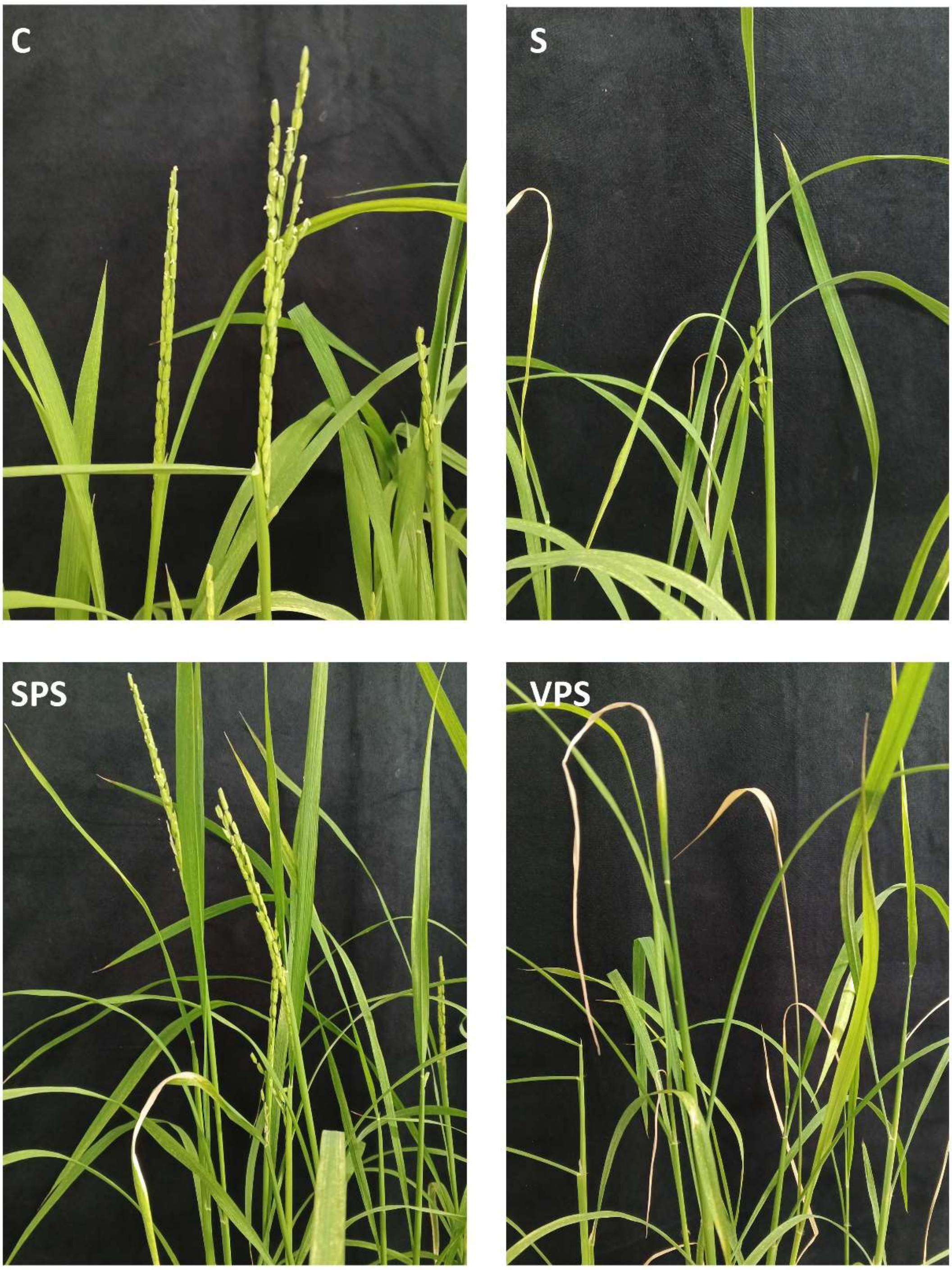


Figure S9 – Visual aspects of tillers of rice plants after 14 days of salt.

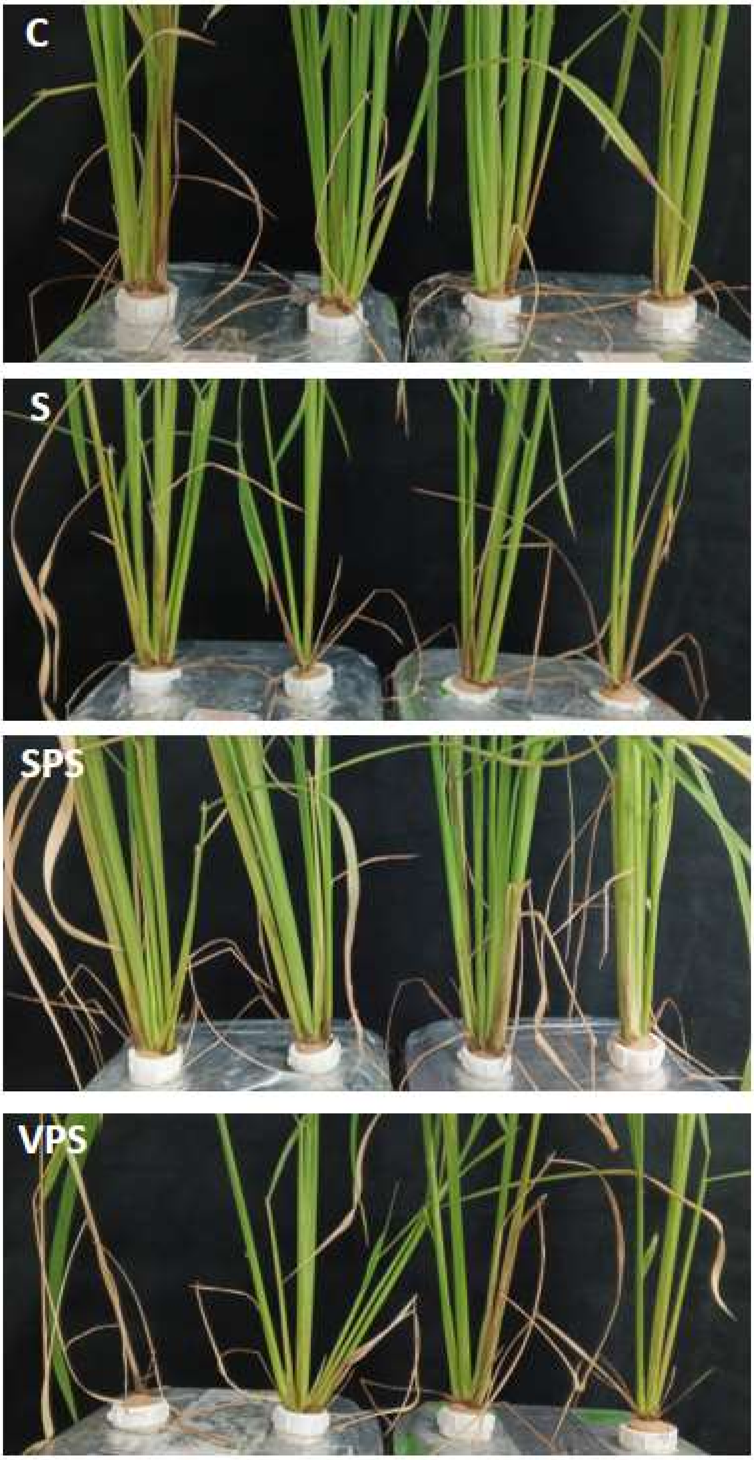
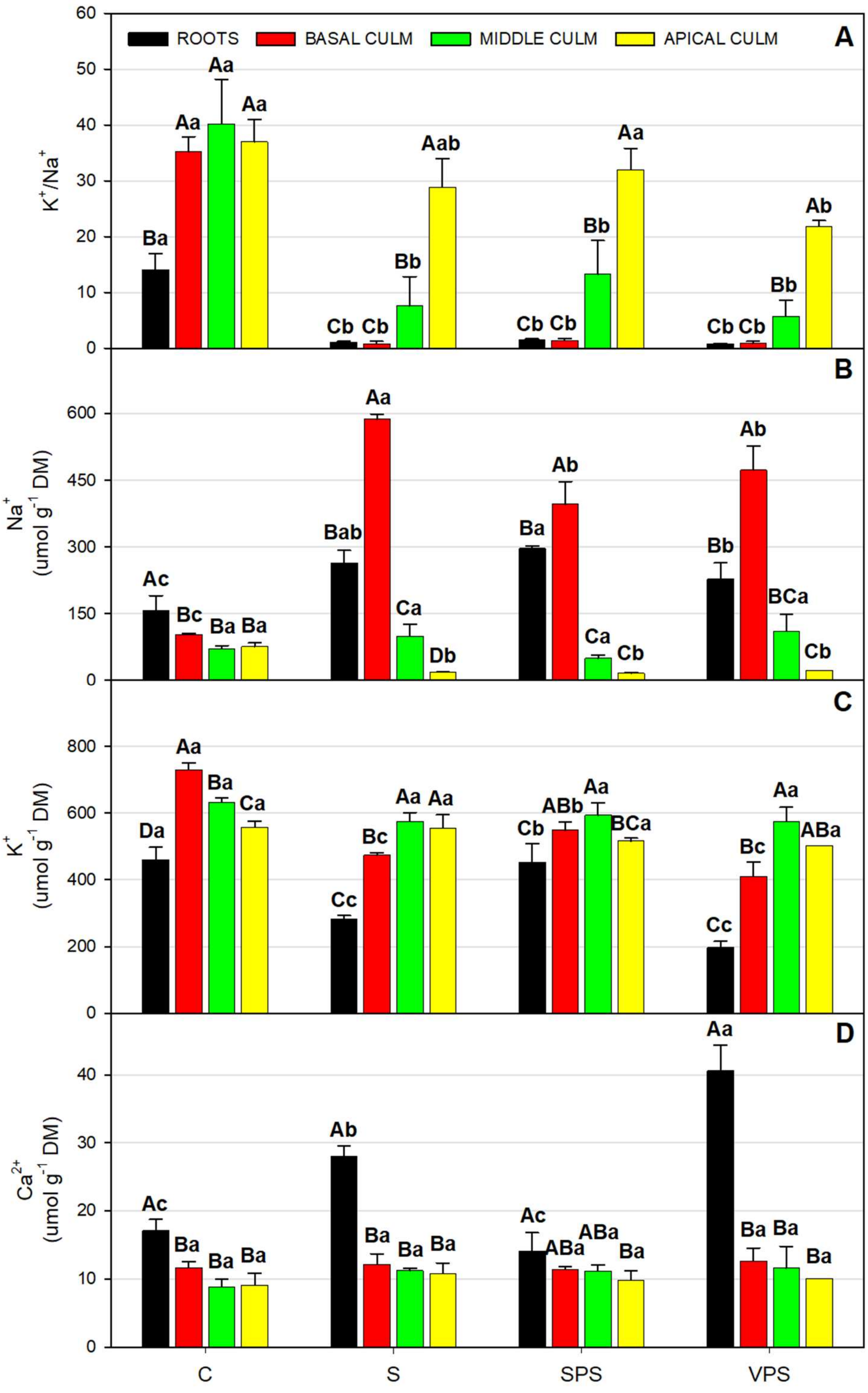


Figure S10 – Spatial distribution of K^+/Na^+ ratio (A) and ionic accumulation of sodium – Na^+ (B), potassium – K^+ (C) and calcium – Ca^{2+} (D) on rice roots and culms after 16 days of salt. Data represents averages from four replicates ± SD and different letters represent significant difference among treatments by Tukey's test ($p<0.05$). Uppercase letters compare within the same treatment, while lowercase letters compare between treatments.



6 FINAL CONSIDERATIONS

The results obtained from our studies reinforce the hypothesis that pre-exposure to moderate saline stress at early life stages can indeed activate mechanisms that lead to a more efficient and rapid acclimation to subsequent stressors, thereby demonstrating the crop's remarkable phenotypic plasticity.

Our findings show that saline stress delivered in stress-recovery cycles induces dose-dependent responses and that the stress intensity is also dependent on the developmental stage of the leaf. The application of low-to-moderate salinity during the vegetative stages (V6) did not severely compromise productivity, suggesting the existence of a stress threshold that can be leveraged for priming purposes. Furthermore, the progressive accumulation of toxic ions in the oldest leaves suggests that the plant strategically manages stress to protect younger, photosynthetically active tissues.

The second experiment elucidated the critical importance of the timing of priming. While the seed priming (SPS) treatment proved highly beneficial, leading to increased dry biomass production and tillering despite a reduction in leaf length, the vegetative-phase priming (VPS) demonstrated a strong distressor effect, resulting in parameters that were inferior even to the salt-alone treatment. This finding highlights that a stressor can function as either a eustressor or a distressor depending on the plant's developmental stage. In addition, the acclimation induced by seed priming was not limited to morphological parameters; our data suggest the activation of a distinct salt-removal mechanism based on Ca^{2+} content, which may be related to the regulation of leaf senescence and contributes to the plant's overall resilience.

Taken together, the results of this thesis validate the hypothesis that rice possesses significant phenotypic plasticity, adapting its responses based on the timing, intensity, and repetition of the stress event. Our discoveries regarding the potential of seed priming open new perspectives for food security by proposing a promising technique to improve rice resilience to salinity. However, to fully unravel the complex mechanisms behind this plasticity, further studies are necessary. Investigating epigenetic mechanisms and their interactions with antioxidative responses in a more detailed spatio-temporal scale is the crucial next step to fully elucidate how this stress "memory" is formed and activated.

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APPENDIX A - ORIGINAL PHOTOS OF EXPERIMENTS

Figure 17 – Effect of the application of 25 mM NaCl during 6 days in rice plants beginning at stage V4.

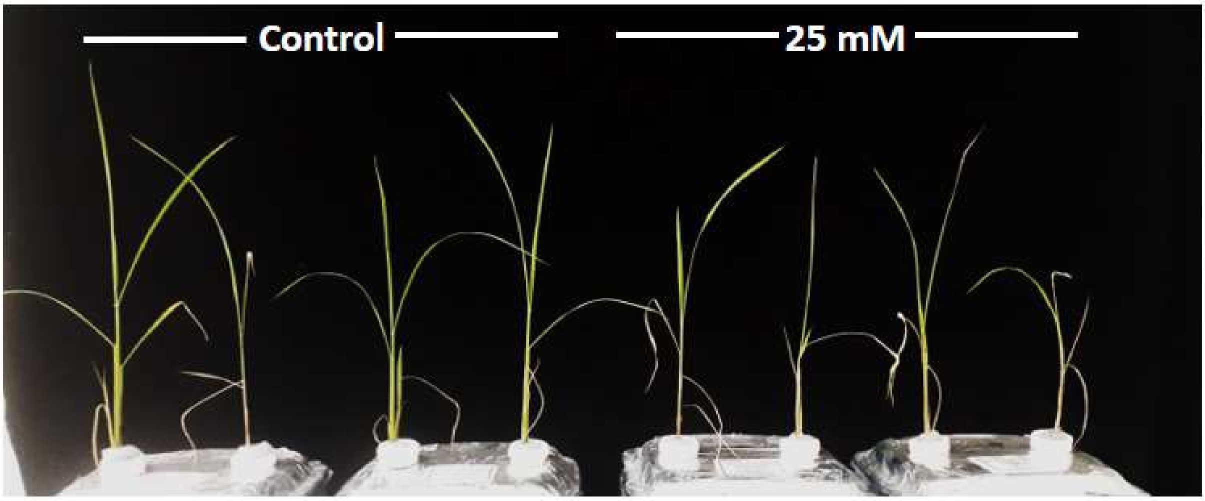

Figure 18 – Effect of the application of 25 mM NaCl during 10 days in rice plants beginning at the stage V4. Control – no salt; Recovery – 7 days salt and 3 days recovery; Continuous – 10 days salt.

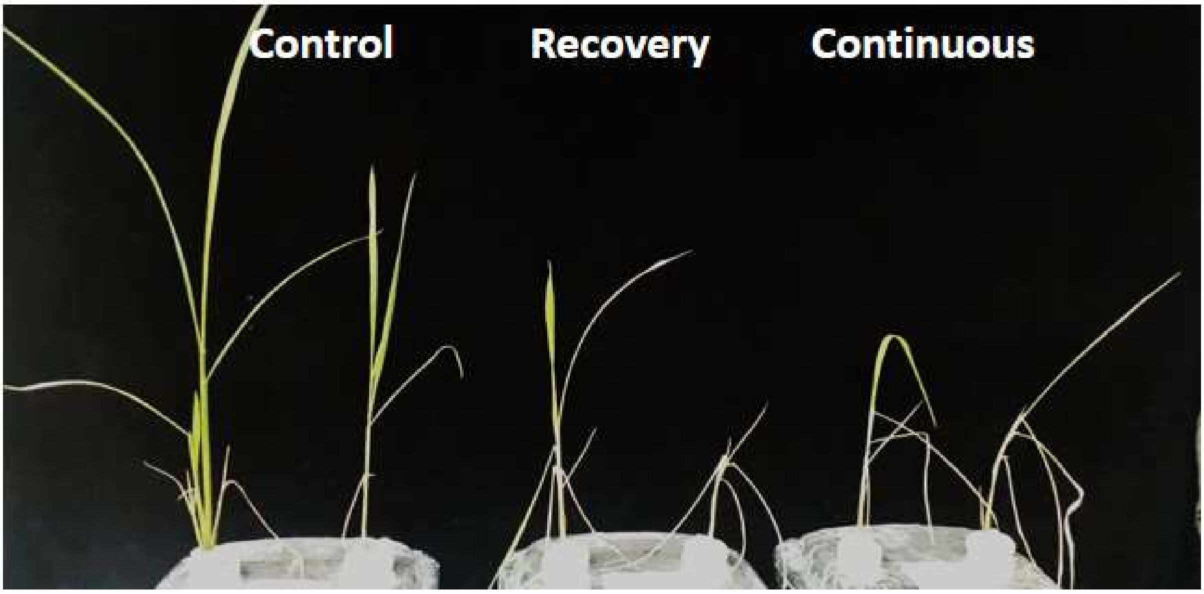

Figure 19 – Symptoms on rice leaves during the 1st (A) and the 2nd (B) recovery cycle resulting from the application of multiple salt doses (0, 16, 25 and 50 mM NaCl) in cycles of 7 days exposure/ 3 days recovery (Chapter 4).

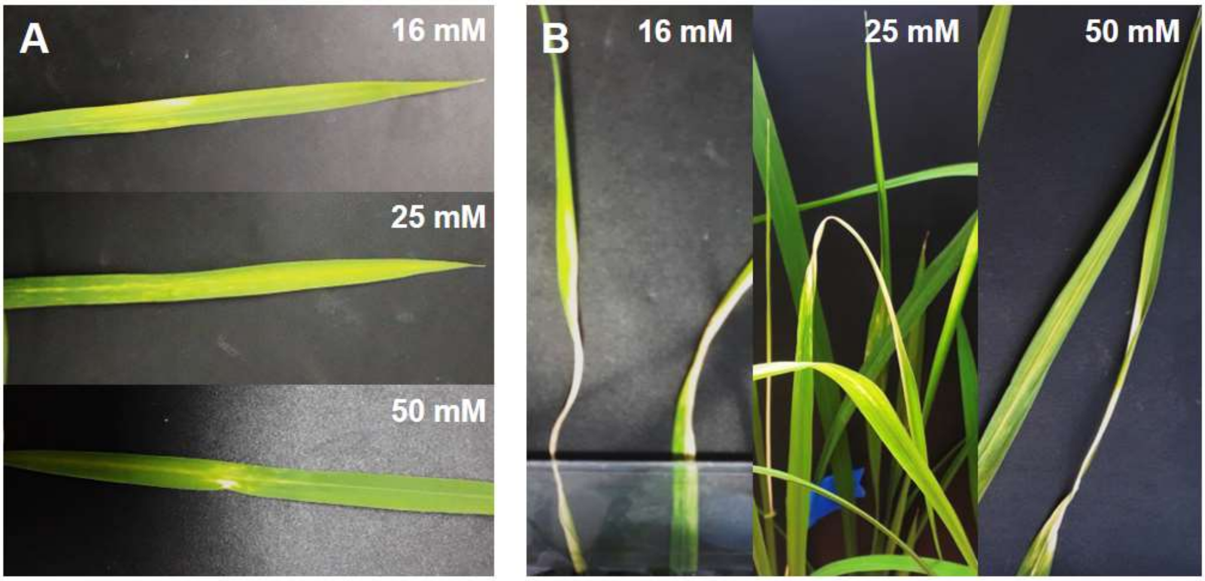

Figure 20 – Dose dependent flag leaf necrosis at the end of the 3rd exposure cycle resulting from the application of multiple salt doses (0, 16, 25 and 50 mM NaCl) in cycles of 7 days exposure/ 3 days recovery (Chapter 4).

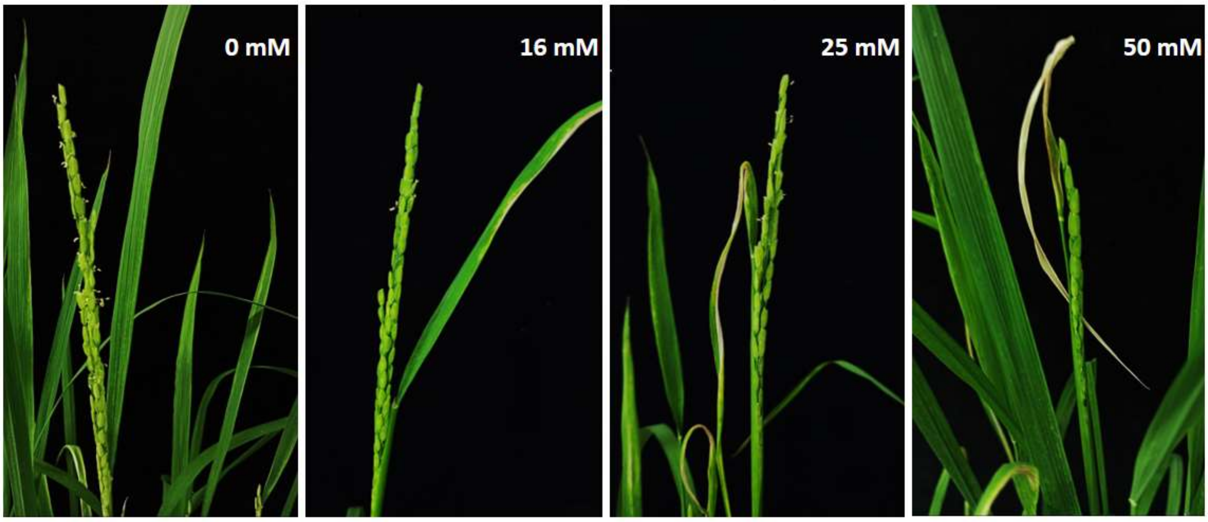

Figure 21 – Seed priming treatment (SPS) – 1st attempt: seedlings on germitest® paper under controlled and priming conditions (25 mM NaCl) at 7th and at 14th day of germination (7 days after priming removal).

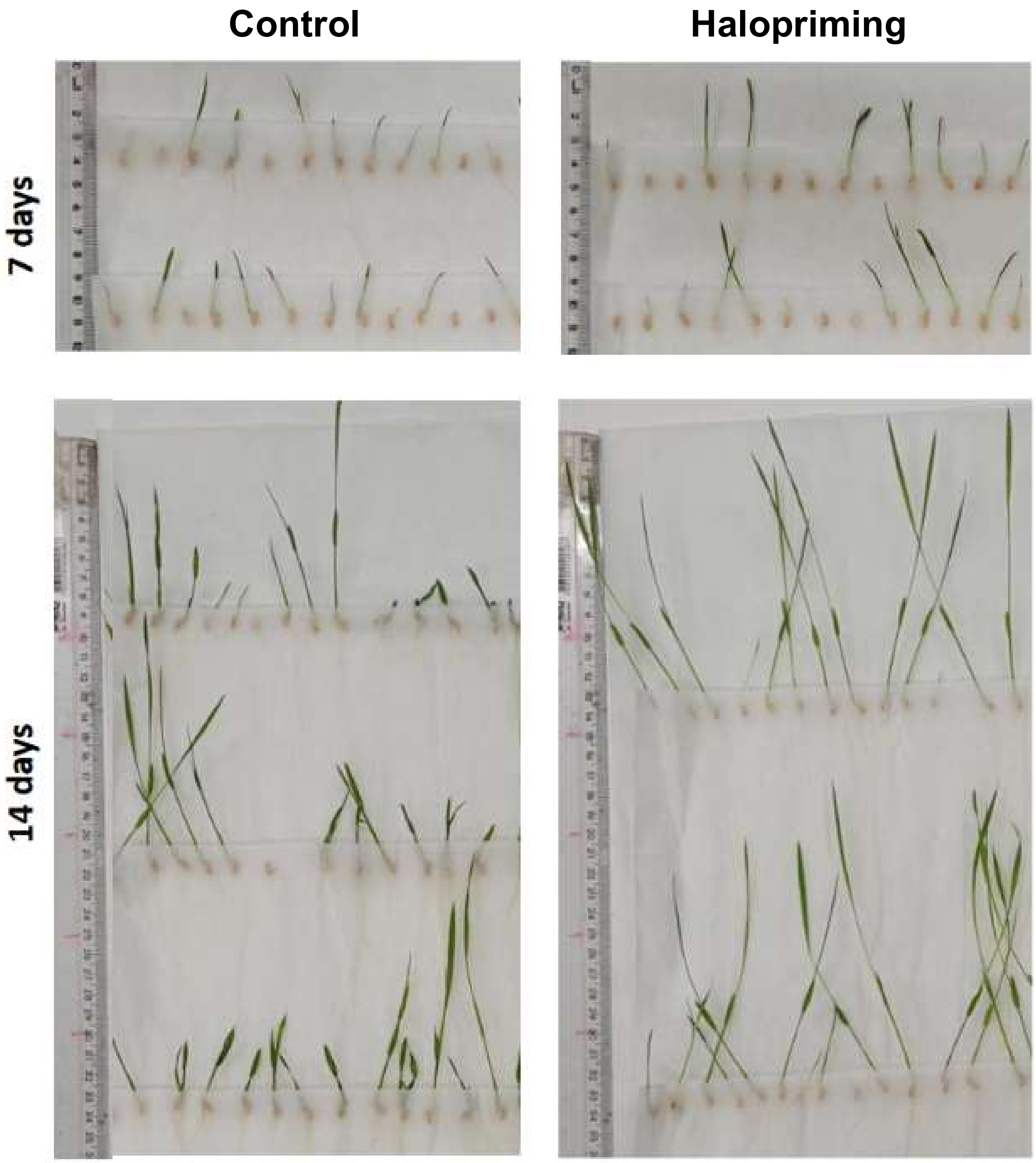

Figure 22 – Visual aspects of whole rice plants at the end of the priming experiment (Chapter 5) – 13 days of salt. 53-day-old rice plants exposed to control (C), 50 mM NaCl (S), seed priming (25 mM), seed priming followed by salt treatment (SPS), vegetative priming (VP) and vegetative priming followed by salt treatment (VPS) for 6 days from the V10 phase.

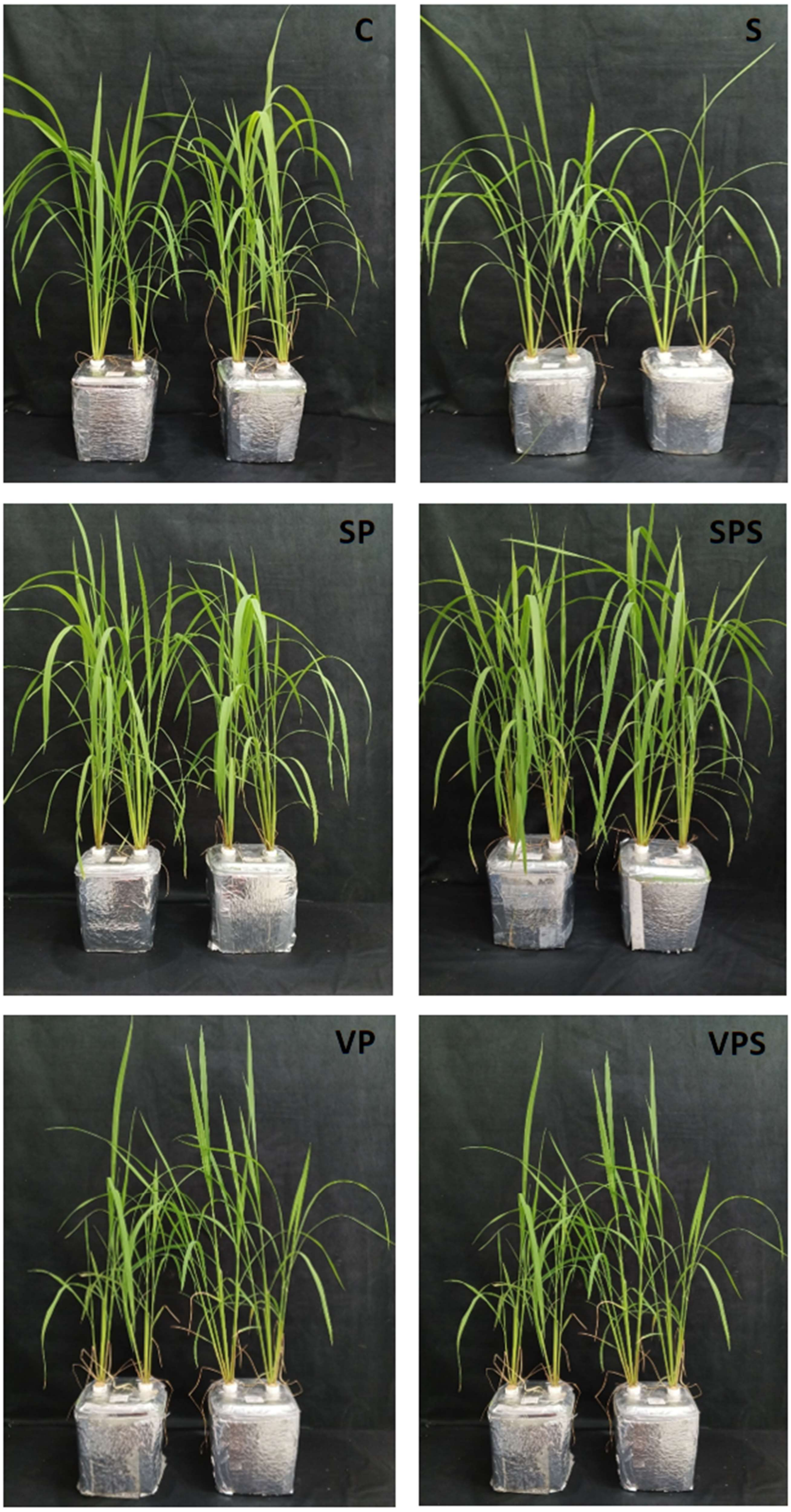

Figure 23 – Visual aspects of whole rice plants at the end of the priming experiment (Chapter 5) – 13 days of salt. 53-day-old rice plants exposed to control (C), 50 mM NaCl (S), seed priming (25 mM), seed priming followed by salt treatment (SPS), vegetative priming (VP) and vegetative priming followed by salt treatment (VPS) for 14 days from the V10 phase.

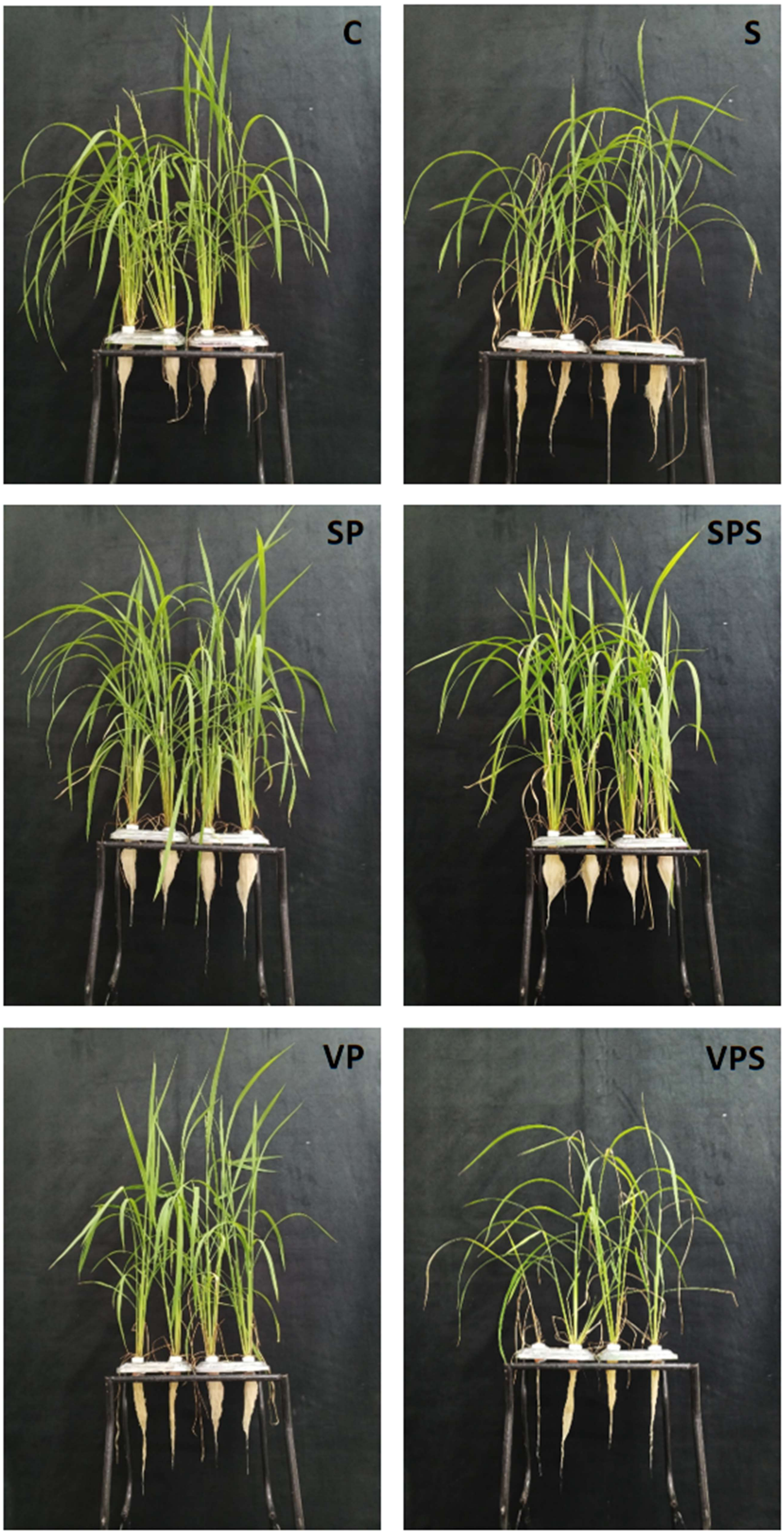

Figure 24 – Visual aspects of whole rice plants panicles at the end of the priming experiment (Chapter 5) – 16 days of salt. 56-day-old rice plants exposed to control (C), 50 mM NaCl (S), seed priming (25 mM), seed priming followed by salt treatment (SPS), vegetative priming (VP) and vegetative priming followed by salt treatment (VPS) for 16 days from the V10 phase.

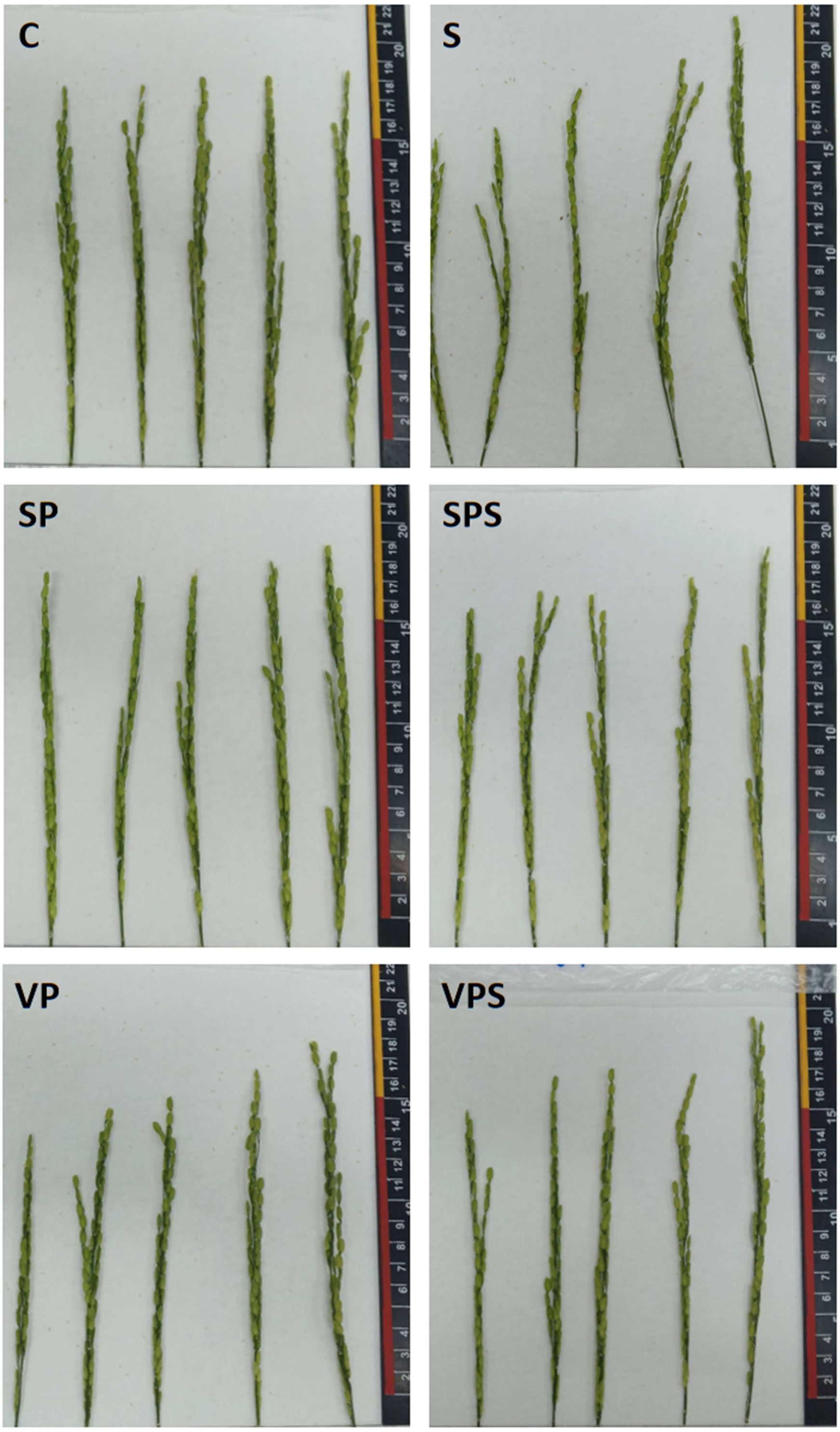

Figure 25 – Symptoms of the widespread presence of mites in the priming experiment.

APPENDIX B - PARTICIPATION IN SCIENTIFIC EVENTS

CERTIFICADO

A Sociedade Brasileira de Fisiologia Vegetal, inscrita no CNPJ n° 80.919.590/0001-90 DECLARA que ***ALLANA RAYRA HOLANDA SOTERO****, CPF n° 058.276.793-80 participou do* ***V SIMPÓSIO DE ECOFISIOLOGIA SISTÊMICA (V SES -2023)****, ocorrido em FORTALEZA/CE , 05 a 08 de dezembro de 2023*

Rio Claro, 01 de Setembro de 2025.

Danilo M. Daloso
Danilo de Menezes Daloso
Presidente da SBFV

Certificado emitido em 10/01/2024 15:30:06

Verifique sua autenticidade em
https://portal.sbfv.org.br/certificado/verificador/12876ad9-b894-4f91-b463-d47453ea0462

CERTIFICADO

CERTIFICO que **Allana Rayra Holanda Sotero** apresentou o resumo no **V SIMPÓSIO DE ECOFISIOLOGIA SISTÊMICA (V SES -2023)** em **05 a 08 de dezembro de 2023** realizado em **Fortaleza/CE** com o título: **"Priming" em sementes favorece maior influxo de K+ em plantas de arroz sob estresse salino severo.**.

Este resumo teve como co-autores:

- Allana Rayra Holanda Sotero, Allana R. H. S., UFC - (Apresentador);
- Matheus Vasconcelos Monteiro, Matheus V. M., UFC ;
- Kellyane da Rocha Mendes, Kellyane R. M., UFC ;
- Magna Pereira dos Santos, Magna P. S., UFC ;
- Joaquim Albenisio Gomes da Silveira, Joaquim A. G. S., UFC ;

Rio Claro, 1 de setembro de 2025

Danilo de Menezes Daloso
Presidente

Certificado emitido em 1 de setembro de 2025

Verifique sua autenticidade em
https://portal.sbfv.org.br/resumo/verificador/106

CERTIFICADO

CERTIFICO que **Allana Rayra Holanda Sotero** apresentou o resumo no **V SIMPÓSIO DE ECOFISIOLOGIA SISTÊMICA (V SES -2023)** em **05 a 08 de dezembro de 2023** realizado em **Fortaleza/CE** com o título: **Efeito potencial da salinidade como euestressor em plantas de arroz.**.

Este resumo teve como co-autores:

- Allana Rayra Holanda Sotero, Allana R. H. S., UFC - (Apresentador);
- Matheus Vasconcelos Monteiro, Matheus V. M., UFC ;
- Lívia Leitão Cardoso, Lívia L. C., UFC ;
- Kellyane da Rocha Mendes, Kellyane R. M., UFC ;
- Joaquim Albenisio Gomes da Silveira, Joaquim A. G. S., UFC ;

Rio Claro, 1 de setembro de 2025

Danilo de Menezes Daloso
Presidente

Certificado emitido em 1 de setembro de 2025

APPENDIX C - PUBLISHED ABSTRACT (CO-AUTHOR)

XVIII
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PLANT PHYSIOLOGY
I Ibero-latinamerican Congress
of Plant Biology

Nitrate alleviates ammonium toxicity and favor salinity acclimation by improving oxidative protection and CO_2 assimilation in *Jatropha curcas*

Kellyane da Rocha Mendes, Rafael Magalhães de Aragão, Raysa Mayara De Jesus Sousa, Rachel Hellen Vieira de Sousa Lima, Allana Rayra Holanda sotero e Joaquim Albenisio Gomes da Silveira

University Federal of Ceará, UFC- Brazil

PLANT SYSTEMS BIOLOGY: INTEGRATING SCALES AND TIMES PLANT SYSTEMS BIOLOGY: INTEGRATING SCALES AND TIMES

Abstract: Nitrogen nutrition is crucial for photosynthetic efficiency under salinity and a suitable balance between the NO_3^- and NH_4^+ supply is essential, and it is apparently plant species dependent. We tested the hypothesis that high NO_3^-/NH_4^+ ratios in root medium are able to avoid ammonium toxicity and favour salinity acclimation in a moderately sensitive plant species, *Jatropha curcas*. To this, young plants were exposed to four NO_3^-/NH_4^+ ratios (5.0/0.0 mM; 3.75/1.25 mM; 1.25/3.75 mM; 0/5.0 mM), in presence of 100 mM NaCl or absence (control), for 10 consecutives days. Plants supplied with increased NO_3^- levels, were capable to proportionally stimulate plant growth (root and shoot dry mass). Inversely, these nitrate treatments reduced stress indicators in leaves such as accumulation of reactive oxygen species - ROS (H_2O_2 and TBARS), electrolyte leakage and free total amino acids and NH_4^+ contents. In parallel, high NO_3^-/NH_4^+ ratios – supplied plants also presented higher CO_2 assimilation and stomatal conductance, associated with high electron transport rates at the PSII level. Interestingly, these plants supplied with high NO_3^- levels exhibited lower Na^+ accumulation and higher K^+/Na^+ ratios in leaves as compared with those grown under similar NH_4^+ concentrations. Therefore, our data collectively strongly suggest that *Jatropha curcas* plants, a moderately salt sensitive species, is also a NH_4^+-sensitive species. Moreover, high NO_3^- supply is able to minimize both salt and NH_4^+ toxicities provably by indirect mechanisms favoring the overall N nutrition under these adverse environmental conditions. As a central physiological component of these favorable effects is the photosynthetic process involving stomatal conductance, CO_2 assimilation and photochemical efficiency all associated with an effective oxidative protection.

Keywords: Ammonium, *Jatropha curcas*, nitrate, photosynthesis, salinity.

APPENDIX D - MANUSCRIPT IN PREPARATION

TYPES OF MEMORY AND ACCLIMATION TO SALINITY TRIGGERED BY NACL-PRIMING ARE DEPENDENT ON THE DEVELOPMENT PHASE IN RICE (*Oryza sativa* L.)

Allana R. H. Sotero[1], Marcio O. Martins[1], Matheus V. Monteiro[1] and Joaquim A. G. Silveira[1*]

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ABSTRACT

Aims – We tested the hypothesis that NaCl-priming supplied on germination and earlier seedling establishment and vegetative development phases trigger contrasting effects on memory and salinity acclimation associated with growth and ionic homeostasis performance of rice plants.

Methods – Salt priming of 25 mM NaCl for 7 days was applied throughout germination-seedling establishment transition and vegetative V6 phases to evaluate plant memory and salt acclimation in rice plants. Later, at V10 phase, the plants were exposed to 50 mM NaCl for 16 days until middle reproductive period (R4), to evaluate effectiveness of plant memory and salt acclimation.

Results – Single halopriming supplied on germination-seedling establishment and vegetative phases triggered very contrasting memory effects to salinity responses. These effects were accentuated by plant exposure to 50 mM NaCl indicating strong interaction between priming and salt stress. Halopriming supplied throughout the germination- seedling establishment phases triggered a positive memory followed by a great acclimation to avoid further salinity stress. These favorable effects were associated with a more favorable ionic homeostase (K^+/Na^+ ratios) which positively reflected in higher growth of roots, culms, and leaves. Remarkably, the salt priming supplied at vegetative phase induced a negative memory for salt stress acclimation as indicated by a stunted growth and drastic effects on yield indicators, associated with strong disturbances on ionic homeostasis. These results suggest that the acquired negative memory was associated with more dangerous salinity consequences involving combined salt stress effects of salt previous exposure (priming) and further salinity exposure.

Conclusions – The results strongly suggest that salt memory and acclimation to salinity stress is dependent on the plant developmental stage. NaCl-priming supplied throughout the germination and early seedling development triggers positive memory and effective salt-acclimation to a further salinity stressful condition. In contrast, when the halopriming is supplied throughout the vegetative phase it triggers a negative memory and harmful interaction with salt stress in rice plants.

Keywords – Acclimation, *Oryza sativa,* plant memory, salinity, salt stress.

Highlights:

1. Halopriming supplied throughout the germination-seedling establishment triggers favorable effects (positive memory) for rice better handle with salinity
2. In contrast, halopriming supplied throughout the vegetative phase triggers negative memory, drastically aggravating the negative effects of salinity
3. These contrasting effects caused by halopriming were more accentuated on the growth in both vegetative and reproductive growth
4. The positive memory and favorable effects for salinity acclimation are related with satisfactory K/Na homeostasis in roots and leaves

INTRODUCTION

Cultivated plants are commonly exposed to several adverse environmental factors which negatively has induced impacts on food production worldwide, including excess salt ions in soils and coastal regions. Unfortunately, these problems are expected to worsen in the coming years with the advent of climate change, especially rising temperatures, and reduced water availability. On the other hand, the priming techniques, especially from crop seeds, have been widely used for many years as a simple and effective way to induce uniform, improved growth, and higher yields (…………..). Nevertheless, the physiological mechanisms which are underlying to favorable priming effects in plants are virtually unknown (…………….). More recently, the halopriming technique in rice seeds has been introduced to induce salinity stress resistance in some world regions (………………...).

Since the 2000s, this area of plant physiology has gained enormous progress from advances in genetics, multi-omics, improvements in plant memory theories and, most recently, with the introduction of epigenetics to better understand the relationships between priming-memory-stress-acclimation, contributing to non-genetic plant breeding under stressful conditions (………………...). The most current theories on plant memory establish that different types of priming in plants can induce favorable and positive responses (positive memory) and negative responses (negative memory) to further stressful conditions. There is evidence that these types of responses depend on several factors, especially the stage of plant development where the priming agent is applied, the dose (eustress or distress), and the form of exposure, involving different types of cycle/recovery situations, all acting in a spatio-temporal perspective (…………….).

These memory responses to different priming types or environmental oscillating factors involve epigenetics modifications synchronized with molecular, metabolical and physiological changes (………………….). For the first time, the Israeli scientist GN Amzallag established that plants have developed mechanisms he called “developmental windows” which enable them to be differently sensitized by externals environmental factors involving different acclimation responses. Indeed, when sorghum seedlings (5 to 10 days after germination) were exposed (acclimated) to a lower NaCl concentration (150 mM) and further for a high concentration (300 mM), they exhibited a clear resistance to high salinity (Amzallag, 1999). These favorable adaptative responses were dependent on the developmental stages of sorghum plants. Currently, several works have evidenced that these mechanisms involving priming-memory-stress resistance are general for higher plants and several stress types (…………………..).

These epigenetics mechanisms have opened the perspective of a non-genetic improvement since epigenetic traits can be passed on to future generations (………………). In case of salinity, this approach is promising since several crops, especially rice, are largely affected by this problem worldwide and halopriming technique is suitable and reproducible to be performed (………………..).

Recently, has been proposed that the memory-stress-acclimation relationships in plants involve a complex spatio-temporal-multiscale dynamics which are still scarcely understood (……………..). Priming is also an event of stress and memory as a matter of time. At the whole plant scale the different phenological stages (the unfolding of plant development throughout time) can exhibit different dynamics of responses to the same stimulus and, also, different memory effects. It has been shown that the duration and effect of memory can be linked, among other things, to the phenological stage at which the first stress was applied.

MATERIAL AND METHODS

Plant growth and treatment conditions

Rice seeds (*Oryza sativa* spp. Japonica) from a salt-sensitive cultivar (Nipponbare) were sown in Germitest ® paper during 14 days in a growth chamber (150 mmol m^{-2} s^{-1} photosynthetic active radiation – PAR, 25 °C, 60-70% relative humidity and 12h photoperiod). 14-days after sowing, seedlings were transferred to 3-L pots containing ½ diluted Hoaglang-Arnon nutrient solution (Hoagland & Arnon, 1950) and were grown in a greenhouse (average of maximum photosynthetically active radiation – PAR of 800 µmol m^{-2} s^{-1}; average temperature of 30 °C; 12-h photoperiod; and average relative humidity of 75%). During the growth period, the nutrient solution was completely changed every week. A halopriming treatment was applied at two different sets of plants: one at the germination establishment (GH) and the other at the stage V6 (VH). This treatment will be explained in the next section. When reached stage V10, plants were exposed to the following treatments: salt (S), germination establishment halopriming + salt (GHS) and vegetative halopriming + salt (VHS). Salt treatments mean 50 mM NaCl in the nutritive solution for 14 days. For salinity tolerance assay, plants' measurements were carried out once a week (plants' height, leaves and tillers number). At the end of the experiment, photosynthesis and fresh and dry mass were measured, panicles and roots were harvested and oven-dried at 70 °C until constant mass, leaf samples (20 cm of leaf middle section from non-senescent mature leaves) were harvested and frozen in liquid N_2 and stored at -80 °C for biochemical determinations.

Halopriming treatment

Halopriming was performed by exposing plants to a 25 mM NaCl solution for 7 days, applied at two distinct development stages. The germination establishment halopriming (GH) involved incubating seeds in the saline solution for 7 days. Following this period, the primed seedlings were transferred to a new germitest paper moistened with distilled water for an additional 7 days. In contrast, control seeds (C) were incubated exclusively in distilled water for the full 14-day period. For the vegetative halopriming (VH), a 25 mM NaCl solution was added to the nutrient solution when plants reached stage V6 (28 days after sowing) and was completely replaced with a fresh nutrient solution after 7 days.

Salt treatment

Salt stress was performed by exposing plants to a 50 mM NaCl solution for 7 days applied when plants reached V10 stage (42 days after sowing). Three groups of plants were started: salt (S) group, which received no prior priming; germination establishment halopriming + salt (GHS) group; and vegetative phase halopriming + salt (VHS) group. After 14 days, all plants were harvested for subsequent analysis.

Leaf gas exchange measurements

Gas exchange parameters were measured by using a portable infrared gas analyzer system (LI-6400XT, Li-COR, Lincoln, NE, USA) in mature middle leaves (L12). PPFD and temperature inside the measurement chamber were kept at 1000 μmol m^{-2} s^{-1} and 28°C, respectively. The amount of blue light was set up to 10% of the PPFD to maximize stomatal aperture (FLEXAS *et al.*, 2008), CO_2 partial pressure was kept at 40 Pa and the leaf-to-air vapor pressure difference was 1.85. Measurements were recorded when the total coefficient of variation was lower than 5% and temporal stability was achieved (about 3 min).

Determination of Na^+ and K^+ contents in leaves

Na^+ and K^+ contents were determined as described by (Cavalcanti *et al.*, 2004). Dry leaves were finely grinded, and 50 mg samples were extracted with 20 mL of deionized water at 95 °C for 60 min in hermetically closed tubes. After cooling, the extract was filtered, and the determinations were performed in a flame photometer (Micronal mod. B462).

Leaf hydrogen peroxide content, electrolyte leakage and lipid peroxidation

H_2O_2 content determination was performed using Amplex® red kit in accordance with Zhou *et al.* (1997). 150mg of fresh leaf samples were macerated with liquid N_2 in the presence of 1 ml of 100 mM phosphate buffer (ph 7.5), centrifuged at 12,000g (4 °C), and the supernatant was immediately used for H_2O_2 determination. Aliquots of 200 µL of the supernatant were added to a reaction medium consisting of 200 µL of the amplex red solution and incubated for 30 min at 25° C protected from the light. The absorbance was determined at 560 ηm. Blank was prepared in parallel and subtracted from the sample. H_2O_2 content was calculated from a standard curve and results were expressed as µmol H_2O_2 g^{-1} FM.

Membrane damage (MD) was measured by electrolyte leakage as described previously by Blum & Ebercon (1981). Ten leaf segments (3 cm length from the middle part of leaf 11) were placed in test tubes containing 10 mL deionized water. The tubes were incubated in a shaking water bath at 25°C for 24 h and the electric conductivity of the medium (L1) was measured. After that, segments were boiled at 95°C for 60 min, cooled to 25°C and the electric conductivity (L2) was measured. The relative MD was estimated by $MD = L1/L2 \times 100$.

Lipid peroxidation was determined by measuring the thiobarbituric acid-reactive substances (TBARS), according to Cakmak & Horst (1991), with minor modifications as described by Rosa *et al.* (2010). The TBARS content was calculated using the molar extinction coefficient of 155 mM^{-1} cm^{-1} and the results were expressed as ηmol MDA-TBA g^{-1} FM.

Experimental design and statistical analysis

The experiment was arranged in a completely randomized design, with four independent replicates, each one consisting of one pot containing two plants. Statistical analyses were conducted using SigmaPlot version 14.5 (Systat Software, San Jose, CA, USA). Data were analyzed using ANOVA and means were compared by Tukey's test at the 0.05 level of confidence ± standard deviation.

In addition, the data obtained from the experiment were submitted to the multivariate statistical analysis, performed by principal components analysis (PCA) using the metaboanalyst platform (Chong *et al.*, 2018). The data for PCA was previously transformed and range scaled (mean-centered and divided by the range of each variable) to reduce the scale variability between the data.

RESULTS

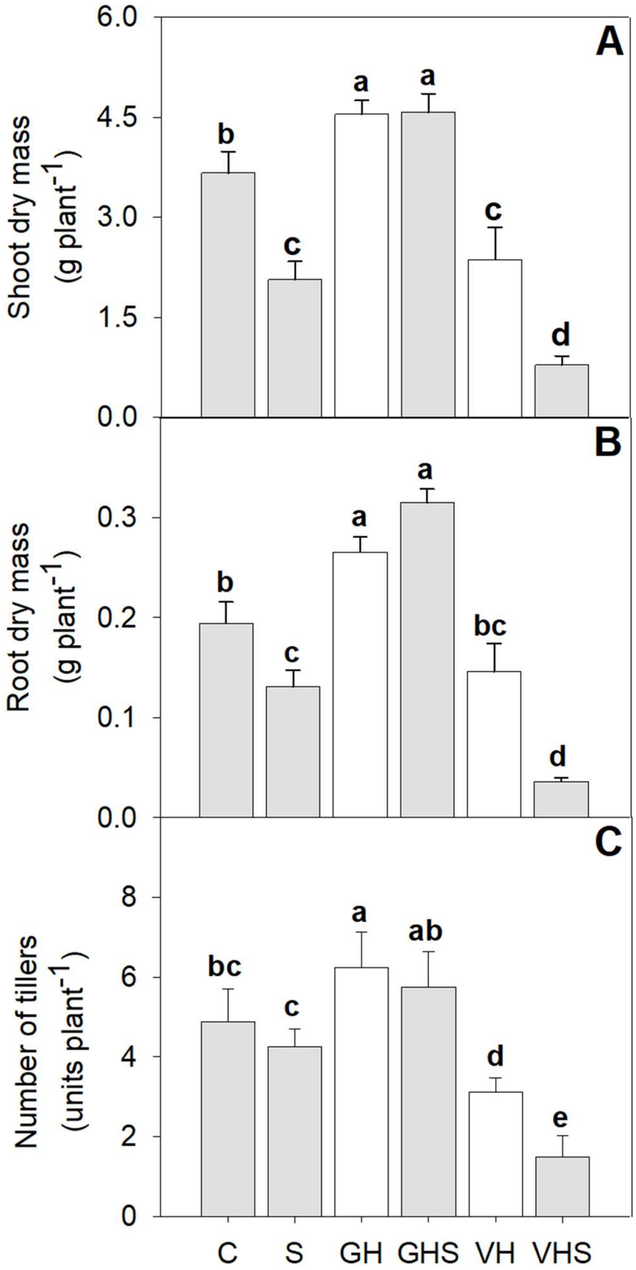

Figure 1. Changes in dry mass of (A) shoots, (B) roots and (C) number of tillers of 56-day-old rice plants exposed to control (C), 50 mM NaCl (S), halopriming (25 mM) throughout germination-seedling establishment phase (GH), halopriming throughout germination-seedling establishment phase followed by salt treatment (GHS), halopriming throughout vegetative phase (VH) and halopriming throughout vegetative phase followed by salt treatment (VHS) for 14 days from the V10 phase. Data represent averages from four replicates ± SD and different letters represent significant difference among treatments by Tukey's test ($p<0.05$).

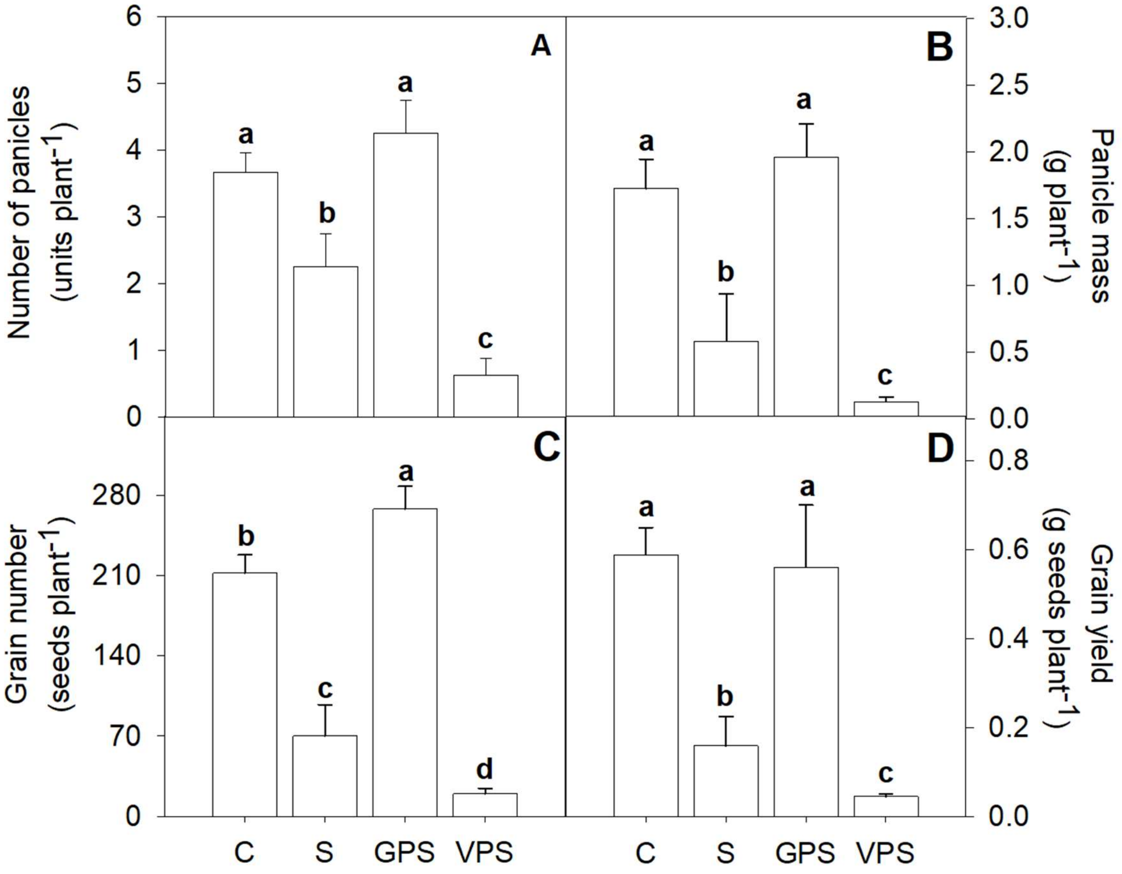

Figure 2. Changes in (A) number of panicles, (B) panicle mass, and (C) mass of 100 seeds of 56-day-old rice plants exposed to control (C), 50 mM NaCl (S), halopriming (25 mM NaCl) throughout germination-seedling establishment phase followed by salt treatment (GHS) and halopriming throughout vegetative phase followed by salt treatment (VHS) for 14 days from the V10 phase. Data represent averages from four replicates ± SD and different letters represent significant difference among treatments by Tukey's test (p<0.05).

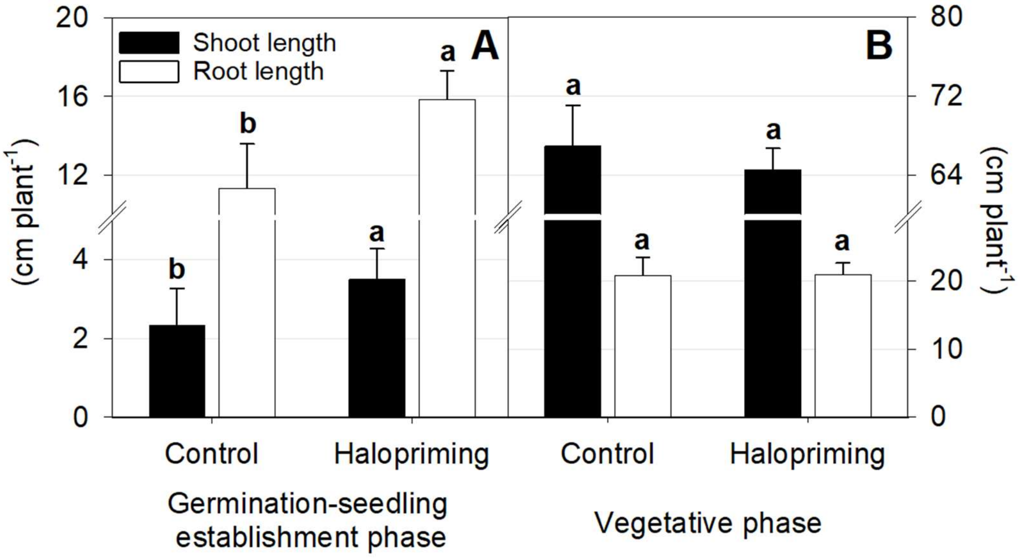

Figure 3. Shoot and root development of rice exposed to halopriming (NaCl 25 mM) for 7 days during (A) germination – seedling establishment and (B) vegetative phase from V6. Seeds and V6 plants suffered previously halopriming as described in M&M and showed in Figure S2.

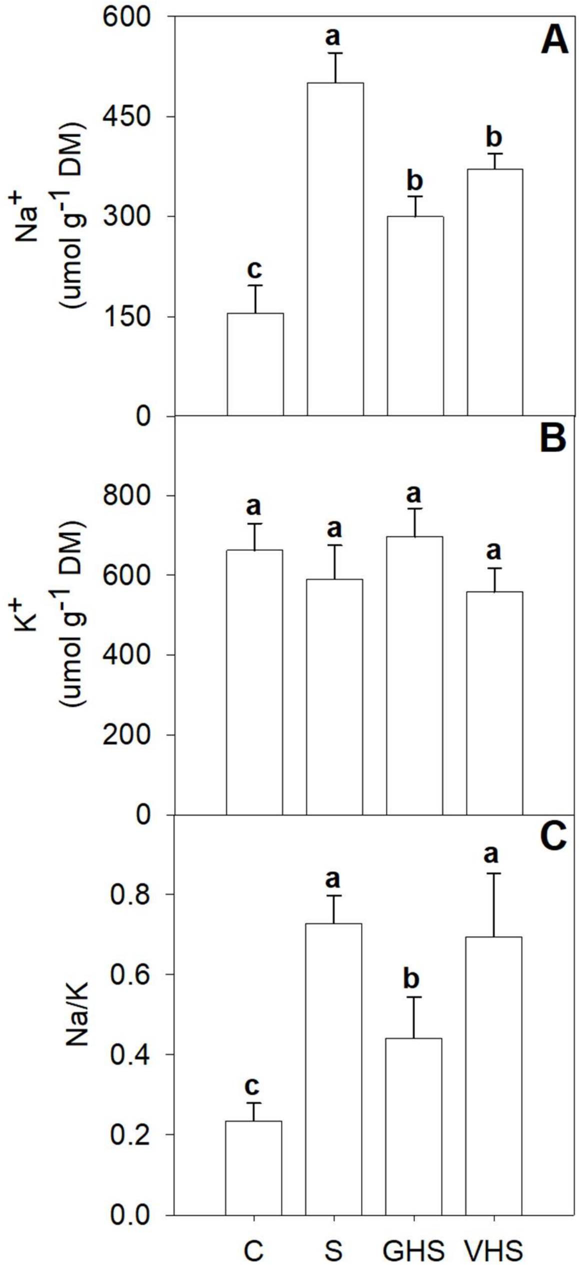

Figure 4. Changes in (A) Na^+ content, (B) K^+ content and (C) Na/K in leaves of 56-day-old rice plants exposed to control (C), 50 mM NaCl (S), halopriming (25 mM NaCl) throughout germination-seedling establishment phase followed by salt treatment (GHS) and halopriming throughout vegetative phase followed by salt treatment (VHS) for 14 days from the V10 phase. Data represent averages from four replicates ± SD and different letters represent significant difference among treatments by Tukey's test ($p<0.05$).

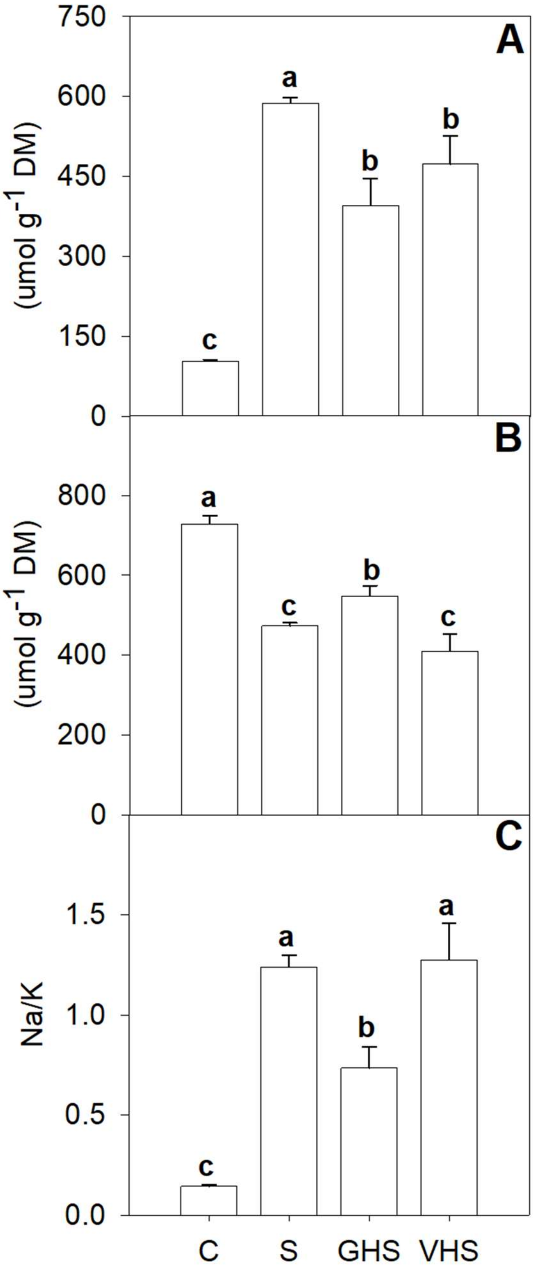

Figure 5. Changes in (A) Na^+ content, (B) K^+ content and (C) Na/K ratios in roots of 56-day-old rice plants exposed to control (C), 50 mM NaCl (S), halopriming (25 mM NaCl) throughout germination-seedling establishment phase followed by salt treatment (GHS) and halopriming throughout vegetative phase followed by salt treatment (VHS) for 14 days from the V10 phase. Data represent averages from four replicates ± SD and different letters represent significant difference among treatments by Tukey's test ($p<0.05$).

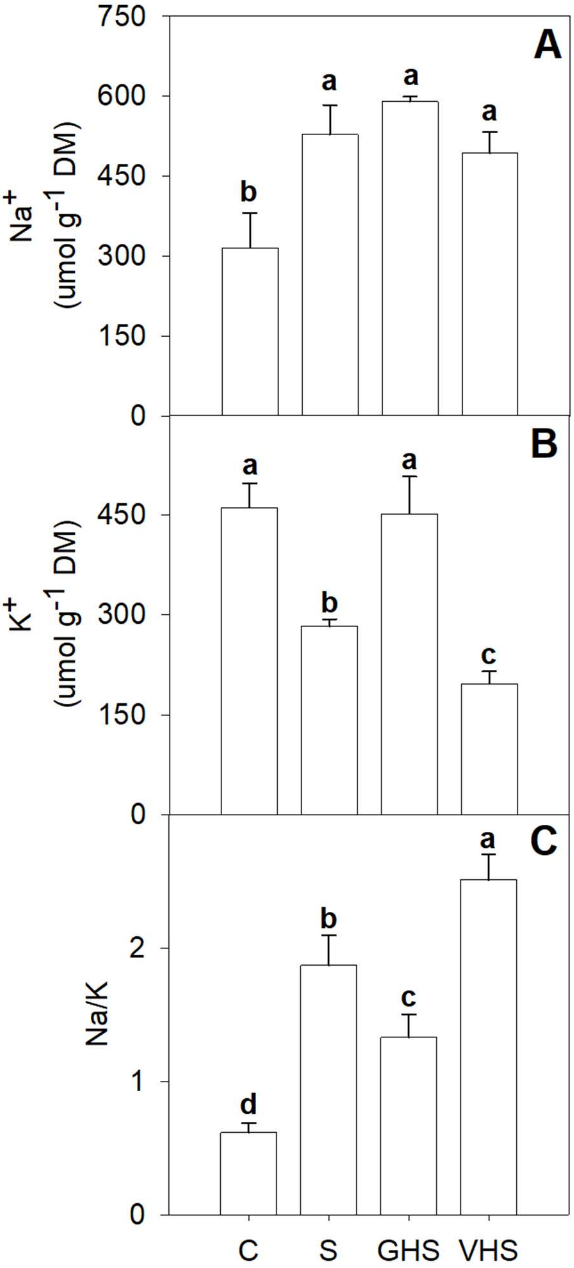

Figure 6. Changes in (A) Na^+ content, (B) K^+ content and (C) Na/K ratios in culms of 56-day-old rice plants exposed to control (C), 50 mM NaCl (S), halopriming (25 mM NaCl) throughout germination-seedling establishment phase followed by salt treatment (GHS) and halopriming throughout vegetative phase followed by salt treatment (VHS) for 14 days from the V10 phase. Data represent averages from four replicates ± SD and different letters represent significant difference among treatments by Tukey's test ($p<0.05$).

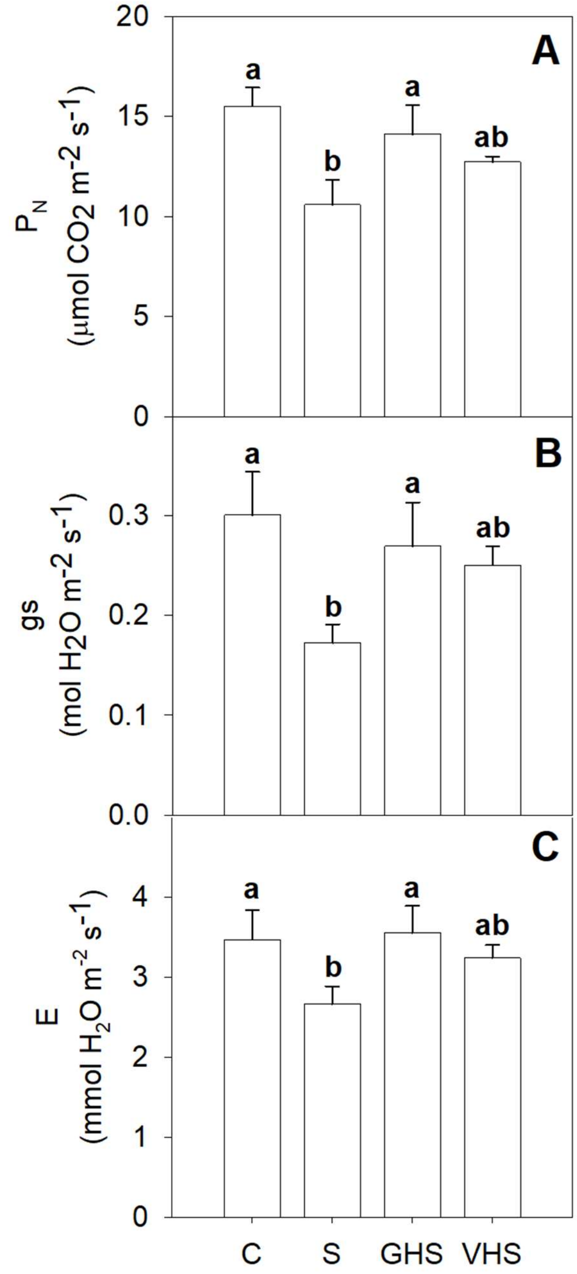

Figure 7. Changes in (A) net CO_2 assimilation (P_N), (B) stomatal conductance (gs) and (C) transpiration (E) in leaves of 56-day-old rice plants exposed to control (C), 50 mM NaCl (S), halopriming (25 mM NaCl) throughout germination-seedling establishment phase followed by salt treatment (GHS) and halopriming throughout vegetative phase followed by salt treatment (VHS) for 14 days from the V10 phase. The gas exchange measurements were performed on the fully expanded L12 leaves. Data represent averages from four replicates ± SD and different letters represent significant difference among treatments by Tukey's test ($p<0.05$).

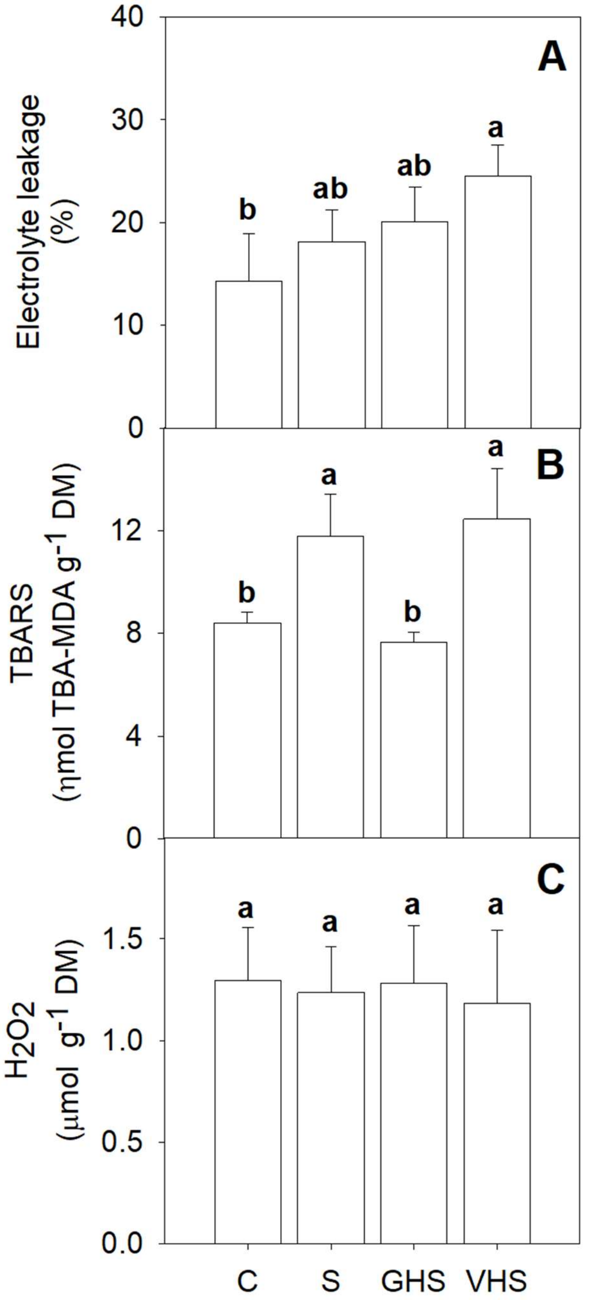

Figure 8. Changes in (A) electrolyte leakage, (B) lipid peroxidation, and (C) H_2O_2 content in leaves of 56-day-old rice plants exposed to control (C), 50 mM NaCl (S), halopriming (25 mM NaCl) throughout germination-seedling establishment phase followed by salt treatment (GHS) and halopriming throughout vegetative phase followed by salt treatment (VHS) for 14 days from the V10 phase. Data represent averages from four replicates ± SD and different letters represent significant difference among treatments by Tukey's test ($p<0.05$).

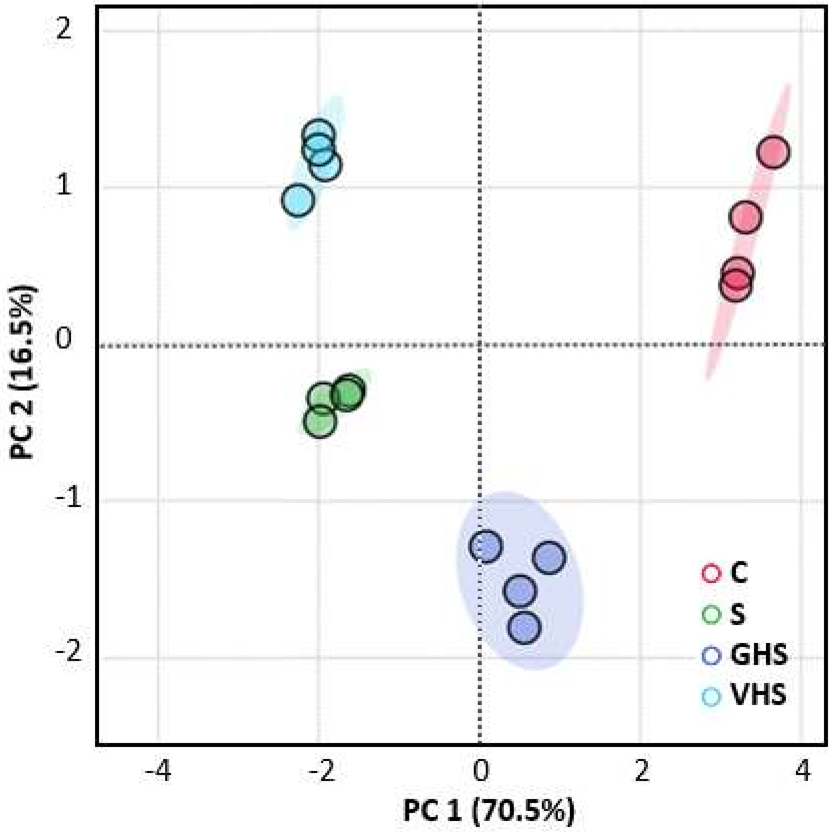

Figure 9. Principal components analysis (PCA) in leaves of rice plants exposed to control (C), 50 mM NaCl (S), halopriming throughout germination-seedling establishment phase followed by salt treatment (GHS) and halopriming throughout vegetative phase followed by salt treatment (VHS) for 14 days from the V10 – R2/R3 phases. PCA was carried out using the Metaboanalyst platform and all measurements performed in the research (Fig. 1 – Fig. 8). PCA reinforce that the four treatments induced very contrasting responses especially between C versus S, C versus VHS, S versus GPS, and GPS versus VPS. In another terms, PCA highlights that halopriming supplied throughout germination-seedling establishment phase has induced positive memory for salt stress whereas halopriming throughout vegetative phase induced a negative memory one in rice plants.

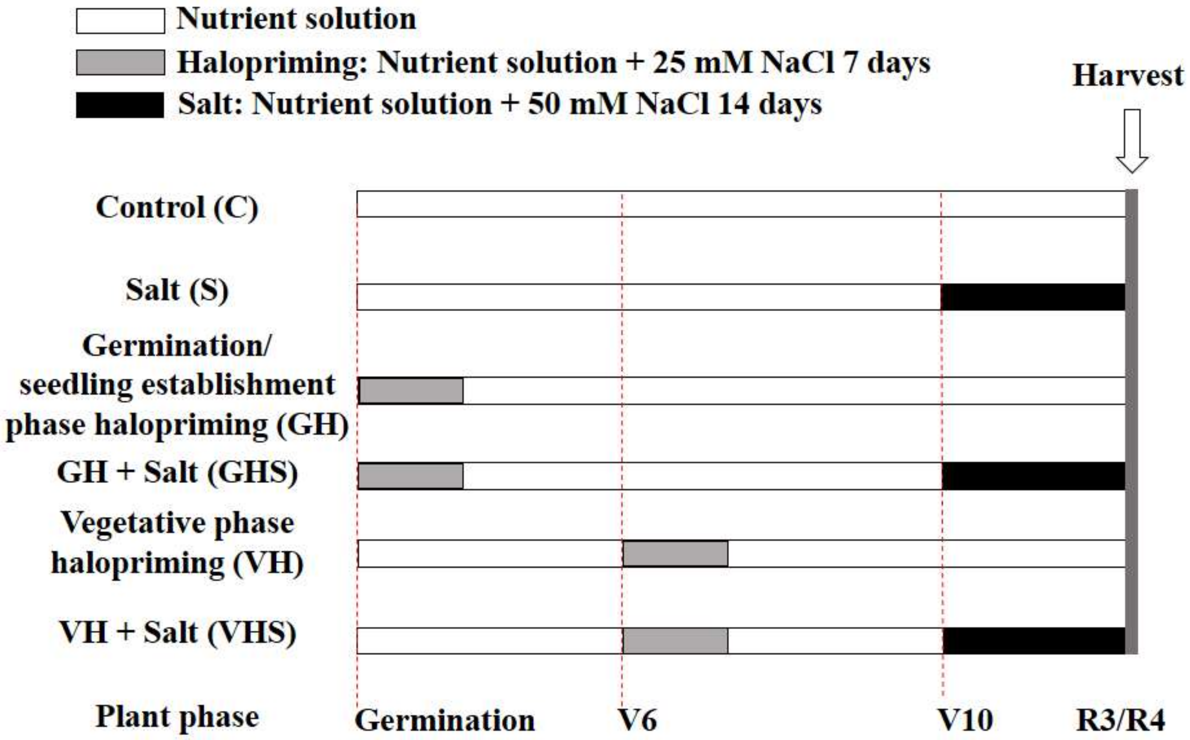

Figure S1. Schematic representation of the halopriming and salt stress treatment schedule in rice plants. This graph details the six experimental treatments arranged in a completely randomized design with four replicates per treatment: Control (salt absence), Salt (50 mM NaCl), Germination – seedling establishment phase halopriming (GH), GH + Salt, Vegetative phase halopriming (VH - initiated at V6), and VH + Salt. Halopriming (indicated by yellow bars) consisted of 7 days at 25 mM NaCl (applied during germination or at V6). The main salt stress (indicated by black bars) was 14 days at 50 mM NaCl, initiated at the V10 stage. The final harvest was conducted at 56 days after sowing.

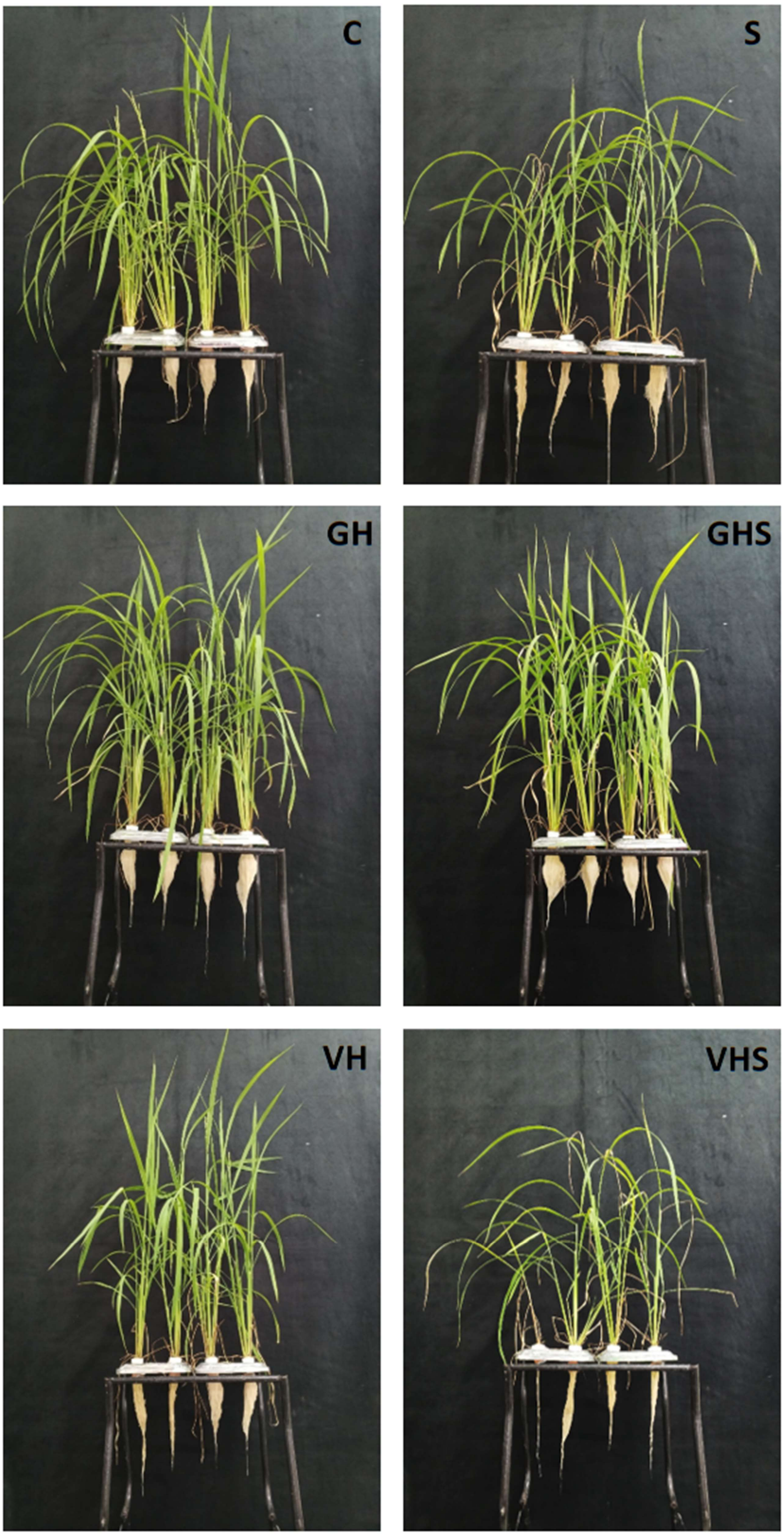

Figure S2. Visual aspects of 56-day-old rice plants exposed to control (C), 50 mM NaCl (S), halopriming (25 mM NaCl) throughout germination-seedling establishment phase (GH), throughout germination-seedling establishment phase followed by salt treatment (GHS), halopriming throughout vegetative phase (VH) and halopriming throughout vegetative phase followed by salt treatment (VHS) for 14 days from the V10 phase.

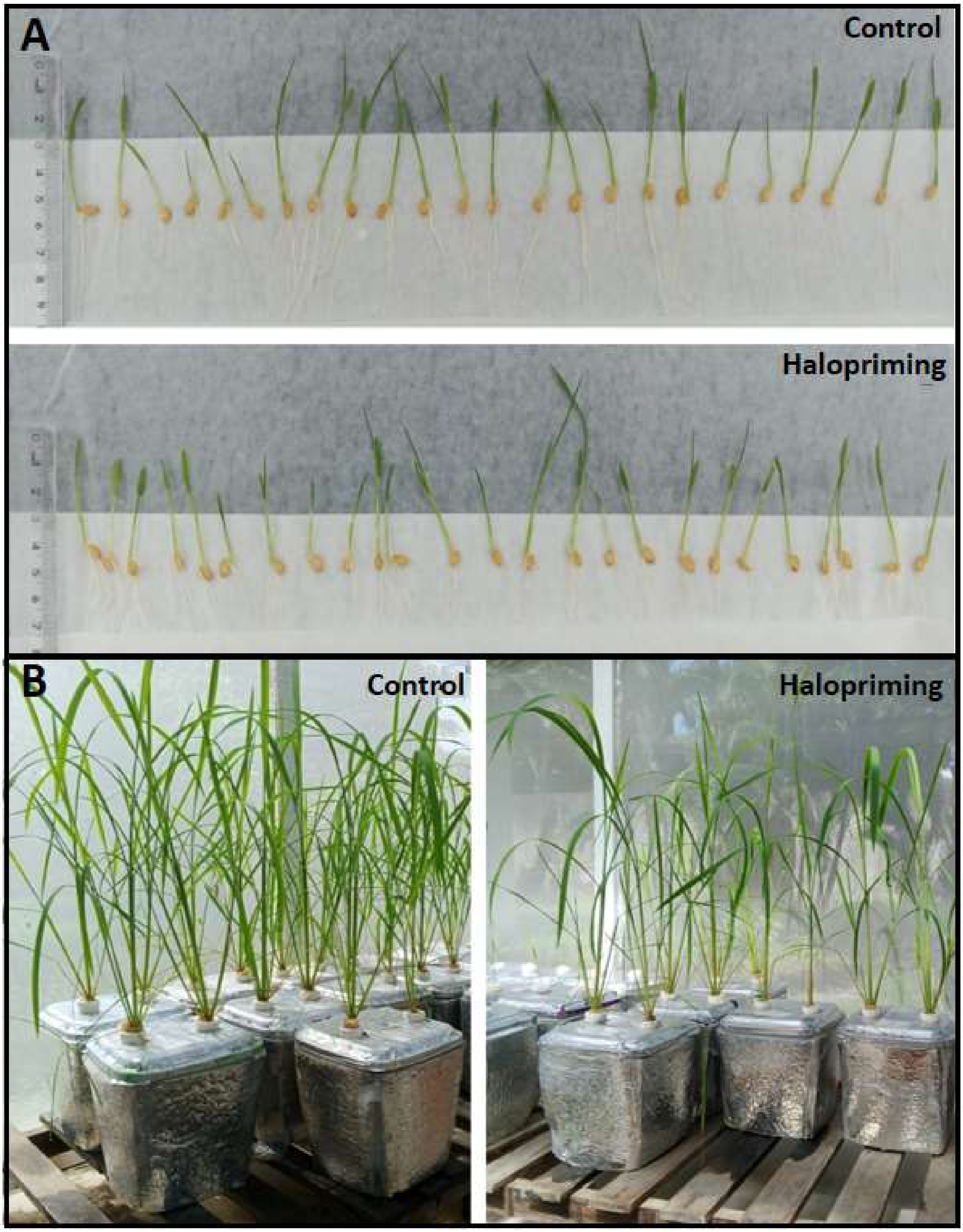

Figure S3. Visual aspects of rice exposed to halopriming (NaCl 25 mM) for 7 days throughout (A) germination-seedling establishment and (B) throughout V6 – V8 phases. Afterwards, these post-germinated primed and control seedlings (A) were transferred to pots containing nutrient solution until the V10 phase whereas (B) post-primed and control plants remained in nutrient solution until V10 phase.

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