UNIVERSIDADE FEDERAL DO CEARÁ
CENTRO DE CIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA DO SOLO

KELLYANE DA ROCHA MENDES

SALT STRESS IS REGULATED BY INTERACTIONS WITH HEAT AND SPATIOTEMPORAL CHANGES IN RICE PLANTS

FORTALEZA
2024

KELLYANE DA ROCHA MENDES

SALT STRESS IS REGULATED BY INTERACTIONS WITH HEAT AND SPATIOTEMPORAL CHANGES IN RICE PLANTS

Tese apresentada ao Programa de Pós-Graduação em Ciência do solo da Universidade Federal do Ceará, como requisito parcial à obtenção do título de doutora em Ciências do solo. Área de concentração: Biologia do solo, adubação e nutrição de plantas.

Orientador: Prof. Dr. Joaquim Albenísio Gomes da Silveira.

FORTALEZA

2024

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Sistema de Bibliotecas
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

M491s Mendes, Kellyane da Rocha.
Salt stress is regulated by interactions whith heat and spatiotemporal changes in rice plants / Kellyane da Rocha Mendes. – 2024.
133 f. : il. color.

Tese (doutorado) – Universidade Federal do Ceará, Centro de Ciências Agrárias, Programa de Pós-Graduação em Ciência do Solo, Fortaleza, 2024.
Orientação: Prof. Dr. Joaquim Albenisio Gomes da Silveira.

1. Heat. 2. intensity stress. 3. Oryza sativa. 4. salinity. 5. spatiotemporal variability. I. Título.
CDD 631.4

KELLYANE DA ROCHA MENDES

SALT STRESS IS REGULATED BY INTERACTIONS WITH HEAT AND SPATIOTEMPORAL CHANGES IN RICE PLANTS

Tese apresentada ao Programa de Pós-Graduação em Ciência do solo da Universidade Federal do Ceará, como requisito parcial à obtenção do título de doutora em Ciências do solo. Área de concentração: Biologia do solo, adubação e nutrição de plantas.

Aprovada em: 18/04/2024.

BANCA EXAMINADORA

Prof. Dr. Joaquim Albenísio Gomes da Silveira (Orientador)
Universidade Federal do Ceará (UFC)

Prof. Dr. Claudivan de Lacerda
Universidade Federal do Ceará (UFC)

Prof.ª Dr.ª Rosilene Mesquita
Universidade Federal do Ceará (UFC)

Prof. Dr. Marcio de Oliveira Martins
Universidade Federal do Ceará (UFC)

Prof. Dr. André Luís Coelho
Universidade Federal do Ceará (UFC)

Aos meus pais Paulino Mendes e Maria Judite,
À minha filha Maitê,
Às minhas irmãs Mirian, Helen Kássia, Kézia e Sheila,
Ao professor Joaquim Albenísio.
Dedico.

AGRADECIMENTOS

A Deus, pela vida, por me conceder oportunidades e me guiar e capacitar para todas as conquistas em minha vida.

A mim, por persistir mesmo durante todas as dificuldades.

A minha família; Aos meus pais Paulino e Judite, por todo o amor e cuidado dedicados, é quem me incentiva e me dar suporte para ir sempre mais longe! Minhas irmãs Mirian, Kássia, Kézia e Sheila, por me amarem e apoiarem, vocês me dão forças na caminhada. Aos meus sobrinhos (as) que alegram e iluminam minha vida.

A minha filha Maitê, que chegou como um furacãozinho, mas também me renovou, me aproximou mais dos verdadeiros amigos, e me deu mais forças para concluir e lutar por nós duas.

Ao Professor Dr. Joaquim Albenísio, pela excelente orientação, e também por todo apoio, humanidade e amizade comigo. Além de um grande mestre com o qual tenho muita honra de conviver e aprender, também se tornou um grande amigo, o qual levarei sempre comigo, com todos os aprendizados acadêmicos e da vida. Muito obrigada por toda ajuda acadêmica, pelos conselhos para a vida e por me apoiar e encorajar. Sem o senhor eu jamais conseguiria.

A toda família Labplant que me trouxe muitos amigos que pretendo levar para a vida inteira, em especial: A Edna Fernandes, muito obrigada por tudo, a amizade e apoio durante toda convivência e ainda nessa reta final me socorrer muitas vezes, abdicando noites e finais de semana para me ajudar. Ao Danny Alex e Rikaelly por todos momentos compartilhados e ajuda nos experimentos e laborátorio. A Allana, Magna, dra. Rachel, Flávio, Raysa, Moaciria, Matheus, Caroline, Eva, Bruno e Humaria, muito obrigada por todo carinho comigo, apoio e convivência, as experiências trocadas dentro e fora do universo acadêmico. Sou muito grata a cada um de vocês pelo aprendizado e convívio desses anos, e mais ainda pela amizade, carinho e palavras de apoio e conforto, principalmente nesses momentos finais, que os mais próximos sabem o quanto precisei e estiveram comigo.

Ao professor Dr. Danilo Daloso, pelo aprendizado no Labplant e contribuição para minha formação e construção dessa tese.

Aos professores participantes da banca examinadora Dra. Rosilene, Dr. Claudivan, Dr. André e Dr. Marcio pelo tempo dedicado e pelas valiosas colaborações e sugestões.

À Instituição Fundação Cearense de apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP), pelo apoio financeiro com a manutenção da bolsa de auxílio.

A Universidade Federal do Ceará por toda estrutura disponibilizada.

Ao PPG Ciências do Solo pela oportunidade, e aos professores do programa por contribuir na minha formação.

Ao secretário Edilson Dote por sua disponibilidade, paciência e carinho nas resoluções durante esse período de doutorado.

Aos motoristas do campus Pici Sr. Joaquim e Sr. Marcos pela amizade e carinho comigo.

A todas as pessoas que estiveram comigo, que acreditaram e acreditam em mim, meu muito obrigada!! Nós conseguimos!!

“Seja forte e corajosa, não temas e nem desanime, pois o Senhor o seu Deus estará com você por onde você andar” (Josué 1:9).

ABSTRACT

This thesis was developed to test the hypotheses that spatiotemporal variation factors (plant parts and developmental stage) and heat interaction differentially affect the effects of salinity on key physiological growth processes in rice plants. In chapter 1, a review study was developed addressing the importance of these themes in plant physiology in the context of rice growth and productivity. In chapter 2, rice plants were challenged with salinity, with 25 mM NaCl at the beginning of the vegetative phase (V4 stage), and 50 mM from V7 until the final reproductive phase of panicle maturation (R9). The objective was to study the interactions between salinity and reproductive phases in spatial variation (plant parts: flag leaves, middle leaves, roots, culms and panicles). The results indicate that the ontogenetic phases of the leaves (age) significantly affect the negative effects of salinity, with older leaves (basal) being more susceptible to the processes of senescence and cell death induced by salinity. Salinity also severely affects rice productivity, including the number of panicles, mass and number of seeds and harvest index. In addition to significantly affecting the quality of seeds indicated by low germination rate and low vigor. These results were associated with Na^+ accumulation and reductions in K^+/Na^+ ratios in these parts of the plant. Total N contents were also differentially affected in response to plant parts and reproductive stages. The oldest and dead leaves showed low fluctuations in total N content in both stages R3 and R7, while the flag leaves showed a high reduction caused by saline stress, especially in R7. Taken together, the data suggest that leaves were intensely affected by saline stress, exhibiting intense senescence associated with protein degradation. Changes in the profile of free amino acids and other metabolites, as well as multivariate analyzes (PCA, principal component analysis) corroborate the results, indicating that the protein degradation process is intensely stimulated in salt stress and that there is an intense variation spatial (different parts of the plant) and temporal (stages of plant development). That is, the metabolite processes and sensitivity to salt stress in terms of rice growth are spatiotemporally dependent. In chapter 3, a manuscript was developed involving the interactions between heat and salinity in rice plants. We tested the hypothesis that heat can stimulate Na^+ transport towards the leaves, aggravating the osmotic and ionic effects. In parallel, the heat-salinity interaction could favor the antioxidant response in rice leaves. Rice plants were previously exposed to 0 and 100 mM NaCl for eight days at 27 °C and subsequently two groups were transferred to high temperature (42 °C) for 10 hours (heat and heat + salt) while two others were transferred to 27

°C (control and salt). The heat-salinity interaction greatly stimulated the accumulation of Na^+ in the leaves, causing an intense decrease in the K^+/Na^+ ratios, inducing significant osmotic and ionic changes. The stomata were closed, causing drastic damage to the assimilation of CO_2 and reducing the efficiency of water use. In contrast, PSII activity was less affected. Unexpectedly, this combined stress partially favors oxidative protection, as indicated by the reduction in H_2O_2 levels and lipid peroxidation associated with the reduction in reduced ascorbate and glutathione contents and increased enzymatic activities. Therefore, high temperature drastically aggravates the negative effects caused by salt stress on photosynthetic efficiency, despite this interaction having partially favored antioxidant defense. Taken together, the work conducted in this thesis reveals that salinity acts on rice plants, and possibly on most species, in an integrative and systemic way, involving multi-environmental and endogenous components, which interact with each other and in a spatial- temporal.

Keywords: heat; intensity stress; *Oryza sativa;* salinity; spatiotemporal variability.

RESUMO

Esta tese foi desenvolvida para testar as hipóteses de que os fatores de variação espaço-temporal (partes da planta e estágio de desenvolvimento) e interação de calor afetam diferencialmente os efeitos da salinidade sobre os processos-chaves fisiológicos e de crescimento em plantas de arroz. No capítulo 1 foi desenvolvido um estudo de revisão abordando a importância desses temas na fisiologia vegetal no contexto do crescimento e produtividade do arroz. No capítulo 2, plantas de arroz foram desafiadas com salinidade, com 25 mM de NaCl no início da fase vegetativa (estágio V4), e 50 mM do V7 até a fase reprodutiva final de maturação das panículas (R9). O objetivo foi estudar as interações entre a salinidade e as fases reprodutivas na variação espacial (partes da planta: folhas bandeira, folhas médias, raiz, colmos e panículas). Os resultados indicam que as fases ontogenéticas das folhas (idade) afetam significativamente os efeitos negativos da salinidade, sendo as folhas mais velhas (basais) mais suscetíveis aos processos de senescência e morte celular induzidos por salinidade. A salinidade também prejudica intensamente a produtividade do arroz, desde o número de panículas, massa e número de sementes e índice de colheita. Além de afetar significativamente a qualidade das sementes indicadas por baixa taxa de germinação e baixo vigor. Esses resultados foram associados ao acúmulo de Na^+ e reduções nas relações K^+/Na^+ nessas partes da planta. Os conteúdos de N total também foram afetados diferencialmente em resposta às partes da planta e aos estágios reprodutivos. As folhas mais velhas e mortas apresentaram baixas oscilações no conteúdo de N-total nos dois estágios R3 e R7, enquanto as folhas bandeira apresentaram alta redução causada pelo estresse salino, especialmente em R7. Em conjunto, os dados sugerem que as folhas foram intensamente afetadas pelo estresse salino, exibindo intensa senescência associada com degradação de proteínas. As alterações no perfil de aminoácidos livres e de outros metabólitos, assim como as análises multivariadas (PCA, análise de componentes principais) corroboram com os resultados, indicando que o processo de degradação de proteínas é intensamente estimulado no estresse salino e que existe uma intensa variação espacial (diferentes partes da planta) e temporal (estágios de desenvolvimento da planta). Ou seja, os processos metabólitos e sensibilidade ao estresse salino em termos de crescimento do arroz são espaço-temporalmente dependentes. No capítulo 3 foi desenvolvido um manuscrito envolvendo as interações entre calor e salinidade em plantas de arroz. Testamos a hipótese de que o calor pode estimular o transporte de Na^+

em direção às folhas, agravando os efeitos osmóticos e iônicos. Paralelamente, a interação calor-salinidade poderia favorecer a resposta antioxidante nas folhas de arroz. Plantas de arroz foram expostas anteriormente a NaCl 0 e 100 mM por oito dias a 27 °C e posteriormente dois grupos foram transferidos para alta temperatura (42 °C) por 10 horas (calor e calor + sal) enquanto outros dois ocorreram a 27 ° C (controle e sal). A interação calor-salinidade estimulou grandemente o acúmulo de Na^+ nas folhas causando intensa diminuição nas relações K^+/Na^+, induzindo significativas alterações osmóticas e iônicas. Os estômatos foram fechados causando prejuízo drástico na assimilação de CO_2 e diminuindo a eficiência do uso da água. Em contraste, a atividade do PSII foi menos afetada. Inesperadamente, esse estresse combinado favorece parcialmente a proteção oxidativa, conforme indicado pela redução nos níveis de H_2O_2 e peroxidação lipídica associada à redução nos conteúdos de ascorbato e glutationa reduzidos e aumento nas atividades enzimáticas. Portanto, a alta temperatura agrava drasticamente os efeitos negativos causados pelo estresse salino na eficiência fotossintética, apesar dessa interação ter favorecido parcialmente a defesa antioxidante. Em conjunto, os trabalhos conduzidos nessa tese, revelam que a salinidade atua em plantas de arroz, e possivelmente na maioria das espécies, de uma forma integrativa e sistêmica, envolvendo multicomponentes ambientais e endógenos, os quais interagem entre si e de uma maneira espaço-temporal.

Palavras-chave: calor; intensidade de estresse; *Oryza sativa*; salinidade; variabilidade espaço-temporal.

LIST OF ABBREVIATIONS AND ACRONYMS

APX	Ascorbate peroxidases
ASA	Reduced ascorbate
CAT	Catalase
Ci	Intercellular CO2 concentration
E	Transpiration rate
EC	Electrical conductivity
ETR	Apparent electron transport rate of PSII
ETR/P_N	Ratios between apparent electron transport rate of PSII and CO2 assimilation
Fo	Initial fluorescence of PSII
Fv/Fm	Maximum potential quantum yield of PSII
GO	Glycolate oxidases
GPOD	Guaiacol peroxidases
GPX	Glutathione peroxidases
GS	Glutamine synthetase
gs	Stomatal conductance
GSH	Reduced glutathione
HI	Harvest index
NPQ	Non-photochemical quenching
P_N/Ci	Instantaneous carboxylation efficiency
qP	Photochemical quenching
1-qP	Fraction of closed PSII reaction centers
PSII	Photosystem II
RWC	Relative water content
SOD	Superoxide dismutases
WUE	Water use efficiency
ΦPSII	Effective quantum yield of PSII

CONTENTS

1 GENERAL INTRODUCTION

Rice (*Oryza sativa*) is widely cultivated throughout the world, covering different regions with differences in climate and soil. Its productivity is highly dependent on several factors, with biotic and abiotic stresses being the most important. The use of rice as a plant model is extremely positive in two crucial aspects: a) biological, given that it is a monocot with a more complex genome than *A. thaliana* and fully sequenced and b) agroeconomic, given its importance as a food source for more than half of the world's population. In tropical regions, this crop faces many problems such as water and mineral deficiencies, high temperature and salinity (NAHAR *et al.*, 2023). These problems are aggravated in semi-arid regions, especially in rainfed crops, where several of these stress factors occur simultaneously (KHORASANINEJAD; ZARE; HEMMATI, 2020).

Salinity and high temperature are very common abiotic stresses (FERREIRA-SILVA *et al.*, 2011; NIKOLIĆ *et al.*, 2023; RIVERO *et al.*, 2014; SUZUKI *et al.*, 2016). Soils affected by salinity represent approximately 20% of the world's total cultivated land area (HUANG *et al.*, 2020), and are distributed in more than 100 countries (PRASERTSUK; WIJITKOSUM, 2021). Excess salts in the soil can cause nutrient deficiencies, thus causing low levels of soil and plant fertility, directly resulting in decreases in crop yield (ZHANG *et al.*, 2021). Excess salts together with high temperature in the soil can cause several biochemical and physiological disorders in plants, of which photosynthesis is one of the most affected processes (NAHAR *et al.*, 2022; SUZUKI *et al.*, 2016).

A good reason for studying salinity combined with high temperature is the extreme interest for the agroeconomic development of the Brazilian northeast region. This region, as well as all tropical-equatorial regions, is subject to an intense input of high temperatures practically throughout the year, which together with high rates of evapotranspiration, low precipitation and irrigation promote an excessive accumulation of salts in the soil (SHRIVASTAVA; KUMAR, 2015). Furthermore, a large part of the region's soils are pedogenetically salinized. Therefore, crop development is constantly challenged by these two stresses in the region, making it necessary to better understand the interrelationships between these abiotic factors, crop yield, photosynthesis and antioxidant metabolism.

In addition to the environmental factors that provoke different responses in plants when exposed to them, within the plant there are several factors that result in changes. Plants are complex living beings, constantly changing, and we can say that they do not present static responses. Currently, one of the biggest difficulties in studying plant metabolism is that the most used techniques are reductionist and only allow us to analyze the event at a steady state moment. Considering plants as open complex systems, they are expected to present different responses to environmental conditions, such as salinity and high temperature, depending on the spatio-temporal scale (NETO *et al.*, 2021). In other words, different phenological stages (temporal change) and different plant organs (spatial) can present different response dynamics to the same stimulus (GALVIZ; SOUZA; LÜTTGE, 2022). Therefore, a study on a spatiotemporal scale contributes to a more realistic understanding of this complexity of plants.

How do spatial and temporal dimensions intensify salinity in rice cultivation? In which parts of the plant and stage is salinity most harmful? How does salinity affect N metabolism and rice yield? How does the salinity + heat interaction act and affect rice plants? How does this interaction differ from isolated stresses? These are important issues that caught the attention of scientists for periods. And although there are some issues already studied there are still many questions to be answered. This thesis aims to help answer these questions adding important information in the assembly of this infinite puzzle called scientific knowledge.

2 OBJECTIVES

2.1 General

The general objective of this thesis is to explore the multifaceted impacts of salinity on plant spatial and temporal variations, vulnerability across stages and organs, changes in metabolic processes, impacts of interactive stresses of salinity and high temperature in rice cultivation, and implications for general performance of the culture. This study seeks to improve our understanding of complex plant responses to environmental stressors, with consequences for improving agricultural resilience and rice production.

2.2 Specifics

1. Determine the most vulnerable parts of the rice plant and growth stages to salinity stress, focusing on identifying the areas and phases where salinity exerts the greatest negative impact.

2. Evaluate the impact of salinity on nitrogen metabolism in rice plants and its consequential effects on crop yield, aiming to elucidate the underlying mechanisms contributing to reduced productivity under saline conditions.

3. Assess the interactive effects of salinity and high temperature on rice plants, comparing the responses to these combined stresses with those observed under isolated conditions, to understand the unique physiological alterations induced by their interaction.

4. Investigate how the interaction between salinity and high temperature affects photosynthesis, antioxidant metabolism, and overall plant performance in rice, providing insights into the complex interplay between abiotic stress factors and plant responses.

5. Contribute to a deeper understanding of the complexity of plant responses to environmental stresses, by carrying out studies considering the interaction of environmental factors and the spatiotemporal scale, with the aim of providing a

more realistic representation of the dynamics of plant stress and improve the base of scientific knowledge in this area.

3 CHAPTER 1: INTERACTION BETWEEN SPATIOTEMPORAL CHANGES AND HEAT AFFECTING SALT STRESS INTENSITY IN RICE PLANTS: A REVIEW

INTRODUCTION

Abiotic factors that may cause environmental stress to plants are constantly associated with reduced crop yields, especially where low technologies exist to control these stresses (JIAO *et al.*, 2021). Among these, salinity is seen as one of the most common environmental stresses, especially in arid and semi-arid regions (KHORASANINEJAD; ZARE; HEMMATI, 2020). Excess salts in the external root growth medium induces an increase in the flow of ions towards the epidermal cells and results in high ionic concentrations. Consequently, this will lead to an increasing influx of saline ions, leading to the establishment of a new ionic homeostasis (Silveira et al., 2016). Salt stress and high ion accumulation alter photosynthesis, respiration and nutrient metabolism (GUILHERME *et al.*, 2019; SILVEIRA *et al.*, 2016).

The salinity effects on plants are complex. Based on plant physiology, different factors are involved in salinity tolerance, such as species-specific traits and also at different scales, from cellular (SILVEIRA *et al.*, 2016). Plants can cope with salt stress conditions through different responses at molecular and cellular levels. The same plant presents diverse and complex physiological, enzymatic and cellular mechanisms under different levels of salinity (AHMADI *et al.*, 2022). However, the plant's response depends on the species, growth stage, period of exposure to saline stress, in addition to specific mechanisms of the cultivars and each cell (PRITTESH *et al.*, 2020).

Among the species, rice is a cereal crop considered sensitive to salt, with electrical conductivity threshold (EC) in the soil of 2 dS m $^{-1}$. EC above threshold mean losses in productivity (HUANG *et al.*, 2020). The intensity of salinity effects is also altered during the growth stages (WEI *et al.*, 2018). The adverse effects of salt stress on grain yield are mainly attributed to osmotic stress, which reduces root water absorption and causes internal dehydration, and the direct excessive accumulation of salts, leading to ionic toxicity that disrupts metabolic processes, particularly in photosynthetic cells (ZHU *et al.*, 2020).

In addition to the different responses that plants present, it is also necessary to consider external factors (ARIF *et al.*, 2020; GEORGIEVA; VASSILEVA, 2023). Plants can be exposed to various combinations of abiotic stress factors that occur sequentially or simultaneously in natural habitats (NAHAR *et al.*, 2023). The interaction between these factors results in unexpected and unpredictable responses when compared to isolated studies ((RADHA *et al.*, 2023). Salinity, in most environments, occurs simultaneously with other stresses, such as high temperature (FERREIRA-SILVA *et al.*, 2011; NIKOLIĆ *et al.*, 2023;

RIVERO *et al.*, 2014; SUZUKI *et al.*, 2016). With the effects of global warming and ongoing climate change, the occurrence and coexistence of these stresses tend to increase. Therefore, understanding the response of plants to simultaneous exposure to heat and salinity becomes essential to promote sustainable agriculture.

Thus, this review aims to address how the effects of salinity on rice plants are modified and/or intensified by:

1- Space-time changes (considering salinity acting on different growth stages of and plant parts), and

2- Combined effects of salinity and heat.

Salt intensity and spatiotemporal throughout rice development

Space and time integration in a plant stress study

In most researches, plants are considered modular organisms in which parts are independents from each other. This is a concept of plants based on a strongly materialist and reductionist view (LÜTTGE, 2021; NETO *et al.*, 2021). However, this vision is not the only way to build consistent and robust plant science. A complementary counterbalance is provided by the perspective of plants as self-organized systems. Plant parts are integrated by complex and coherent signaling systems, allowing the emergence of collective behaviors rather than just localized responses (NETO *et al.*, 2021).

Biotic and abiotic stress responses depend on levels of plant organization, and considering it as a homogeneous event can lead to misinterpretation of the effects of environmental stimuli on the plant as a whole (AULER *et al.*, 2021). Plants are complex systems, organized into multilevel structures, from molecular structures to the entire plant body, from individuals to communities (SOUZA; BERTOLLI; LÜTTGE, 2016). In such systems, non-linear and unpredictable dynamic networks are dominant. Therefore, as plants are self-organized open systems that are in constant interaction with a constantly changing environment, we can state that, indisputably, biological phenomena are spatiotemporal occurrences (GALVIZ; SOUZA; LÜTTGE, 2022).

The plant spatiotemporal response of a species refers to changes in its growth, development and physiological processes in response to external factors, such as environmental stresses (drought, high temperature, salinity and low nutrients availability), or internal, at the cellular and molecular levels. And these responses occur in spatial (different parts of the plant) and temporal (different time scales) dimensions (GALVIZ; SOUZA;

LÜTTGE, 2022; WILHELM; WIRTH, 2015). In plant systems, different levels of regulation can affect each other simultaneously and reciprocally and physiological processes can achieve highly complex non-linear behaviors (KAISER *et al.*, 2015; NETO *et al.*, 2021). Therefore, considering spatiotemporal responses is important to improve the understanding about how plants face their environmental challenges, minimizing stationary and linear responses that do not represent reality.

Spatiotemporal changes during rice plant development under salt stress

During plant development, changes in spatial and temporal responses are evident. The responses of different plant organs are unique and non-stationary, differing from each other. Over time, organs that begin as a drains become a source for younger organs (SOUSA *et al.*, 2021). Leaf age and position is a great example of how space (position) and time (age) affect the responses to different environmental conditions on plants (JAHAN; SHARWOOD; TISSUE, 2023). During its life span, a leaf undergoes several developmental stages (SENEWEERA *et al.*, 2011). Initially, leaf acts as a sink of resources, importing carbon and nitrogen, increasing protein synthesis and cell expansion and accomplishing little net photosynthesis (FIELD, 1987). When fully expanded, leaf photosynthetic capacity typically reaches its maximum and it starts to contribute to the supply of carbon. Mature leaves also present a low level of protein turnover, which remain until internal or external conditions trigger the initiation of senescence (LIM; NAM, 2007).

Leaf senescence can be understood as an integrated response of leaf cells to information about age and other internal and environmental signals (Figure 1). Environmental factors include salinity, high temperature, among others, and internal signals include age, reproductive phase, hormonal level and high salt and solute concentration in general (SOUSA *et al.*, 2021). At this stage, the leaf acts as a source of minerals and biomolecules for the entire plant level, contributing to the maintenance and formation of new organs (JAHAN; SHARWOOD; TISSUE, 2023). Therefore, these leaves present metabolic changes, such as protein degradation, higher levels of ROS and lipid peroxidation, decreased photosynthesis rates and increased membrane permeability (LIU *et al.*, 2021).

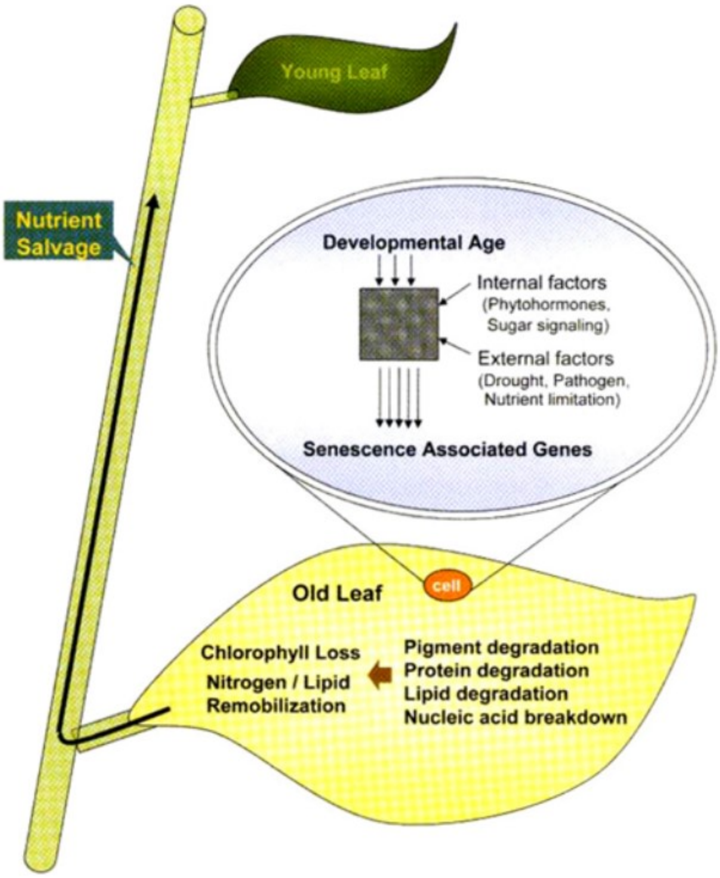


Figure 1: Leaf senescence-related processes (LIM; NAM, 2007).

In addition to variations in responses due to the position and age of plant organs, developmental stages are also examples of spatiotemporal changes in the intensity of the effect of salinity on plants. In rice cultivation, the determining stage for defining productivity is the grain filling phase (WEI *et al.*, 2018). According to Radha *et al* (2023), rice is more resistant to salt during the germination and vegetative phases than in the reproductive phases. In the dynamics of metabolic flows in the plant, strength and sink content linked to the source activity determine the grains filling and quality, which are vital to the productivity of the crop (ZHANG et al., 2021). Abiotic stress' factors, such as salinity, during grain filling, affect the grains quality and calcination, as well as protein and starch composition (RADHA *et al.*, 2023).

Heat and salt stress interaction

Plants often need to deal with several abiotic factors simultaneously, such as the interaction of temperature, drought, radiation, salinity, among others (ARIF *et al.*, 2020; GEORGIEVA; VASSILEVA, 2023; NAHAR *et al.*, 2022; ZHANG *et al.*, 2022). Increased temperature and salinity levels are consistently reported as frequent and simultaneous environmental conditions (NAHAR *et al.*, 2023) and are among the most critical limiting factors for crop production worldwide, especially for plants grown in regions arid and semi-arid (BILAL *et al.*, 2023). As consequences, these impacts can trigger secondary stresses. One of the most common examples of secondary stress in plants is oxidative stress, caused by the overproduction and accumulation of Reactive Oxygen Species (ROS) due to an imbalance in their internal homeostasis. When subjected to these stressors, plants develop complex regulatory networks to detect and react to these stresses efficiently (ZHANG *et al.*, 2022).

Heat stress triggers a series of changes in plants, such as protein denaturation and membrane instability, resulting in oxidative stress and ROS accumulation (GEORGIEVA; VASSILEVA, 2023) . Furthermore, membrane fluidity in plants can trigger the malfunction of membrane-bound ion transporters. Dysfunction of ion transporters due to thermal stress, such as H^+-ATPase or H^+-PPase, can lead to imbalance in ionic and metabolic homeostasis, thus inhibiting plant growth and development (LIU *et al.*, 2021; RIVERO *et al.*, 2014). In rice plants, high temperature stress has a greater impact on grain quality attributes, including reducing the sensory qualities of milled rice. Heat also decreases photosynthesis and photorespiration, decreasing total biomass production (RADHA *et al.*, 2023).

Saline stress, caused by high soil salinity, initially results in osmotic and ionic stress. The osmotic stress leads to reduced water content in the plant, causing dehydration. Ionic stress occurs with the triggering of Na^+ toxicity, which results in disturbances in K^+/Na^+ homeostasis, and changes the plant's metabolism. These changes can increase ROS levels due to impaired enzymatic activity and metabolic processes, resulting in the development of oxidative stress as a secondary stress (LIANG *et al.*, 2018; RUBIO *et al.*, 2020; SAHIN *et al.*, 2018). Furthermore, it causes nutritional imbalances, impairing nutrient absorption and interfering with the activity of ion transporters and antiporters, such as high-affinity K^+ transporters and Na^+/K^+ antiporters.

When plants are individually exposed to heat and salinity they demonstrate deterioration at physiological and biochemical levels, subsequently affecting growth and development (NAHAR *et al.*, 2023). However, responses to multiple stress factors cannot be predicted from single effect responses (NAHAR *et al.*, 2022; NIKOLIĆ *et al.*, 2023; RILLIG *et al.*, 2019). Therefore, adaptation strategies are necessary for the development of agronomic traits that increase plant tolerance to combined stresses (FRANCINI; SEBASTIANI, 2019). As emphasized by Rillig et al. (2019), studies of stress as a single factor continue to be important for discovering mechanisms of plant responses, but global change biology needs to embrace the multiplicity of factors that naturally affect ecosystems.

When combined, the adverse effects of salt stress can be exacerbated with heat stress, as increased transpiration can amplify salt uptake by the upper parts of the plant (SUZUKI *et al.*, 2016). The intensity and extent of simultaneously occurring abiotic stresses can exert a more negative impact on plant physiology than single stresses (SUZUKI *et al.*, 2016). Nahar *et al.* (2022) pointed out detrimental impacts of salt and heat interactions on physiological, biochemical and transcriptional responses associated with stress resistance in rice plants. On the other hand, some plants have greater resistance and can even benefit from the combination of these stresses, when compared to their effect alone (FERREIRA-SILVA *et al.*, 2011). Nikolić et al. (2023) emphasized the specificity of responses under the salinity and heat interaction, and observed that increasing salinity levels negatively impacted the germination and growth of rice seedlings, but higher temperatures partially alleviated these effects.

Concluding remarks and future perspectives

Rice (*Oryza sativa* L.) is the staple food consumed by a large part of the world's population, being cultivated mainly in tropical climates, where water scarcity, high temperatures, salinity and nutrient deficits can significantly reduce productivity. The combination of several abiotic stresses affects plant production, from seedling establishment to biomass and grain production. Exposure to stress factors such as salinity and high temperature during the vegetative and reproductive phase compromise panicle development and grain filling, impacting overall grain production and being a problem for global food security (ZHANG *et al.*, 2021).

Most current studies and research ignore important factors such as the multiplicity of stress factors to which plants are exposed, as well as the changes occurring within the plant in different organs or collection time. The most comprehensive studies considering the systemic responses and complexity of plants as living beings are the answers to get us closer to reality in the field (GALVIZ; SOUZA; LÜTTGE, 2022; RILLIG *et al.*, 2019). This change in the forms of research is essential to initially make us aware of this need for a broader vision and in the future to develop methods that better enable such a range of results. Additional studies involving next-generation sequencing platforms and high-throughput phenotyping could help identify new candidate genes responsible for regulating grain development in situations of combined stress and identify stages more susceptible to stress, for the development of crops more prepared for spatiotemporal changes and climate variations (KILASI *et al.*, 2018; RADHA *et al.*, 2023).

REFERENCES

AHMADI, Fatemeh *et al.* Cell-type-specific H+-ATPase activity and antioxidant enzymes improve the Echinacea purpurea L. Moench tolerance to salinity stress at different NO3-/NH4+ ratios. **Industrial Crops and Products**, [*s. l.*], v. 186, n. June, p. 115199, 2022. Disponível em: https://doi.org/10.1016/j.indcrop.2022.115199.

ARIF, Yamshi *et al.* **Salinity induced physiological and biochemical changes in plants: An omic approach towards salt stress tolerance**. [*S. l.*]: Elsevier Masson SAS, 2020.

AULER, Priscila Ariane *et al.* Stress memory of physiological, biochemical and metabolomic responses in two different rice genotypes under drought stress: The scale matters. **Plant Science**, [*s. l.*], v. 311, n. July, 2021.

BILAL, Saqib *et al.* Efficacy of endophytic SB10 and glycine betaine duo in alleviating phytotoxic impact of combined heat and salinity in Glycine max L. via regulation of redox homeostasis and physiological and molecular responses. **Environmental Pollution**, [*s. l.*], v. 316, n. July 2022, 2023.

FERREIRA-SILVA, Sérgio Luiz *et al.* High temperature positively modulates oxidative protection in salt-stressed cashew plants. **Environmental and Experimental Botany**, [*s. l.*], v. 74, n. 1, p. 162–170, 2011.

FRANCINI, Alessandra; SEBASTIANI, Luca. Abiotic stress effects on performance of horticultural crops. **Horticulturae**, [*s. l.*], v. 5, n. 4, 2019.

GALVIZ, Yutcelia; SOUZA, Gustavo M.; LÜTTGE, Ulrich. The biological concept of stress revisited: relations of stress and memory of plants as a matter of space–time. **Theoretical and Experimental Plant Physiology**, [*s. l.*], v. 34, n. 2, p. 239–264, 2022. Disponível em: https://doi.org/10.1007/s40626-022-00245-1.

GEORGIEVA, Mariyana; VASSILEVA, Valya. **Stress Management in Plants: Examining Provisional and Unique Dose-Dependent Responses**. [*S. l.*]: MDPI, 2023.

GUILHERME, Eliezer A. *et al.* Nitrogen-utilization efficiency during early deficiency after a luxury consumption is improved by sustaining nitrate reductase activity and photosynthesis in cotton plants. **Plant and Soil**, [*s. l.*], v. 443, n. 1–2, p. 185–198, 2019.

HUANG, Jie *et al.* Effects of nitric oxide on nitrogen metabolism and the salt resistance of rice (Oryza sativa L.) seedlings with different salt tolerances. **Plant Physiology and Biochemistry**, [*s. l.*], v. 155, n. November 2019, p. 374–383, 2020. Disponível em: https://doi.org/10.1016/j.plaphy.2020.06.013.

JAHAN, Eisrat; SHARWOOD, Robert Edward; TISSUE, David T. Effects of leaf age during drought and recovery on photosynthesis, mesophyll conductance and leaf anatomy in wheat leaves. **Frontiers in Plant Science**, [*s. l.*], v. 14, n. June, p. 1–15, 2023.

JIAO, Peipei *et al.* Short-term transcriptomic responses of Populus euphratica roots and leaves to drought stress. **Journal of Forestry Research**, [*s. l.*], v. 32, n. 2, p. 841–853, 2021. Disponível em: https://doi.org/10.1007/s11676-020-01123-9.

KAISER, Elias *et al.* Dynamic photosynthesis in different environmental conditions. **Journal of Experimental Botany**, [*s. l.*], v. 66, n. 9, p. 2415–2426, 2015.

KHORASANINEJAD, Sarah; ZARE, Farshad; HEMMATI, Khodayar. Effects of silicon on some phytochemical traits of purple coneflower (Echinacea purpurea L.) under salinity. **Scientia Horticulturae**, [*s. l.*], v. 264, n. 3, 2020.

KILASI, Newton Lwiyiso *et al.* Heat stress tolerance in rice (oryza sativa L.): Identification of quantitative trait loci and candidate genes for seedling growth under heat stress. **Frontiers in Plant Science**, [*s. l.*], v. 871, n. November, p. 1–11, 2018.

LIANG, Wenji *et al.* Plant salt-tolerance mechanism: A review. **Biochemical and Biophysical Research Communications**, [*s. l.*], v. 495, n. 1, p. 286–291, 2018. Disponível em: https://doi.org/10.1016/j.bbrc.2017.11.043.

LIU, Jiahao *et al.* Cerium oxide nanoparticles improve cotton salt tolerance by enabling better ability to maintain cytosolic K+/Na+ ratio. **Journal of Nanobiotechnology**, [*s. l.*], v. 19, n. 1, p. 1–16, 2021.

LÜTTGE, Ulrich. Integrative emergence in contrast to separating modularity in plant biology: views on systems biology with information, signals and memory at scalar levels from molecules to the biosphere. **Theoretical and Experimental Plant Physiology**, [*s. l.*], v. 33, n. 1, p. 1–13, 2021.

NAHAR, Lutfun *et al.* Impact of Single and Combined Salinity and High-Temperature Stresses on Agro-Physiological, Biochemical, and Transcriptional Responses in Rice and Stress-Release. **Plants**, [*s. l.*], v. 11, n. 4, 2022.

NAHAR, Lutfun *et al.* Tolerance with High Yield Potential Is Provided by Lower Na+ Ion Accumulation and Higher Photosynthetic Activity in Tolerant YNU31-2-4 Rice Genotype under Salinity and Multiple Heat and Salinity Stress. **Plants**, [*s. l.*], v. 12, n. 9, p. 1–25, 2023.

NETO, Milton C.Lima *et al.* Understanding photosynthesis in a spatial–temporal multiscale: The need for a systemic view. **Theoretical and Experimental Plant Physiology**, [*s. l.*], v. 33, n. 2, p. 113–124, 2021. Disponível em: https://doi.org/10.1007/s40626-021-00199-w.

NIKOLIĆ, Nebojša *et al.* Effects of the salinity-temperature interaction on seed germination and early seedling development: a comparative study of crop and weed species. **BMC Plant Biology**, [*s. l.*], v. 23, n. 1, p. 1–14, 2023.

PRITTESH, Patel *et al.* Amelioration effect of salt-tolerant plant growth-promoting bacteria on growth and physiological properties of rice (Oryza sativa) under salt-stressed conditions. **Archives of Microbiology**, [*s. l.*], v. 202, n. 9, p. 2419–2428, 2020. Disponível em: https://doi.org/10.1007/s00203-020-01962-4.

PYUNG, Ok Lim; HONG, Gil Nam. Aging and senescence of the leaf organ. **Journal of Plant Biology**, [*s. l.*], v. 50, n. 3, p. 291–300, 2007.

RADHA, Beena *et al.* **Physiological and molecular implications of multiple abiotic stresses on yield and quality of rice**. [*S. l.*]: Frontiers Media S.A., 2023.

RILLIG, Matthias C. *et al.* The role of multiple global change factors in driving soil functions and microbial biodiversity. **Science**, [*s. l.*], v. 366, n. 6467, p. 886–890, 2019.

RIVERO, Rosa M. *et al.* The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. **Plant, Cell and Environment**, [*s. l.*], v. 37, n. 5, p. 1059–1073, 2014.

RUBIO, Francisco *et al.* Doing 'business as usual' comes with a cost: evaluating energy cost of maintaining plant intracellular K+ homeostasis under saline conditions. **New Phytologist**, [*s. l.*], v. 225, n. 3, p. 1097–1104, 2020.

SAHIN, Ustun *et al.* Effects of individual and combined effects of salinity and drought on physiological, nutritional and biochemical properties of cabbage (Brassica oleracea var. capitata). **Scientia Horticulturae**, [*s. l.*], v. 240, n. June, p. 196–204, 2018. Disponível em: https://doi.org/10.1016/j.scienta.2018.06.016.

SENEWEERA, Saman *et al.* New insight into photosynthetic acclimation to elevated CO2: The role of leaf nitrogen and ribulose-1,5-bisphosphate carboxylase/oxygenase content in rice leaves. **Environmental and Experimental Botany**, [*s. l.*], v. 71, n. 2, p. 128–136, 2011. Disponível em: http://dx.doi.org/10.1016/j.envexpbot.2010.11.002.

SILVEIRA, Joaquim A.G. *et al.* Mecanismos biomoleculares envolvidos com a resistência ao estresse salino em plantas. **Manejo da salinidade na agricultura: Estudos básicos e aplicados**, [*s. l.*], v. 1, p. 161–18, 2010.

SOUSA, Rikaely T. *et al.* Ammonium overaccumulation in senescent leaves as a novel exclusion mechanism to avoid toxicity in photosynthetically active rice leaves.

Environmental and Experimental Botany, [*s. l.*], v. 186, n. February, p. 104452, 2021. Disponível em: https://doi.org/10.1016/j.envexpbot.2021.104452.

SOUZA, Gustavo M.; BERTOLLI, Suzana C.; LÜTTGE, Ulrich. Hierarchy and Information in a System Approach to Plant Biology: Explaining the Irreducibility in Plant Ecophysiology. [*s. l.*], p. 167–186, 2016.

SUZUKI, Nobuhiro *et al.* ABA is required for plant acclimation to a combination of salt and heat stress. **PLoS ONE**, [*s. l.*], v. 11, n. 1, p. 1–21, 2016.

WEI, Huanhe *et al.* Sink-source relationship during rice grain filling is associated with grain nitrogen concentration. **Field Crops Research**, [*s. l.*], v. 215, n. May 2017, p. 23–38, 2018. Disponível em: https://doi.org/10.1016/j.fcr.2017.09.029.

WILHELM, Christian; WIRTH, Christian. Physiodiversity - New tools allow physiologist to embrace biodiversity and reconstruct the evolution of “physiologies”? **Journal of Plant Physiology**, [*s. l.*], v. 172, p. 1–3, 2015.

ZHANG, Huiming *et al.* Abiotic stress responses in plants. **Nature Reviews Genetics**, [*s. l.*], v. 23, n. 2, p. 104–119, 2022.

ZHANG, Jing *et al.* Response of grain-filling rate and grain quality of mid-season indica rice to nitrogen application. **Journal of Integrative Agriculture**, [*s. l.*], v. 20, n. 6, p. 1465–1473, 2021. Disponível em: http://dx.doi.org/10.1016/S2095-3119(20)63311-1.

ZHU, Guanglong *et al.* Optimum nitrogen management enhances growth, antioxidant ability and yield performance of rice in saline soil of coastal area of China. **Chilean Journal of Agricultural Research**, [*s. l.*], v. 80, n. 4, p. 629–639, 2020.

4 CHAPTER 2: SALT STRESS INTENSITY IS DEPENDENT ON SPATIO-TEMPORAL-SCALE VARIATIONS THROUGHOUT REPRODUCTIVE PHASE IN RICE

Abstract – The cause–effects relationships induced by salinity throughout the reproductive phase of grass plants involving growth, N metabolism and salt sensitivity in different parts of the plant (spatial variation) are unknown. In this work we use rice as a model to test the hypothesis that salinity supplied at begin of vegetative phase induces differential effects on different parts of rice plants (root, culm, oldest leaves, middle leaves and flag leaves) in terms of salt sensitivity, dry mass accumulation, ionic homeostasis and N metabolism which could be also dependent on the vegetative phases. A high dose of NaCl (50 mM) was supplied in hydroponic medium. In the vegetative phase V4 (25 mM NaCl) and in V7 increased to 50 mM, being maintained until the end of the reproductive phase. Harvests were carried out at stages R3, R5, R7 and R9. Salinity caused several physiological disturbances already during the vegetative phase as indicated visually by reduction in plant size, tiller number, chlorosis followed by death cellular signs on the oldest leaves and chlorotic stains on the flag leaves at begin of reproductive phase. The negative effects caused by salt stress were partially correlated with Na^+ accumulation intensity and reduction in K^+/Na^+ ratios, except to flag leaves and roots, which exhibited lower and higher changes, respectively, but associated with higher and lower disturbances on dry matter accumulation and N metabolism, respectively. At begin of the reproductive phase (R3), salt stress affected drastically almost completely all the oldest leaves (L5-L6-L7-L8-L9) inducing senescence followed by cellular death and necrosis. In parallel, the dry mass of other parts was differently reduced compared to control: flag leaves (59%), culm (55%), middle leaves (43%) and root (34%). At end of the reproductive phase (R7 and R9), these parts were more intensely affected by cumulative salt stress effects highlighting that leaves and culm were more affected than roots. In R9 phase (maturation), the yield indicators were also drastically affected by salt stress as indicated by intense reduction in panicle number, mass, number and size of seeds, harvest index, while the seed number per panicle was not changed. Salinity also affected drastically the N metabolism as indicated by strong changes on GS activity, nitrogenous fractions and metabolomics profiles. These differential spatio-time-scale variations involving diverse parts of the plant and reproductive phases were corroborate by distinct grouping displayed by PCA multivariate analysis.

Keywords: intensity stress, metabolism nitrogen, salinity, spatiotemporal variations, *Oryza sativa.*

INTRODUCTION

The leaf ontogenetic development of leaves in grass species presents a spatiotemporal variation where bottom parts (oldest leaves) and basal portions of leaves are progressively aging from bottom to upper in the canopy and from basal to tip regions (Lee & Masclaux-Daubresse, 2021; Sousa *et al.*, 2021). This process is more evident near to end of the vegetative phase and begin of reproductive development, which can be accelerated in presence of stressful conditions (SOUSA *et al.*, 2021). That spatiotemporal-ontogenetic process involves some mechanisms related to aging such as senescence, cellular death and apoptosis induced or physiologically programed. Indeed, when rice plants are challenged with toxic NH_4^+ concentrations they trigger a rapid senescence localized in the oldest leaves and concentrate on the basal parts as an efficient exclusion mechanism capable to avoid ammonium toxicity in the youngest, preserving their metabolism and physiological activities (POUCET *et al.*, 2021; SOUSA *et al.*, 2021).

Salt stress affects the plant development and growth by a complex way and its effects are multifaceted involving spatiotemporal and multiscale changes which are ontogenetically dependent and require a systemic and emergent approach for a satisfactory comprehension (SILVEIRA; SOUSA, 2024). Several works have reported that the most grass plant species are more salt sensitive during the reproductive e grain filling phases by mechanisms still not completely understood (Alencar *et al.*, 2019; Silveira & Carvalho, 2016). In some of these species or cultivars, during the reproductive phase immense amounts of reserves are mobilized from vegetative parts to the filling grain process (Silveira & Machado, 1990; Zhang *et al.*, 2021). In this course, the leaf senescence process is crucial to mobilize reserves or even sugars and amino acids originate from degradation of essential biomolecules in order to allow grain filling in adverse stressful conditions.

Nitrogen together carbon compounds are quantitatively the most important nutrient source to grain filling in grass species, as under normal conditions as well as in stressful environments like salinity (SOUSA *et al.*, 2021). During “natural” and induced senescence proteins are largely degraded to amino acids which are mobilized from vegetative parts (roots, culms and oldest leaves) to filling grain and protein synthesis (LEE; MASCLAUX-DAUBRESSE, 2021; SILVEIRA *et al.*, 1989). The salt stress effects on the N metabolism involving nitrate and ammonia assimilation, amino acids metabolism, synthesis/degradation of other nitrogenous compounds and synthesis/degradation of proteins are extremely complex and only partially know (SILVEIRA *et al.*, 2003; SILVEIRA; CARVALHO, 2016; VIEGAS; SILVEIRA, 1999). Overall, the salt stress of leaves stimulate protein and chlorophyl degradation and reducing N assimilation, generating as a consequence reduction in the content of total-N and protein content and accumulation of free amino acids and NH_4^+ (DA ROCHA *et al.*, 2012; VIEGAS; SILVEIRA, 1999; VIÉGAS *et al.*, 2003).

In rice the salt stress affects intensely several key physiological processes such as tiller emergence, shoot growth and photosynthesis, which drastically can reduce grain filling and yield (LIMA-MELO *et al.*, 2016). Moreover, salinity also affect N assimilation in leaves decreasing the levels of proteins and stimulating the activity of proteases, inducing accumulation of free amino acids and ammonia (LEE; MASCLAUX-DAUBRESSE, 2021; VIEGAS; SILVEIRA, 1999; VIÉGAS *et al.*, 2003). Additionally, the presence of high levels of ammonia can trigger an early senescence in rice leaves, affecting photosynthesis and grain filling (ALENCAR *et al.*, 2019). However, the differential effects of salinity supplied on the initial vegetative phase of rice plants on different stages of reproductive period and on distinct parts of the plant (spatial variation) in terms of growth and N metabolism are scarcely known.

This work was carried out to test the hypothesis that salinity supplied during initial vegetative phase of rice plants induces differential stress intensity which is dependent on spatial localization (plant modules) and also dependent on the reproductive development stage (ontogenetic – temporal context). Our results corroborate this hypothesis, evidencing that salt stress intensity in terms of Na^+ accumulation, reduction in K^+/Na^+ ratios, relative reduction in dry mass and disturbances in N metabolism (GS activity, contents of total-N, free amino acids, and metabolic profiles) are differently affected in root, culm, oldest leaves, mature leaves, and flag leaves. These responses are also dependent on the reproductive stage, since the initial ones R3 until the seed maturation (R9).

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of rice plants (*Oryza sativa* ssp. japonica cv. Nipponbare) no-transformed plant (NT) were germinated in Germitest® paper under controlled conditions (25 ºC, 70 % relative humidity, 100 µmol photons m^{-2} s^{-1} and 12 h photoperiod). After 11 days of age, they were transplanted into 3.6 L plastic pots filled with ¼ diluted nutrient solution, with concentration increased weekly to $^1/_2$ and undiluted solution (HOAGLAND; ARNON, 1950). The pH of the nutrient solution was adjusted every two days to 6.0 ± 0.5 with 1 M KOH or 1 M HCl and completely changed weekly.

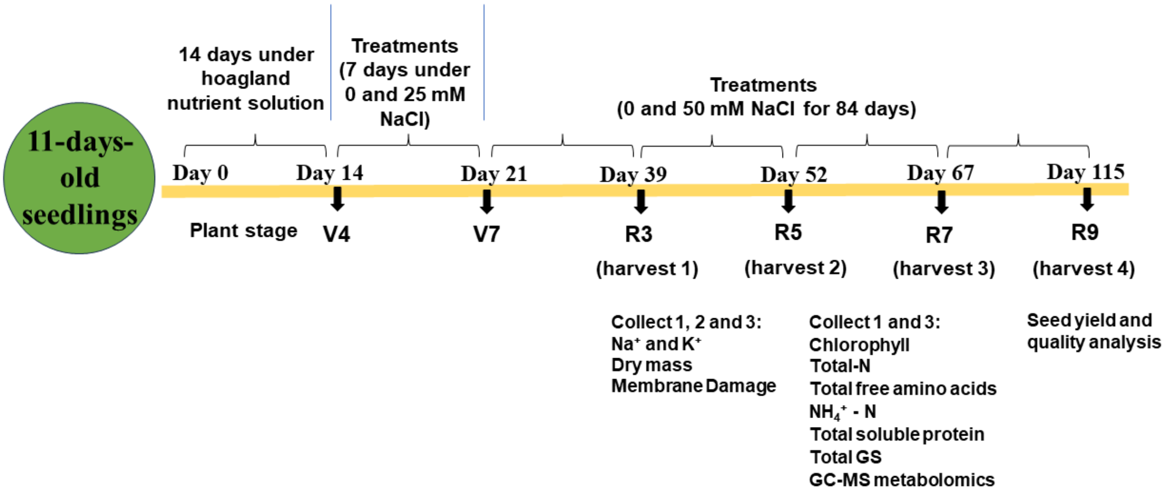


Figure 1. Experimental scheme including time harvest, plant stages, treatments, and variable determinations. Source: Elaborated by the author.

Plants were cultivated in a greenhouse under natural conditions as follow: day/night average temperature of 32/25 °C, mean relative humidity of 65%, maximum photosynthetic photon flux density (PPFD) approximately 800 µmol m^{-2} s^{-1} at noon, and a photoperiod of 12 h. 14 days after transplanting, when the plants reached the V4 stage, the following treatments were started: Control and salt (with 25 mM NaCl). One week later, 21 days after transplanting and at stage V7, the saline concentration was increased to 50 mM and maintained there until the end of production (+ 84 days in total). Plant harvest were carried out at the reproductive stages: R3, R5, R7 and R9 as shown in figure 1, for temporal evaluation.

Reproductive development consists of 10 growth stages based on discrete morphological criteria ranging from R0- Panicle initiation to R9- Complete grain maturation.

The collection stages represent: In R3 - panicle effort (when the panicle begins to stretch out of the formation collar), R5- Expansion of the length and width of the grains, R7- Filling and beginning of grain drying, R8-maturity of at least a single grain and R9-complete maturity of the panicle (COUNCE; KEISLING; MITCHELL, 2000). To spatially evaluate the parts flag leaves (L13), middle leaves (L10, L11 and L12 - fully expanded leaves), dead/senescent leaves (L6, L7, L8 and L9), culms, roots and panicles were separated. Below we are showing the experimental scheme including harvest time and treatments (Figure 1).

Determination of biomass partition and membrane damage

To analyze long-term effects of salt supply on stress indicators, growth, ionic homeostasis, N metabolism and yield throughout reproductive phases, different parts of the rice plants (root, culm, oldest leaves, middle leaves, and flag leaves) were harvested. Plants were collected and separated in different parts, the fresh mass was measured immediately and stored in a freezer at -80 °C for later physiological and biochemical analyzes. Membrane damage (MD) was measured by electrolyte leakage as described previously by Blum & Ebercon (1981). Twenty segments (2.0 cm long) of the leaves (flag, middle and dead/senescent) were placed in test tubes containing 20 mL of 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 $MD = L1/L2 \times 100$.

Determination of Na^+ and K^+ contents

Na^+ and K^+ contents were determined as described by Cavalcanti et al. (2004) The dry mass was 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). Afterwards, the K^+/Na^+ ratio was calculated.

Determination of the contents of total N, ammonium, total free amino acids and soluble proteins

The determination of total nitrogen will be carried out in the presence of concentrated sulfuric acid and under heating (BAETHGEN; ALLEY, 1989). To quantify ammonium and free amino acids contents, samples were incubated with distilled water at 90 °C for 1 h and filtered to obtain a crude extract, ammonium concentration was determined using the phenol-hypochlorite method (FELKER, 1977) and the total free amino acids were measured according to Yemm et al. (1955). The N-forms concentration was expressed g^{-1} DW. The total soluble proteins were measured according to the Bradford’s method (BRADFORD, 1976).

Determination of glutamine synthetase activity (GS) and total chlorophyll content

Fresh leaves were ground until obtaining a fine powder in presence of liquid N_2, 200 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA and 1 mM $MgCl_2$. All extraction stages were carried out at 4 °C. The activity of total GS (EC 6.3.1.2) was determined by the hydroxamate biosynthetic method (HIREL; GADAL, 1980). The assay buffer consisted of 50 mM Tris–HCl buffer, pH 7.8 containing 5 mM ATP, 12.5 mM MgSO4, and 25 mM Na-glutamate. The concentration of the brown complex was determined by measuring the absorbance at 540 ηm. A standard curve was made with γ-glutamyl hydroxamate and the GS activity was expressed as μmol γ-glutamyl hydroxamate (GGH) g FM^{-1} h^{-1}.

To determine the chlorophyll content, 50 mg of fresh rice leaf material was first homogenized with 2 mL of ice-cold 80% acetone. The homogenate was centrifuged at 16,000 g for 5 min at 4 ºC. The supernatant obtained was used for spectrophotometric quantification. of chlorophyll a and b. Absorbance was measured at two wavelengths, 646 and 663. The total content of these pigments was calculated using the proposed equations by Lichtenthaler & Wellburn (1983). Total chlorophyll was expressed in micrograms per grams of fresh mass.

Determination of grain filling and yield indicators and seed vigor

Samples from four random plants were collected at full maturity to determine the harvest index and germination percentage. All plants were separated into vegetative and reproductive (panicles) parts. Plant parts were oven-dried at 65 °C and dry weights were recorded. After drying, the panicles were threshed manually and grain weights were recorded. The harvest index was calculated as the ratio between the grain weight and the total dry weight of the harvest above ground (LI *et al.*, 2012). Seed germination was performed 72 h after sown, under controlled conditions. The formula used to analyze this parameter was: G (%) = (N/A) x 100, where: N = number of germinated seeds; A = total number of seeds sown. Seed vigor was adapted from Mckersie and Tomes (1981). Three replicates containing 100 seeds immersed in 5 mL of deionized water were homogenized at room temperature (25º C). Conductivity (L1) was measured 24 h after homogenization. The vigor of the grains was calculated by dividing the electrical conductivity (L1) of the sample (g). The weight of 100 seeds was determined by weighing the grains, randomly selecting 100 dry seeds from each treatment and recording the weight.

GC-MS-based metabolomics analysis

Extraction, derivatization and gas chromatography coupled to mass spectrometry (GCMS) analysis were performed as previously described (LISEC *et al.*, 2006). Briefly, leaf samples were sprayed with liquid nitrogen, weighed, and divided into 2 mL tubes (50 mg fresh weight per sample). The extraction was carried out by adding 700 µL of pure methanol and 30 µL of ribitol, which is used as an internal quantitative standard. Samples were shaken (10 min, 70°C, 950 rpm) and centrifuged (10 min, 11,000 g). The supernatant was transferred to a tube (2 mL), where 375 µl of cold chloroform and 750 µL of water were added and subsequently centrifuged (15 min, 10,000 g). Then, 150 µL of the upper (polar) phase was transferred to a tube (1.5 mL) and dried in a speed vac. The samples were derivatized by adding 40 µL of methoxamine hydrochloride (20 mg mL^{-1}) dissolved in pure pyridine, shaking (950 rpm, 2 h, 37 °C) and adding 70 µL of N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA). Samples were shaken again for 30 min at 37 °C and then transferred to glass vials for GC-MS analysis. Chromatograms were analyzed using the X-calibur 2.1 software (Thermo Fisher Scientific). Metabolite identification was performed using the Golm Metabolome database (http://gmd.mpimp-golm.mpg.de/) (KOPKA *et al.*, 2005), and also with the help of the MS-DIAL software (TSUGAWA *et al.*, 2015).

Experimental design and statistical analysis

The experiment was arranged in randomized blocks, using two different treatments (control x salt), four harvest (stages R3, R5, R7 and R9) and four replicates. One factorial: 2 Trat x 4 col. x 4 reps. =36 pots. Each biological replicate was represented by a pot containing two plants. For statistical analysis, the means were compared by Tukey test at 5% probability ($p \leq 0.05$) and analyzes were performed using the SigmaPlot 14.0 program (Systat Software, San Jose, USA). Metabolite profiling data were analysed by principal component analysis (PCA) using the *Metaboanalyst* platform (CHONG; WISHART; XIA, 2019). Heat maps were created using the MeV 4.9.0 software.

RESULTS

Long-term salinity induces modular effects on dry mass accumulation throughout reproductive development of rice plants

To investigate the ontogenetic-spatiotemporal effects induced by long-term salt stress on different plant modules (spatial variation), throughout the different phases of the reproductive period (ontogenetic-temporal) of rice plants, the differential dry mass accumulation was evaluated (Table 1 and Fig S1). To archive this, the parts of the plants (root, culm, oldest leaves in advanced senescence - "dead leaves" – a mixture of L5, L6, L7, L8 and L9 leaves, mature and full expanded leaves – "middle leaves" – a mixture of L10 – L11 – L12 leaves, flag leaves – L13 and panicles and grains), were harvested at different reproductive stages (R3, R5, R7 and R9). The dry mass accumulation in salt stressed parts compared with the control, showed significant contrasts in response to part types and development time.

Table 1. Effects of salinity on the dry mass partitioning in different parts of the rice plants in distinct stages of the reproductive development stages R3 and R7. Values are presented in g plant^{-1} and percentage indicates dry matter accumulated in each plant part in comparison to control.

	Plant stage			
Part of the plant	**R3**		**R7**	
	Control	Salt	Control	Salt
Flag Leaves	0.43	0.17 (39%)	0.54	0.15 (28%)
Middle Leaves	0.82	0.46 (57%)	1.08	0.33 (31%)
Dead leaves	0.19	0.34 (173%)	0.11	0.23 (176%)
Culms	2.31	1.05 (45%)	6.97	1.16 (17%)
Roots	0.32	0.21 (66%)	0.62	0.25 (39%)
Shoot	3.76	2.02 (55%)	8.72	1.87 (28%)

(Stage R3= beginning of panicle elongation and R7= grain filling and beginning of drying).

The root was the lesser salt sensitive part displaying reductions of 34% and 61%, at R3 and R7 stages, respectively, compared with shoot parts. The culm exhibited relative decreases of 55% and 83%, respectively in the periods, while flag leaves showed reductions of 61% and 72 and middle leaves exhibited decreases of 43% and 69%, respectively. Interestingly, the oldest leaves (L2-L3-L4-L5) were drastically affected by salt stress, senescing – dying –

drying – falling still during the vegetative phase. In R3 phase (begin of the reproductive period) the other oldest leaves (L5 – L6 – L7 – L8 – L9), which were in advanced senescence induced by salinity, presented drastic reduction in dry mass compared to control, reaching 173% and 176% more dry mass (Table 1). Thus, the oldest leaves triggered a fast and accentuated senescence mechanism induced by salt stress followed by cellular death.

Senescence and dry mass accumulation are partially related to Na^+ accumulation, decrease in the K^+/Na^+ ratios, and membrane damage

The membrane damage in leaves, indicated by the percentage of electrolyte leakage, was drastically affected by salt stress since the vegetative development period. At R3 stage, flag and middle leaves presented values of approximately 40% compared with 10% of control leaves. Interestingly, the dead leaves already showed values of approximately 45% in control and 66% under salt stress conditions, reenforcing that this plant part was most salt sensitive and, additionally, the non-salt treated plants also exhibited signals of physiological disturbances possibly induced by natural senescence process (Figures 2A-C). These adverse physiological effects were more accentuated at the final of the vegetative period (R7) in all analyzed leaf types.

The flag leaves exhibited lower Na^+ accumulation in salt stressed plants during all reproductive period R3-R5-R7 compared with root, culm, middle leaves and dead leaves (Figure 3A-D and S3A). Moreover, the dead leaves exhibited a great Na^+ accumulation, reaching values near to 1,600 µmol g^{-1} DW compared to 250 µmol g^{-1} DW in flag leaves, indicating that this toxic cation is more intensely accumulated on oldest leaves and relatively excluded from the youngest ones. Interestingly, the trend of K^+/Na^+ ratios inversely accompanied the Na^+ accumulation in different plant parts and in distinct reproductive phases since the K^+ content was slightly decreased in response to increased Na^+ content (Figures 4A-D and S2A-D). The panicles exhibited increasing Na^+ contents from R3 (approximately 200 µmol g^{-1} DW) to R7 (400 µmol g^{-1} DW), but an increment also occurred in control plants and the values reached at R7 stage did not were so high as indicated by the values reached by K^+/Na^+ ratios in comparison with other plant parts. Thus, interestingly, the flag leaves displayed lower Na^+ content and lower reduction in K^+/Na^+ ratios induced which were positively related to membrane integrity but not dry mass reduction, compared with the other plant parts.

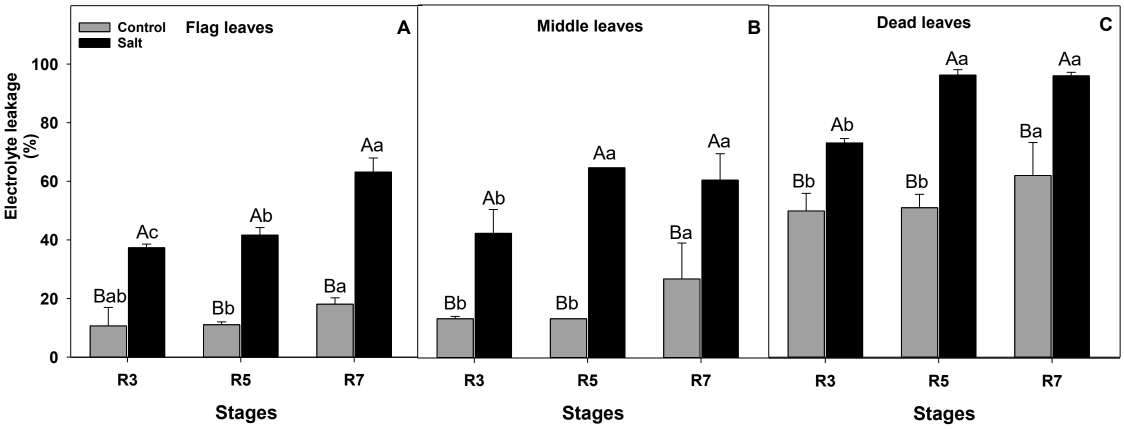


Figure 2. Changes in electrolytes leakage (%) in different leaves of rice plants exposed salt and control and treatments in distinct phases of reproductive development. (A) flag leaves, (B) middle leaves, and (C) dead leaves. Different capital letters represent significant differences among treatments within development stage and different lowercase letters indicate significant differences within treatment at different stages. Data are means of tree replicates and the averages were compared by Tukey's test ($p \leq 0.05$).

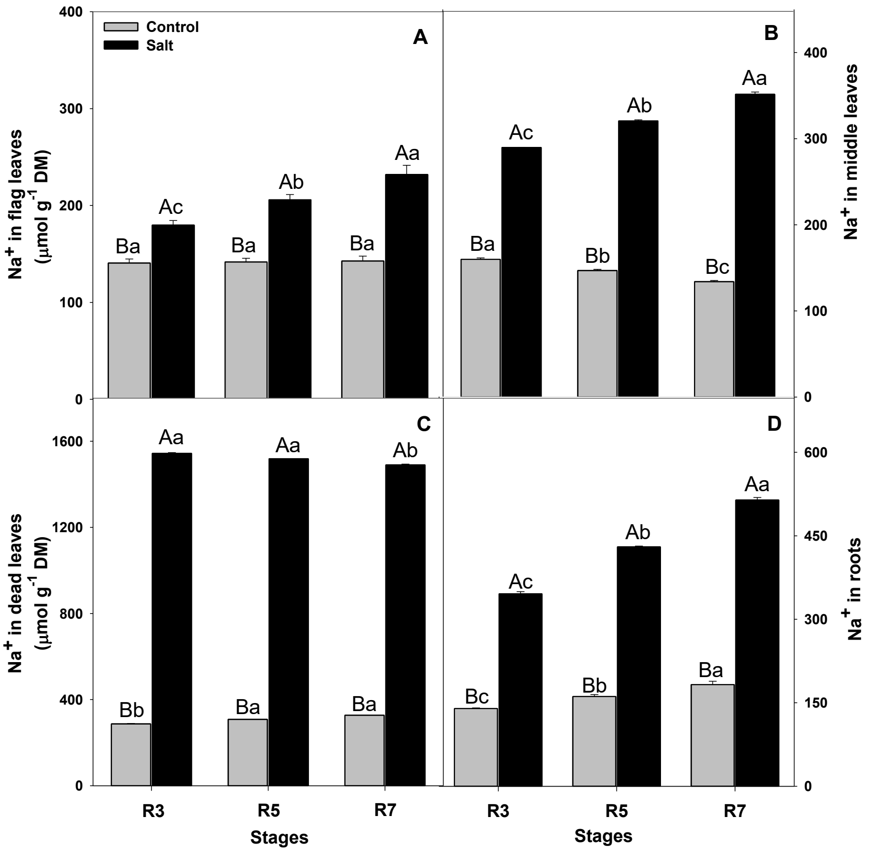


Figure 3. Changes in Na^+ accumulation in different parts of rice plants exposed salt and control and treatments in distinct phases of reproductive development. (A) flag leaves, (B) middle leaves, (C) dead leaves, and (D) roots. Different capital letters represent significant differences among treatments within development stage and different lowercase letters indicate significant differences within treatment at different stages. Data are means of tree replicates and the averages were compared by Tukey's test ($p \leq 0.05$).

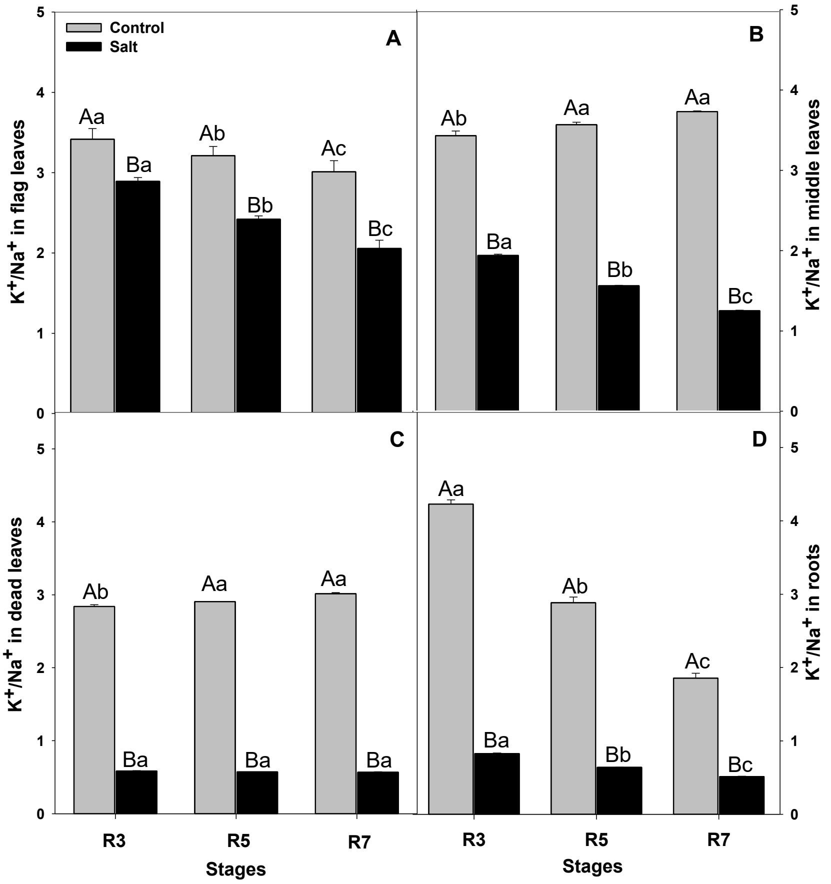


Figure 4. Changes in K^+/Na^+ ratios in different parts of rice plants exposed salt and control and treatments in distinct phases of reproductive development. (A) flag leaves, (B) middle leaves, (C) dead leaves, and (D) roots. Different capital letters represent significant differences among treatments within development stage and different lowercase letters indicate significant differences within treatment at different stages. Data are means of tree replicates and the averages were compared by Tukey's test ($p \leq 0.05$).

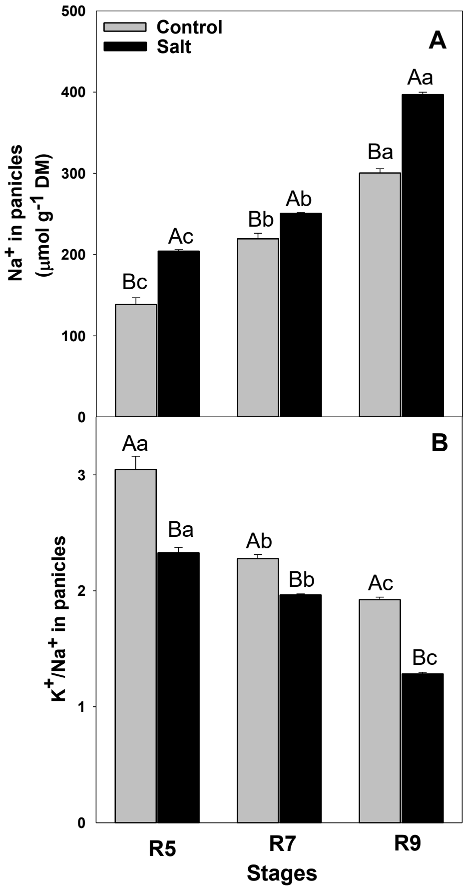


Figure 5. Changes in (A) Na^+ accumulation and (B) K^+/Na^+ ratios in panicles of rice plants exposed salt and control and treatments in distinct phases of reproductive development. Different capital letters represent significant differences among treatments within development stage and different lowercase letters indicate significant differences within treatment at different stages. Data are means of tree replicates and the averages were compared by Tukey's test ($p \leq 0.05$).

Disturbances in accumulation of total-N, free amino acids, ammonium and GS activity in salt stressed plants are intensely dependent on development stage and plant parts

In order to understand the intensity of disturbances on N metabolism induced by salt stress in different parts of rice plants, in response to progressive reproductive stages, we evaluated firstly changes on the contents of total-N, free amino acids and ammonium. Unexpectedly, it is possible to note that the salt-induced disturbances on these nitrogenous fractions in some organizational modules (parts of the plant) were more intense at R3 stage than in R7 ones (Table 2). Indeed, in R3 the content of total-N on the “non-senescing” leaves (flag and middle leaves) was lesser affected by salt stress whereas in dead leaves and roots the content was reduced 34% and 46%, respectively. In contrast, at R7 stage, the flag and middle leaves exhibited an intense decrease of 40% and 19%, respectively whereas dead leaves and roots showed a discrete reduction of 4% and -1%, respectively. These results indicate at R7 stage in salt stressed plants these last parts are relatively stabilized in terms of N mobilization to other sink parts.

The total free amino acids fraction, which during the reproductive period are produced in vegetative parts are essentially originated from protein degradation assuming that inorganic-N assimilation in that period is restrict and senescence-induced protein hydrolysis is stimulate by salt stress. Indeed, flag and middle leaves of salt stressed plants, which act as N source parts, exhibited levels of free amino acids exceptionally increased at R3 stage, by 480% and 247%, respectively (Table 2). In this same stage, dead leaves and roots showed increases of 117% and 138%, respectively, in comparison to control. This result indicates that at R3 stage flag and middle leaves are most important sources to amino acids exportation and that possibly the dead leaves could have lost that trait during some period of vegetative phase.

At R7 phase the flag and middle leaves of salt stressed plants displayed increases in free amino acids of 242% and 109%, respectively, indicating that flag leaves acted as a most N-source even in that advanced reproductive phase. In that same phase, dead leaves and roots presented 184% and 115% in relation to control, evidencing that senescent-dead leaves presented a high accumulation of free amino acids in the N-total pool. Differently of the free amino acids content, NH_4^+ accumulation occurred in a minor extension in flag and middle leaves of salt stressed plants at R3 phase: 114% and 141%, respectively whereas dead leaves and roots reached 83% and 148%, respectively. (Table 2).

Table 2. Effect of salinity on the contents of total-N, total free amino acids and ammonium in different parts of the rice plants in distinct stages of the reproductive development stages R3 and R7. Values are presented in μmol g^{-1} DM and percentage indicates the accumulated in each plant part in comparison to control. Different capital letters represent significant differences among treatments within development stage and different lowercase letters indicate significant differences within treatment in different parts of the plant. Data are means of tree replicates and the averages were compared by Tukey's test ($p \leq 0.05$).

Part of the plant	**R3**		**R7**	
	Control	**Salt**	**Control**	**Salt**
Total-N (µmol g^{-1} DM)				
Flag leaves	2961.67 Ab	2667.49 Bb (90%)	3629.37 Aa	2184.89 Bb (60%)
Middle leaves	2536.92 Ac	2758.39 Ba (109%)	2363.37 Ac	1925.43 Bc (81%)
Dead leaves	3586.39 Aa	2366.69 Bc (66%)	2717.07 Ab	2601.38 Ba (96%)
Culms	1694.04 Ad	1212.4 Be (71%)	1413.07 Ae	1303.99 Be (92%)
Roots	1646.00 Ad	1427.95 Bd (54%)	1659.33 Ad	1669.24 Bd (101%)
Total free amino acids (µmol g^{-1} DM)				
Flag leaves	50.43 Bd	242.28 Ac (480%)	177.94 Be	431.23 Ac (242%)
Middle leaves	88.10 Bc	217.35 Ad (247%)	334.43 Bc	365.73 Ad (109%)
Dead leaves	289.27 Ba	338.10 Ab (117%)	304.78 Bd	559.86 Aa (184%)
Culms	129.25 Bb	222.86 Ad (172%)	478.18 Aa	264.88 Be (55%)
Roots	283.43 Ba	392.39 Aa (138%)	420.79 Bb	484.26 Ab (115%)
Ammonium (µmol g^{-1} DM)				
Flag leaves	21.51 Bc	24.44 Ad (114%)	32.24 Bc	46.29 Ab (144%)
Middle leaves	21.54 Bc	30.24 Ac (141%)	32.37 Bc	37.03 Ac (114%)
Dead leaves	60.68 Aa	50.42 Ba (83%)	50.69 Aa	48.89 Ab (96%)
Culms	17.98 Bd	45.83 Ab (254%)	39.9 Ab	30.37 Bd (76%)
Roots	27.97 Bb	41.36 Ab (148%)	52.32 Aa	55.65 Aa (106%)

(Stage R3= beginning of panicle elongation and R7= grain filling and beginning of drying).

Interestingly, at the R7 stage, inversely to observed for accumulation of free amino acids, flag leaves exhibited higher NH_4^+ content (144%) than middle leaves (114%), in relation to control. Differently of the R3 stage, dead leaves and roots practically did not change their NH_4^+ contents in R7 stage. On the other hand, the culm exhibited a salt stress response distinct from all other plant parts. Indeed, at R3 stage, salt stressed plants showed a total-N content decreased in 29% compared to control whereas in R7 stage the reduction was of 9%. In the same periods, the total free amino acids content changed from 172% and 55% (a decrease of 45%), respectively, in comparison to control. The high amino acids accumulation in R3 stage was positively related to NH_4^+ content which was increased to 254% and further decreased 76% in R7 stage compared to control (Table 2).

During the reproductive phase the effects of salt stress on the contents of nitrogenous fractions were oscillating (Figure 6). At the R5 phase, total-N decreased, total free amino acids did not significantly change and NH_4^+ content decreased almost 2-fold all in comparison to control. In R7 phase, total-N of control was decreased in relation to R5 but in salt stressed the N levels were maintained unchanged. In maturation phase (R9), the N-total content of salt stressed plants and control were similar. In the same R5 and R7 periods, the total free amino acids content of control and salt stressed plants were slightly different but at R9 that N fraction decreased more than 2-fold by effect of salt stress compared to control. The response of NH_4^+ content was highly oscillating, decreasing for more than 2-fold in panicles of salt stressed plants at R5 and R7 stages in relation to control but, interestingly, was strongly increased at R9 stage (Figure 6).

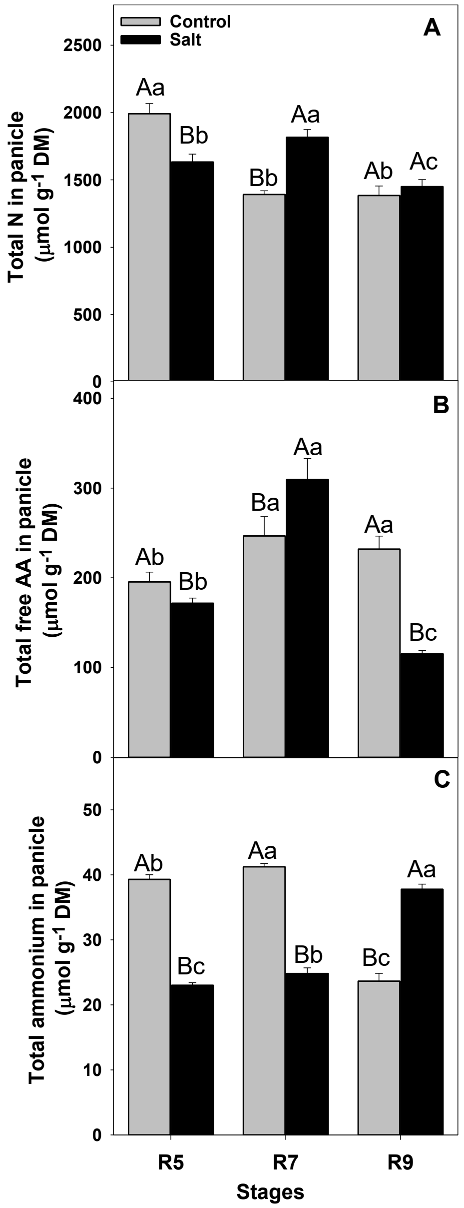


Figure 6. Effect of salinity on the contents of total-N, total free amino acids and ammonium in panicles of the rice plants in distinct stages of the reproductive development stages (R5, R7 e R9). Different capital letters represent significant differences among treatments within development stage and different lowercase letters indicate significant differences within treatment at different stages. Data are means of tree replicates and the averages were compared by Tukey's test ($p \leq 0.05$).

The GS activity in flag leaves of salt stressed plants did not changed at R3 phase but was increased by approximately two-fold at R7 stage whereas differently of dead leaves it was strongly decreased in R3 stage by salt effect and decreased in control at R7 stage. Similar to dead leaves, in roots GS activity was drastically decreased in roots at R3 stage and displaying low activities in non-stressed roots (Figure 7A-C). Thus, the GS activity was not well correlated with the levels of free amino acids and ammonium in all parts of the plants involving the two contrasting reproductive stages R3 and R7.

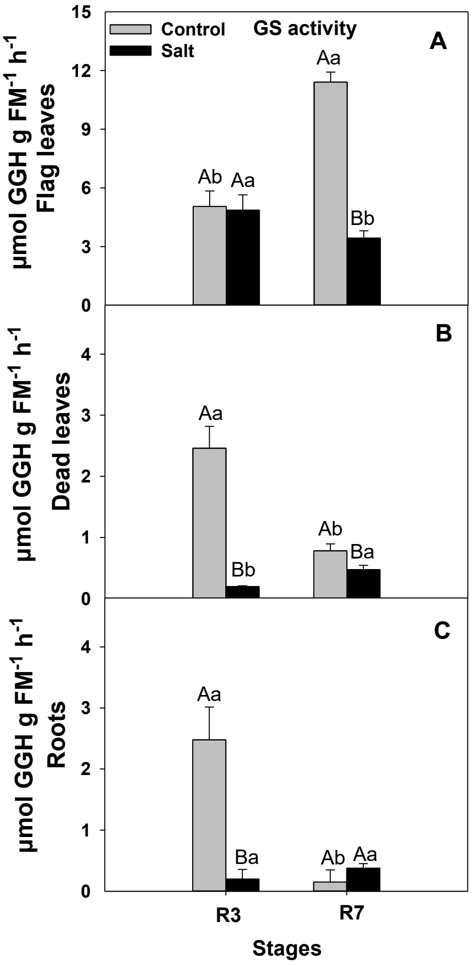


Figure 7. Glutamine synthetase activity in different parts of rice plants exposed to control and salinity. (A) flag leaves, (B) dead leaves and (C) roots in two reproductive phases. Different capital letters represent significant differences among treatments within development stage and different lowercase letters indicate significant differences within treatment at different stages. Data are means of tree replicates and the averages were compared by Tukey's test ($p \leq 0.05$).

Metabolomic and multivariate analyses reinforce that salt stress effects are dependent on ontogenetic, plant modules and observation scale (spatio-time-scale)

Our GC-MS-based metabolite profiling analysis revealed that the effect of salt stress was mainly observed on flag and middle leaves, rather than in dead leaves and roots, as evidenced by the clear separation of control and salt treatments in these organs by the PC1 of the principal component analysis (PCA) from both stages R3 and R7, with a greater separation at the R7 stage (Figures 8A-B, 9A-B). These results are in agreement of the total free amino acids data (Table 2). By contrast, the effect of salt stress on the metabolism of dead leaves, culms and roots was stage-dependent, in which salt stress mainly affected most intensely dead leaves at the R3 stage and culms and roots at the R7 stage, as demonstrated by the separations by the PC1 in these organs/stages (Figures 8C-D, 9C-D; Figure S5). Similarly, no difference was observed between control and salt stress in panicles by PCA (Figure S5).

Comparing the organs/part of the plant, PCA clearly distinguished source and sink tissues at the R3 stage, in which flag and middle leaves formed a separated cluster from culms and roots (Figure 10A). However, culms were surprisingly clustered with the source tissues (flag and middle leaves) at the R3 stage under salt stress, suggesting a transition from sink to source induced by the stress (Figure 10B). These responses seem to be dependent on the developmental stage, since this was not observed at the R7 stage, in which no clear separation among leaves, panicles, culms and roots was observed (Figure 10C-D). Interestingly, dead leaves were not clustered with any of these organs and were separated from them by the PC1 under control or salt stress at both R3 and R7 stages (Figures 10A-D), suggesting that dead leaves have a particular metabolism that do not resemble sink or source tissues.

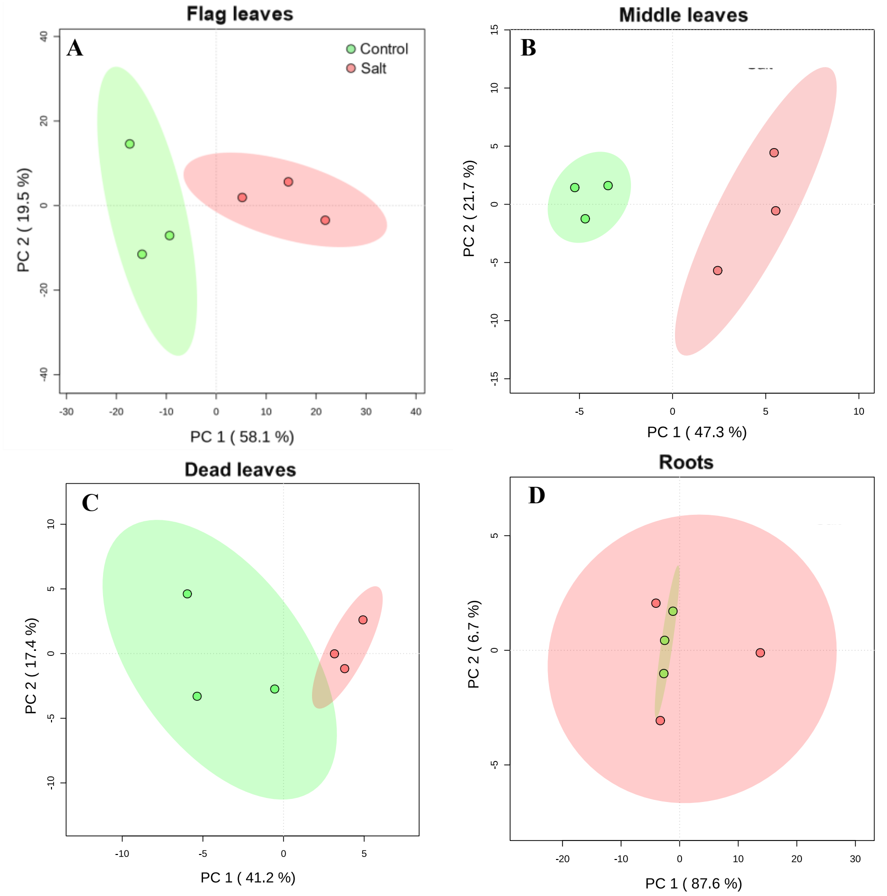


Figure 8. Principal components analysis (PCA) using metabolomics data from flag leaves (A), middle leaves (B), dead leaves (C) and root (D) of rice plants of different treatments of control and salt, in stage R3 (beginning of panicle elongation). PCA was carried out using the Metaboanalyst platform.

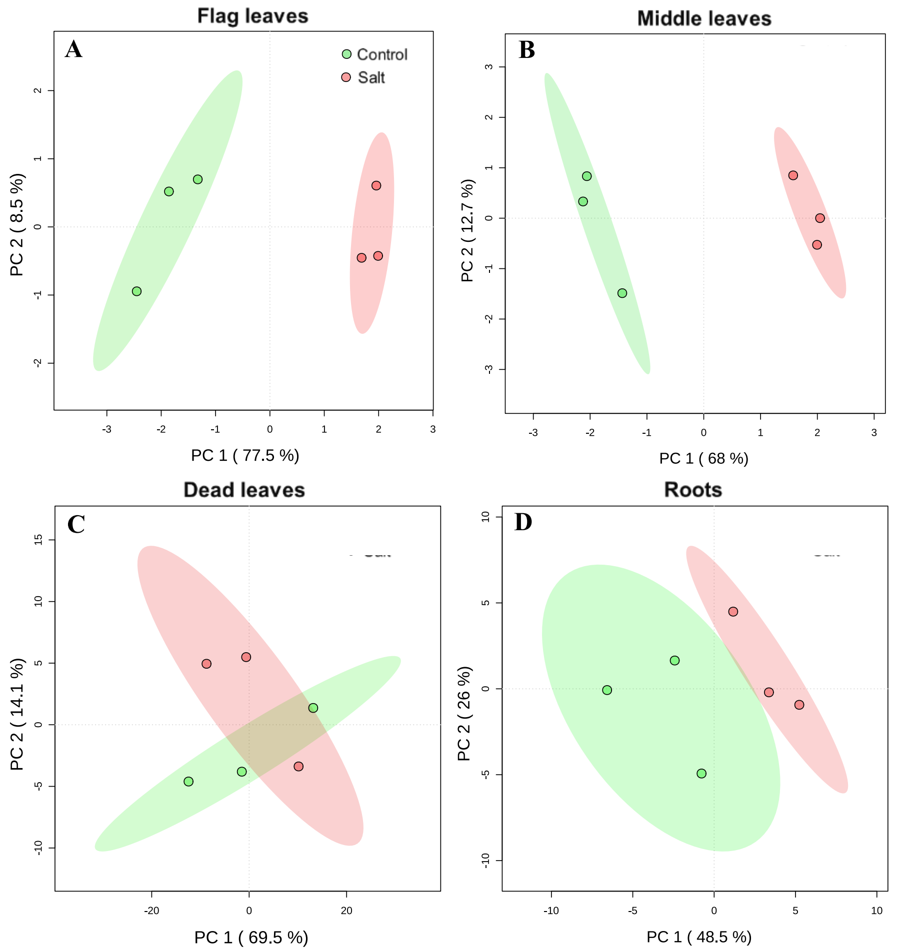


Figure 9. Principal components analysis (PCA) using metabolomics data from flag leaves (A), middle leaves (B), dead leaves (C) and root (D) of rice plants of different treatments of control and salt, in stage R7 (grain filling and beginning of drying). PCA was carried out using the Metaboanalyst platform.

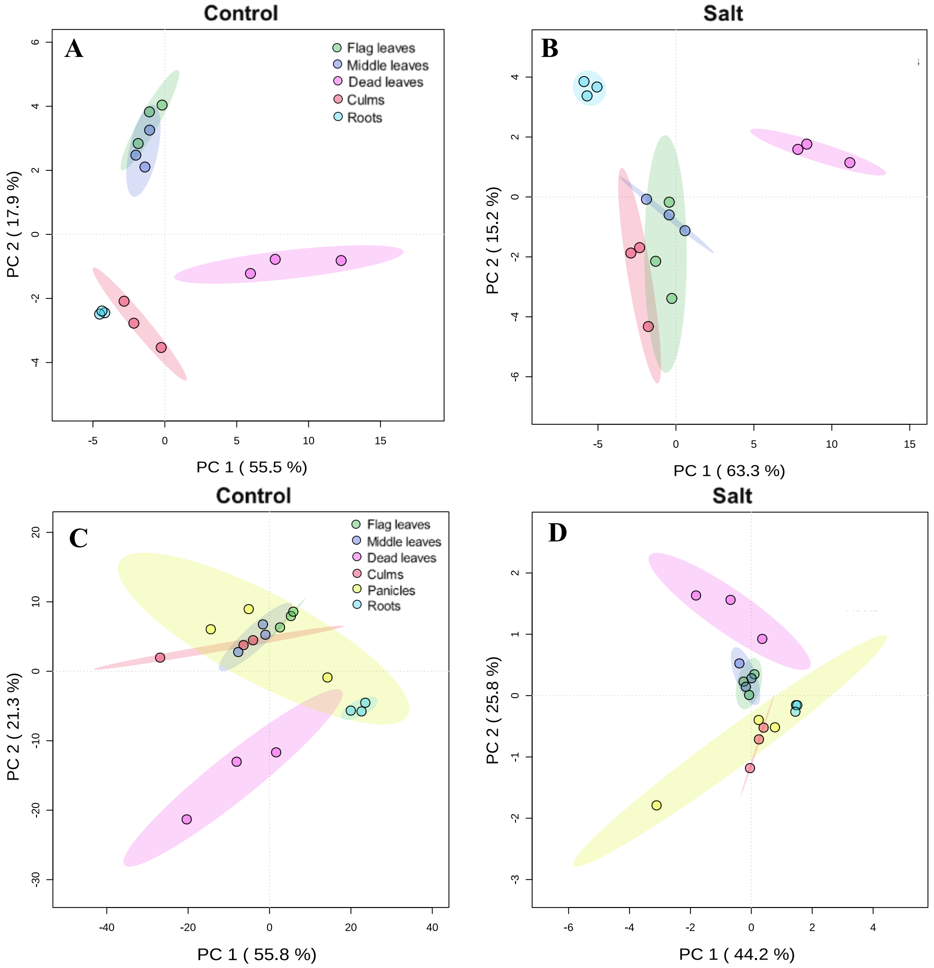


Figure 10. Principal component analysis (PCA) using metabolomic data from flag leaves, middle leaves, dead leaves, roots and stems in the R3 stage of (A) control and (B) salt treatments. Flag leaves middle leaves, dead leaves, roots, culms and panicles in stage R7 of treatments (C) control and (D) salt. PCA was carried out using the Metaboanalyst platform.

The differences between source (flag and middle leaves) and sink part (culms and roots) parts were further evidenced by the analysis of the content of the primary metabolites identified, in which the increases in several metabolites were higher in leaves than culms and roots. Furthermore, the metabolic alterations induced by salt stress seem to be stage-independent in leave and stage-dependent in culms and roots. This is evidenced by the fact that several metabolites showed a similar pattern of increase or decrease in both R3 and R7 stages in both flag and middles leaves, while opposite trends were observed in roots and culms between R3 and R7 stages.

For instance, whilst lysine, phenylalanine, serine, valine and sucrose (among others) increased under salt stress in both roots and culms at the R3 stage, they have lower level than the control at the R7 stage in these organs (Figure 11). In dead leaves, only serine showed this behaviour of higher and lower level under salt stress than the control at the R3 and R7 stages, respectively (Figure 11). Interestingly, the salt stress increased the level of all metabolites identified in panicles (Figures 11, S7). Our metabolic analyses collectively suggest that the effect of salt stress is organ and developmental stage dependent, indicating that salt stress metabolic biomarkers and the metabolism-mediated mechanisms for salt stress acclimation may be spatially and temporally regulated.

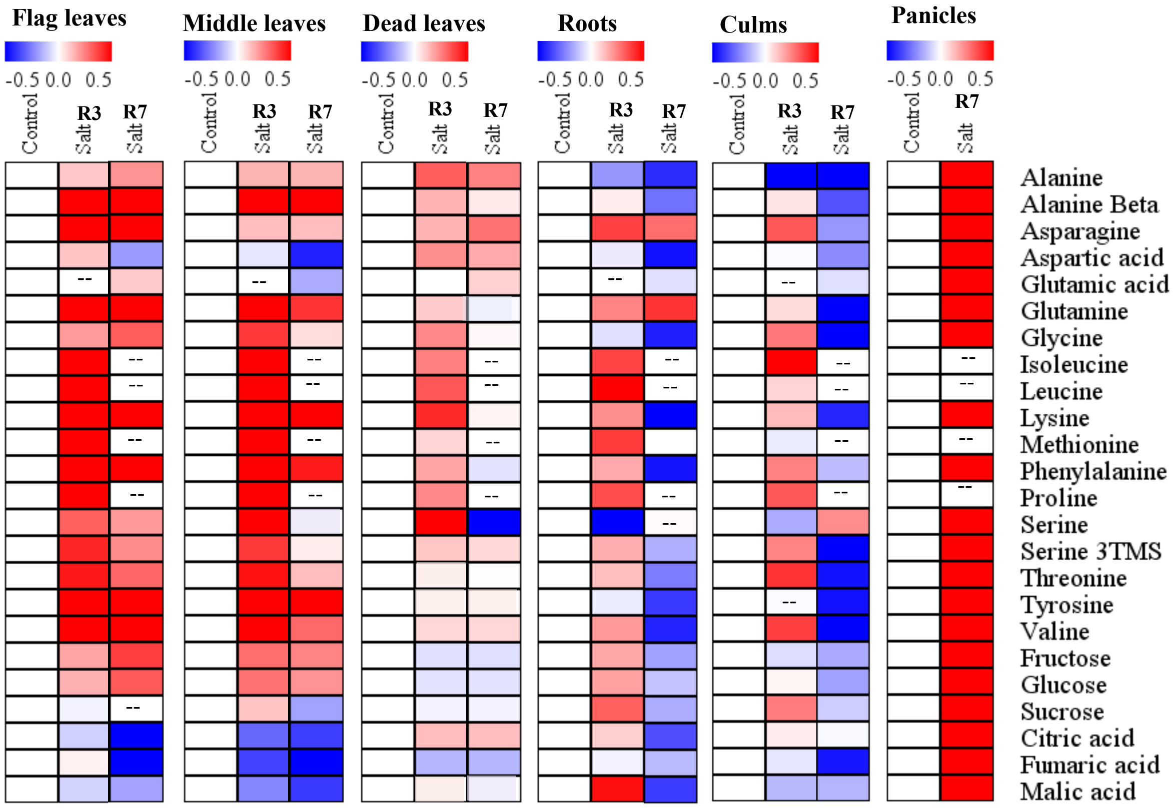


Figure 11. Heatmap representation of absolute differences in the content of metabolites (amino acids, sugars and organic acids), in the parts of rice plants (flag leaves, middle leaves, senescent leaves, roots and culms), at stages R3 and R7 and panicles (stage R7). * -- indicates that said metabolite was not identified at the corresponding stage. Heat map was created using MeV software.

Salinity affects yield indicators and seed quality

To observe how salinity affects the yield and seed quality, a series of analyzes were carried out at the R9 stage, with full seed maturation. The number of panicles per plant (Fig. 12A) was drastically reduced in the saline treatment, and as a result of this, a huge reduction in seeds per plant was also observed (Fig. 12B), where, in both results, it shows an average reduction of 3x. The number of seeds per panicle did not show significant differences (fig 12C). To obtain a grain yield parameter, the harvest index (HI) was calculated. HI is defined as the proportion between harvestable parts (seeds, grains) and biomass, being a measure of success in partitioning photosynthates to seeds. The harvest index for rice varies from about 0.18 to 0.5 depending on the variety (SAITO *et al.*, 2021; YANG; ZHANG, 2010). With a statistically significant difference, the harvest index observed in our results was 0.29 for control plants and 0.16 for plants treated with salt, a reduction of almost 50% (Fig. 12 D).

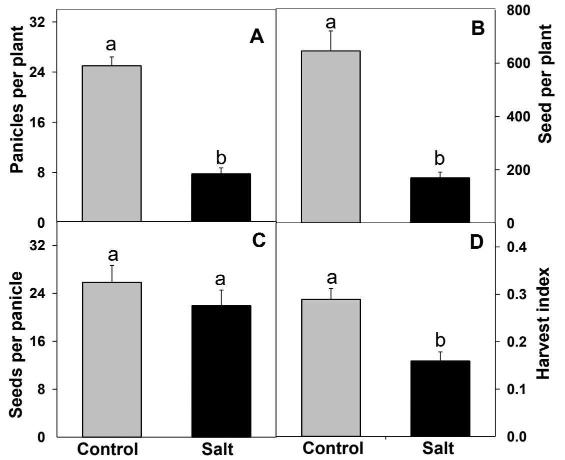


Figure 12. Number of panicles per plant (A), number of seeds per plant (B), number of seeds per panicle (C) and harvest index (D) in rice plants, collected at stage R9 (total maturation). Different letters represent significant differences between the treatments (control and salt). Data are means of four replicates and the averages were compared using the Tukey's test ($p \leq 0.05$).

The weight of 100 seeds, which is another yield indicator widely used in grain production, was also statistically reduced under salinity, from 1.94 to 0.96 (Fig 13A). This reduction was drastically greater when looking at the total weight of seeds per plant, de 5.49 to 0.94 (Fig 13B). This results from little panicle production in plants under stress conditions, as observed (Fig 12). Despite seed production, the germination test demonstrated that few seeds were viable, and of the seeds produced by plants under saline treatment, only 3%

germinated (Fig 12C). Furthermore, these seeds demonstrated high electrolyte leakage in the vigor test carried out (fig 12D).

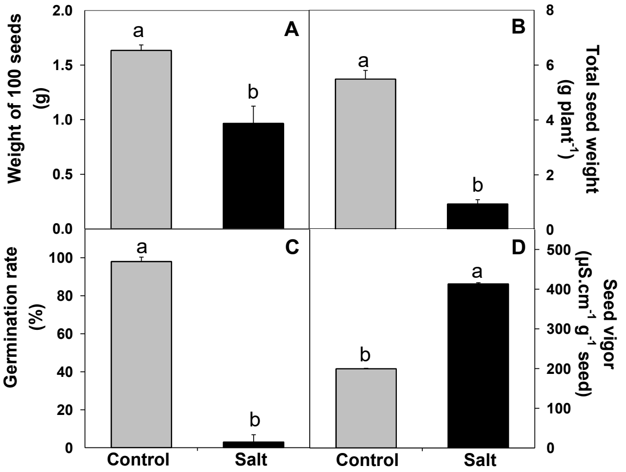


Figure 13. Weight 100 seeds (A), total seed weight (B) germination rate (C), and seed vigor (D) in rice plants, collected at stage R9 (total maturation). Different letters represent significant differences between the treatments (control and salt). Data are means of four replicates and the averages were compared using the Tukey's test ($p \leq 0.05$).

DISCUSSION

Salt sensitivity is module-time-scale dependent throughout the rice reproductive phases

In this current work we firstly demonstrate that salt sensitivity, as indicated by relative reduction in dry mass, Na^+ accumulation and decreases in K^+/Na^+ ratios throughout the reproductive phases of rice development, is dependent on parts of the plant or modules, development reproductive stages, and also of the observation scale (growth, physiological or biochemistry scales). In this perspective, clearly the oldest leaves were the parts more "salt sensitive" since they exhibited higher Na^+ accumulation, higher reduction in K^+/Na^+ ratios, associated with higher relative decrease in the biomass production compared with all other plant parts. These responses are expected in rice plants since salinity and other stress types are capable to induce senescence and cellular death firstly on the oldest tissues (LIMA-MELO *et al.*, 2016; POUCET *et al.*, 2021; SILVEIRA; CARVALHO, 2016; SILVEIRA; SOUSA, 2024; SOUSA *et al.*, 2021).

Interestingly, the samples constituted by middle and flag leaves which represent a mixture of mature and spading leaves, are relatively preserved from membrane damages associated with lower Na^+ accumulation and lower reduction in K^+/Na^+ ratios, present high reduction in biomass possibly associated with salt-induced senescence and reserve mobilization to grain growth (SILVEIRA; SOUSA, 2024). Other parts of the plant (roots and shoot) also intensely reduce their biomass under salt stress conditions provably associated with growth reduction since the biomass allocated to panicle and grain growth is drastically reduced in that circumstances. Other mechanism by which salt stress drastically affect the yield are the low tilling rates in shoot *(*DA ROCHA *et al.,* 2012*;* ZHANG *et al*., 2021). N and C compounds are major constituents of biomass of vegetative and reproductive parts of rice (HUANG *et al*., 2020; MACHADO; SILVEIRA, 1990; SILVEIRA; MACHADO, 1990).

During the reproductive phase, high amounts of these nutrients are mobilized from vegetative parts to panicle formation and grain filling mainly from triggering of the natural senescence in those parts (HUANG *et al*., 2020; LEE; MASCLAUX-DAUBRESSE, 2021; SILVEIRA *et al*., 1989; SILVEIRA; MACHADO, 1990). It's important to note that actually the content of a nutrient like total-N content represents a balance (ratio) between the total of N compounds allocated and/or pre-existing in a specific part of the plant divided by a unit of dry mass, which, in turn, is dependent on biomass allocation and/or produced in that specific part of the plant. Thus, a total-N content of 3,000 μmol g^{-1} DM indicate that exist 3,000 μmol of nitrogenous compounds in each g of dry biomass. Thus, when total-N content is reduced by salt stress signify that this ratio can have decreased by changes in the input/output of N and biomass in that specific plant part.

Throughout the reproductive phase salt stress affected differentially the relative accumulation of biomass in the different parts of the plant and this process occur in an unbalanced way compared to N fractions. As a result, proteins are degraded intensely, and N mobilized from middle and flag leaves by senescence process compared to oldest/dead leaves, culm and roots. Unfortunately, the data discussed here don't allow to stablish cause-effect relationships involving disturbances on the source-sink relations at level of the different plant parts, including grain filling and yield. Indeed, our data suggest the salt stress induced since early vegetative phase affects drastically and differently all source parts associated with drastic reductions on C and N assimilation and growth (SILVEIRA *et al.*, 2003; WEI *et al.*, 2018).

Our data evidence that negative effects of salt stress affect drastically all parts of the plant but the consequences were especially more harmful on the yield components and seed quality. Indeed, yield and harvest index associated with seed vigor are most intensely affected than the vegetative organs root, culm and leaves. The long-term salt stress is a cumulative process, reaching progressively all key physiological processes such overall growth, tilling, photosynthesis, N assimilation, and reserve mobilization, causing a total and irreversible collapse in plant homeostasis (SILVEIRA; SOUSA, 2024).

Metabolomics and multivariate analyzes corroborate that salt stress effects are spatio-temporal-scale dependents

When the metabolomics data are analyzed by PCA approach, especially the free amino acids differently accumulated in different parts of the plant throughout the reproductive phases, they clearly demonstrate that salt stress trigger spatio-time-scale responses in rice plants. Metabolic responses were most altered by salt in flag and middle leaves. Therefore, the youngest leaves were more spatially differentiated from the other organs and showed less temporal variation. The total spatial separation of dead leaves in the R3 stage suggests a particular metabolism of these, being intermediate to the organs source (flag and middle leaves) and sink (root and stem). Those results also reinforce previous data obtained by distinct scale, that is, dry mass partitioning, nitrogenous fractions partitioning and GS activity, for instance, clearly indicating that oldest leaves samples display role and response completely different from other leaves and other organs (LEE; MASCLAUX-DAUBRESSE, 2021).

These results also indicate that some individual amino acids can act as metabolic markers to stress intensity and to source-sink relations in response to salt stress. For example, salt stress induced an increase in phenylalanine, mainly in the flag and middle leaves, which is one of the precursors for the synthesis of secondary metabolites that include several antioxidants important for plant defense against biotic and abiotic stresses (FEDURAEV *et al.*, 2020). Moreover, the PCA analysis also indicate that these responses throughout the reproductive phases are dynamics in a spatiotemporal perspective.

Our results also evidence interesting metabolic alterations induced by salt stress. In accordance with the yield and seed quality data, which show that salinity affect drastically these processes, the metabolic profile in panicles also is deeply altered where most metabolites are intensely accumulated. These alterations can reveal indications of deep metabolic alterations on the starch and protein synthesis in seed what could explicate the intense reduction in seed growth, seed vigor and germination (VOIGT *et al.*, 2009).

CONCLUSIONS

Salt stress affects metabolism and physiology of rice plants in a spatial-time-scale manner. Spatial because the cause-effect mechanisms are dependent on the plant organization parts or modules (roots, culms, oldest, middle and flag leaves). Time and ontogenetic dependent since these responses are also subject to the reproductive development phases. And scale dependent as these differential responses are also dependent on the observation scale employed, that is, biochemical (enzymatic activity, nitrogenous compounds contents, and metabolomics profiles), module physiology (dry mass accumulation in parts of the plant) and the whole physiology (whole plant growth and total biomass accumulation). Salinity also affects drastically the N metabolism as indicated by strong changes on GS activity, nitrogenous fractions and metabolomics profiles. These integrate and systemic approaches are essential to understand the cause-effects mechanisms associated with salt stress on the growth, N metabolism, and yield and seed quality in rice and other crops.

SUPLEMENTARY MATERIAL

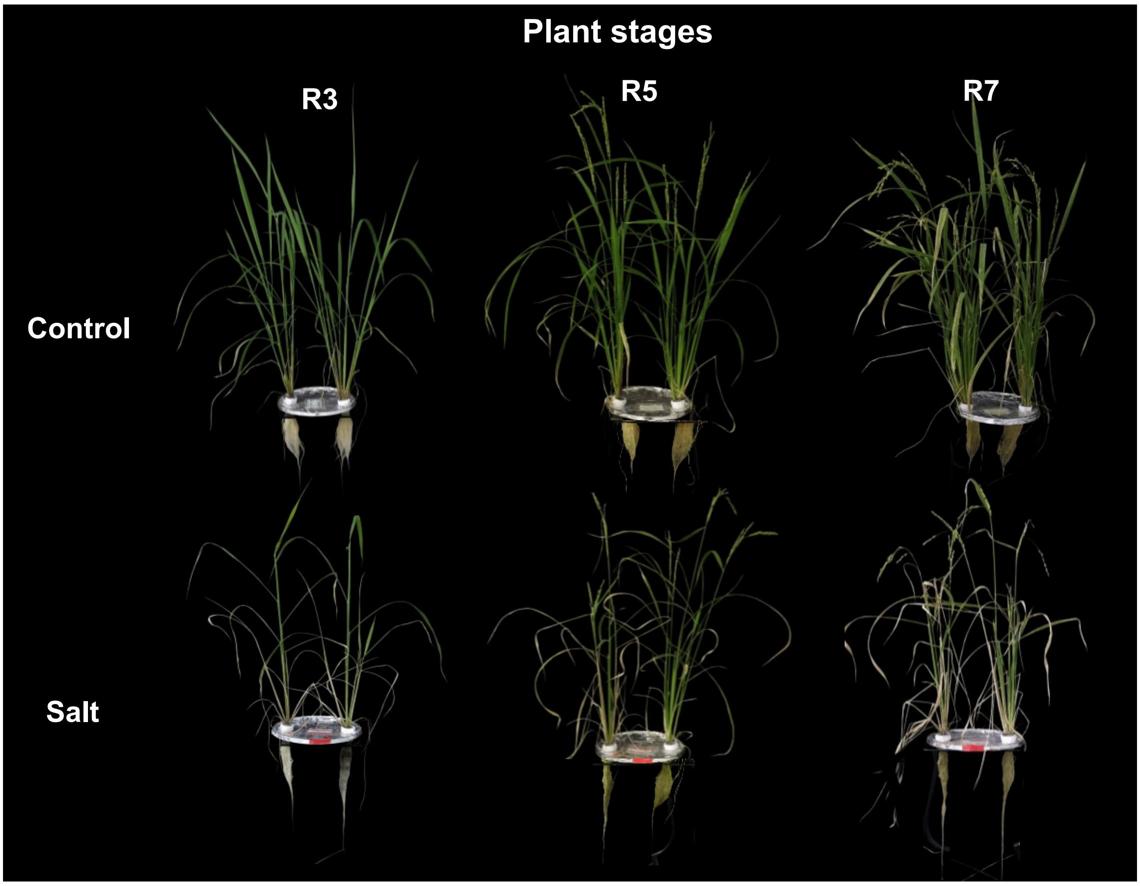


Figure S1. Morphological characterization of rice plants (shoot and root) at different reproductive stages R3 (beginning of panicle formation), R5 (beginning of panicle elongation), and R7 (grain filling and begging of drying) in control plants. Pictures show visual symptoms indicating reduction in shoot tilling, shoot and root size, senescence followed by drying concentrate on the oldest leaves of salt stressed plants. The pots are representative from four independent replicates.

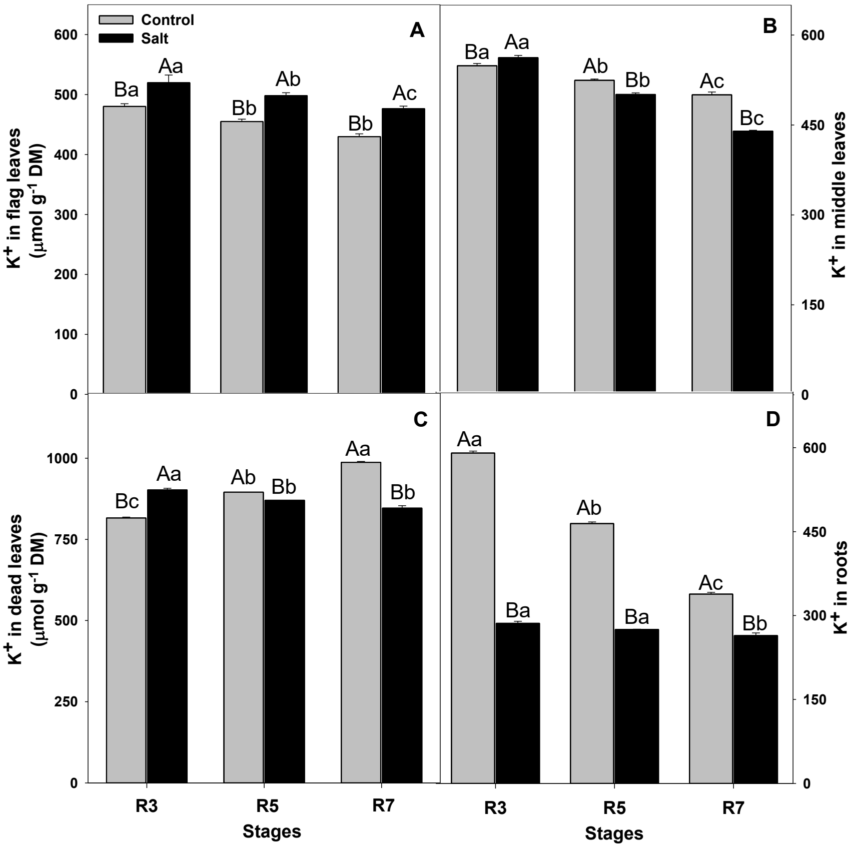


Figure S2. Content K^+ in rice plants exposed to treatment control or salt, flag leaves (A), middle leaves (B), senescent leaves (C) and roots (D). Different capital letters represent significant differences between the treatments (Control and salt), and different lowercase letters represent significant differences within treatment at different stages. Data are means of tree replicates and the averages were compared using the Tukey's test ($p \leq 0.05$).

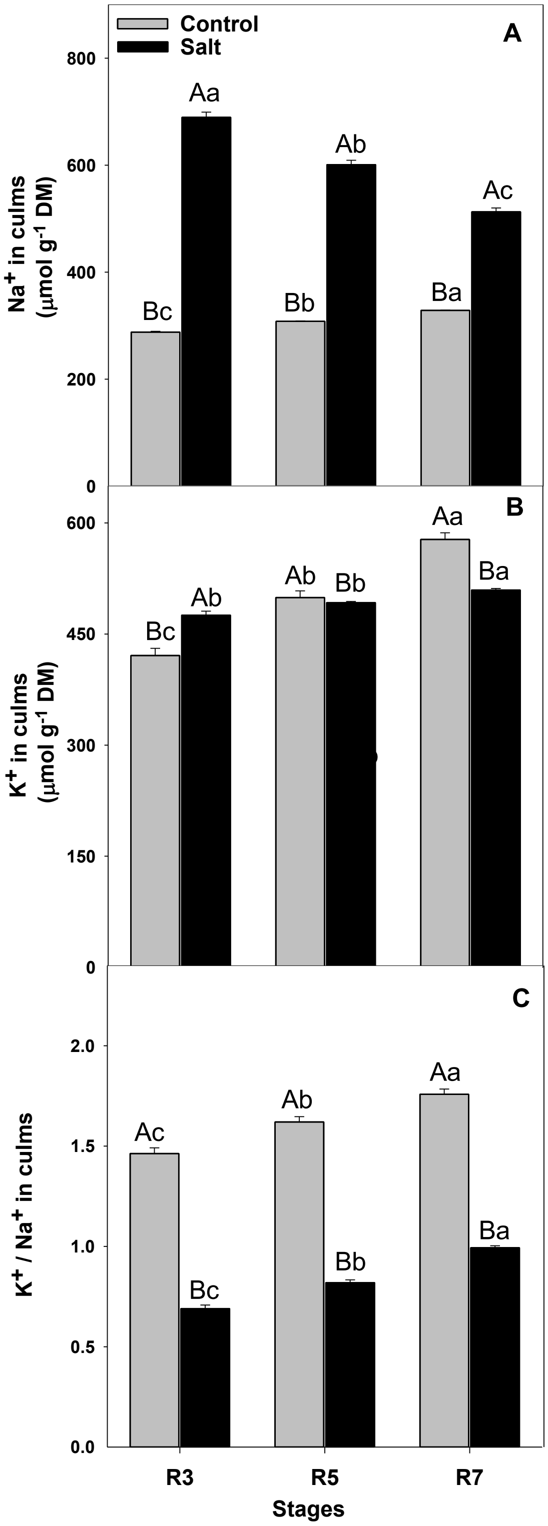


Figure S3. Content Na^+ (A), content K^+ (B) and K^+/Na^+ ratios (C) in culms of rice plants exposed to treatment control or salt. Different capital letters represent significant differences between the treatments (Control and salt), and different lowercase letters represent significant differences within treatment at different stages. Data are means of tree replicates and the averages were compared using the Tukey's test ($p \leq 0.05$).

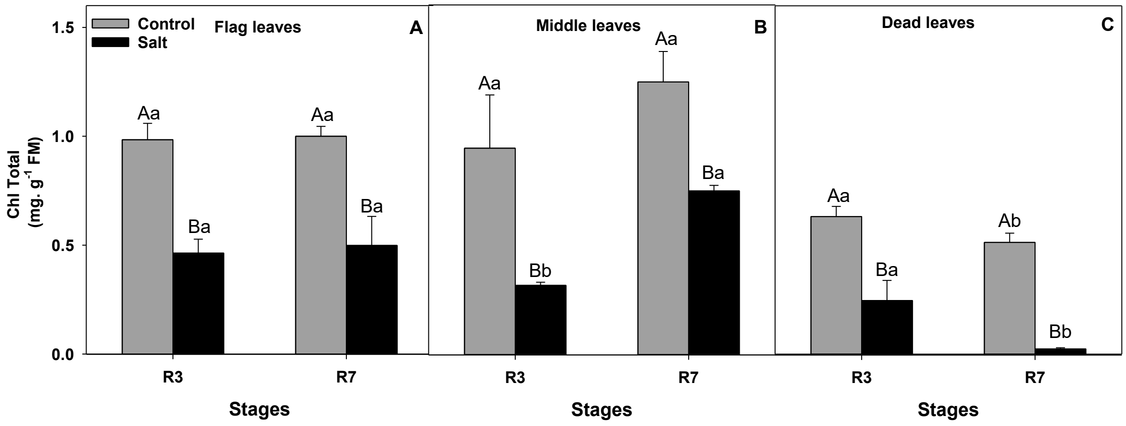


Figure S4. Total chlorophyll in rice plants exposed to treatment control or salt, flag leaves (A), middle leaves (B) and dead leaves (C). Different capital letters represent significant differences between the treatments (Control and salt), and different lowercase letters represent significant differences within treatment at different stages. Data are means of tree replicates and the averages were compared using the Tukey's test ($p \leq 0.05$).

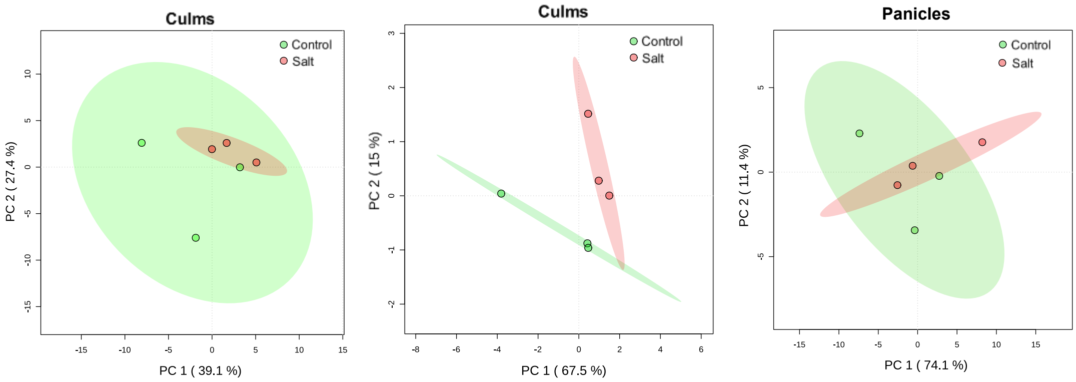


Figure S5. Principal components analysis (PCA) using metabolomics data from culms in stage R3 and R7. R3= beginning of panicle elongation and R7 = grain filling and beginning of drying), and panicles in stage R7 of rice plants of different treatments of control and salt. PCA was carried out using the Metaboanalyst platform.

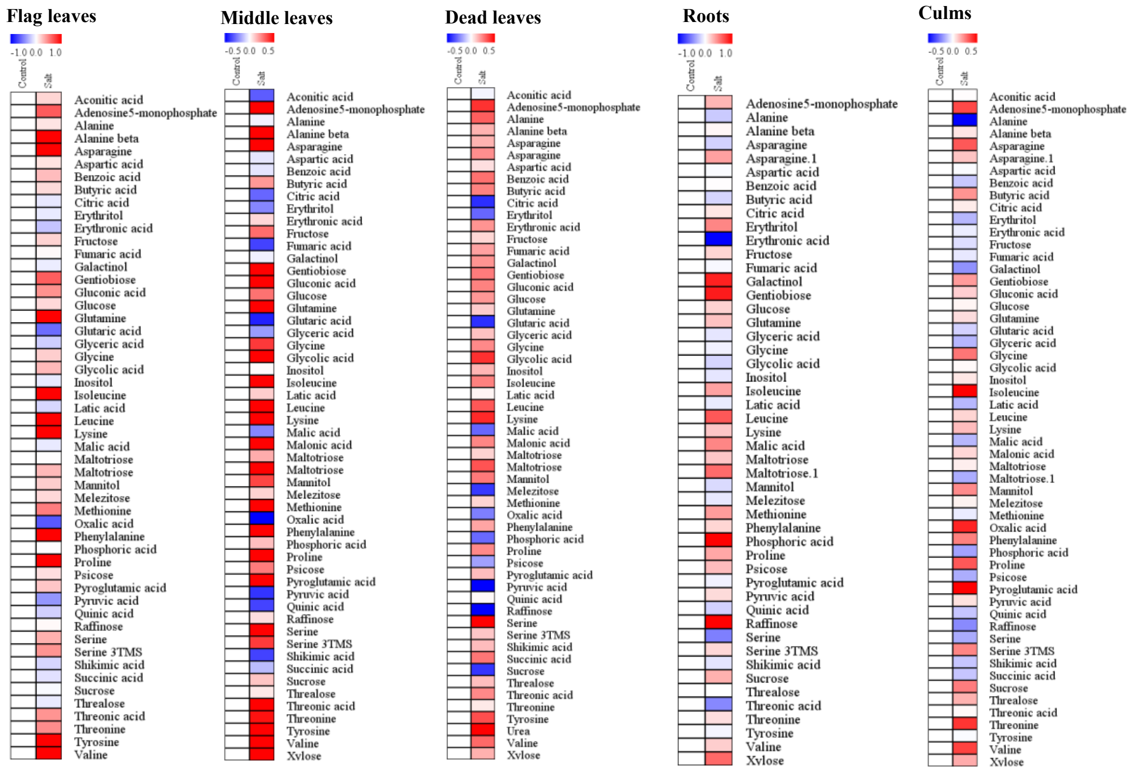


Figure S6. Heat map representation of the absolute differences in the content of metabolites in flag leaves, middle leaves, dead leaves, root and culms of rice plants, in stage R3 (beginning of panicle elongation). Heat map was created using MeV software.

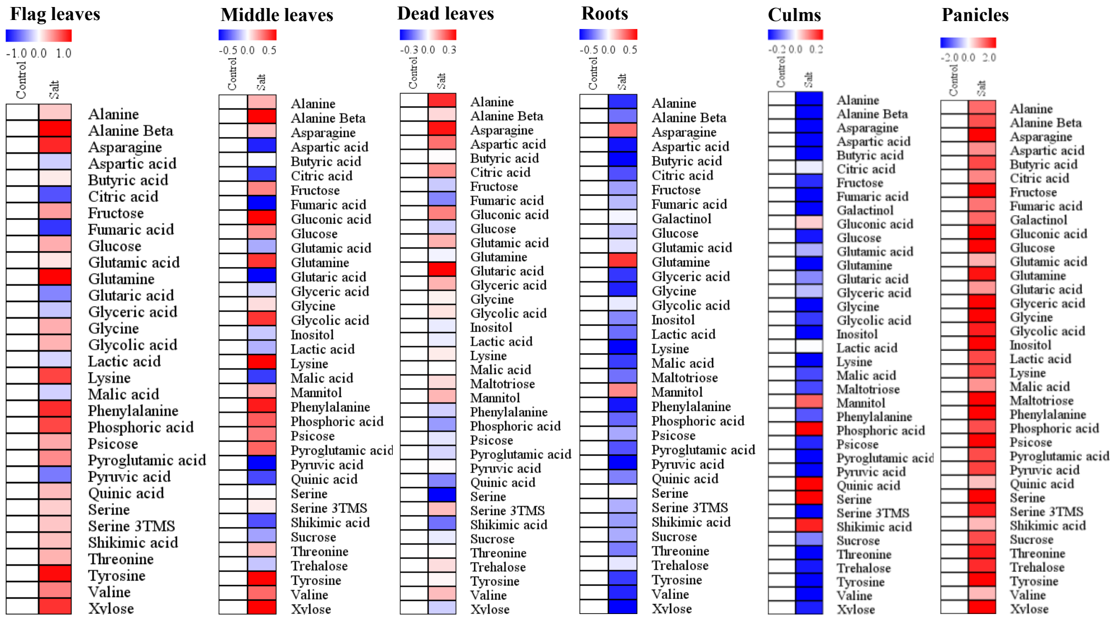


Figure S7. Heat map representation of the absolute differences in the content of metabolites in flag leaves, middle leaves, dead leaves, root, culms and panicle of rice plants, in stage R7 (grain filling and beginning of drying). Heat map was created using MeV software.

Table S1. Soluble proteins in rice plants parts (flag leaves, dead leaves, culm e roots) exposed to treatment control or salt in distinct stages of the reproductive development stages R3 and R7.

Soluble proteins (mg/g fresh mass)								
	Flag leaves		Dead leaves		Culms		Roots	
Stages	Control	Salt	Control	Salt	Control	Salt	Control	Salt
R3	14.405 A	9.688 A	9.461 A	3.320 A	6.074 A	5.468 A	0.871 B	0.548 B
R7	14.566 A	11.89 A	4.031 B	3.079 A	5.619 A	5.970 A	1.656 A	0.702 A

(Stage R3= beginning of panicle elongation and R7= grain filling and beginning of drying).*There was significant differences between treatments for all variables evaluated. Data are means of tree replicates and the averages were compared using the Tukey's test ($p \leq 0.05$).

REFERENCES

ALENCAR, V. T.C.B. *et al.* High ammonium supply impairs photosynthetic efficiency in rice exposed to excess light. **Photosynthesis Research**, [*s. l.*], v. 140, n. 3, p. 321–335, 2019. Disponível em: http://dx.doi.org/10.1007/s11120-019-00614-z.

BAETHGEN, W. E.; ALLEY, M. M. A manual colorimetric procedure for measuring ammonium nitrogen in soil and plant kjeldahl digests. **Communications in Soil Science and Plant Analysis**, [*s. l.*], v. 20, n. 9–10, p. 961–969, 1989.

BLUM, A.; EBERCON, A. Cell membrane stability as a measure of drought and heat tolerance in wheat. **Crop Science**, [*s. l.*], v. 21, n. 1, p. 43–47, 1981.

BRADFORD, Marion M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye biding. **Analytical Biochemistry**, [*s. l.*], v. 72, p. 248–254, 1976.

CAVALCANTI, Fabio Rossi *et al.* Superoxide dismutase, catalase and peroxidase activities do not confer protection against oxidative damage in salt-stressed cowpea leaves. **New Phytologist**, [*s. l.*], v. 163, n. 3, p. 563–571, 2004.

CHONG, Jasmine; WISHART, David S.; XIA, Jianguo. Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. **Current Protocols in Bioinformatics**, [*s. l.*], v. 68, n. 1, p. 1–128, 2019.

COUNCE, Paul A.; KEISLING, Terry C.; MITCHELL, Andrew J. A uniform, objectives, and adaptive system for expressing rice development. **Crop Science**, [*s. l.*], v. 40, n. 2, p. 436–443, 2000.

DA ROCHA, Iza Marineves Almeida *et al.* Exogenous ornithine is an effective precursor and the δ-ornithine amino transferase pathway contributes to proline accumulation under high N recycling in salt-stressed cashew leaves. **Journal of Plant Physiology**, [*s. l.*], v. 169, n. 1, p. 41–49, 2012. Disponível em: http://dx.doi.org/10.1016/j.jplph.2011.08.001.

FEDURAEV, P. *et al.* Phenylalanine and Tyrosine as Exogenous Precursors of Wheat (Triticum aestivum L.) Secondary Metabolism through PAL-Associated Pathways. **Plants**, [*s. l.*], v. 9, n. 4, p. 476, 2020.

FELKER, Peter. Microdetermination of Nitrogen in Seed Protein Extracts with the Salicylate-Dichloroisocyanurate Color Reaction. **Analytical Chemistry**, [*s. l.*], v. 49, n. 7, p. 1080, 1977.

HIREL, Bertrand; GADAL, Pierre. Glutamine Synthetase in Rice. **Plant Physiology**, [*s. l.*], v. 66, n. 4, p. 619–623, 1980.

HOAGLAND, D. R.; ARNON, D. I. Preparing the nutrient solution. **The Water-Culture Method for Growing Plants without Soil**, [*s. l.*], v. 347, p. 29–31, 1950.

HUANG, Jie *et al.* Effects of nitric oxide on nitrogen metabolism and the salt resistance of rice (Oryza sativa L.) seedlings with different salt tolerances. **Plant Physiology and Biochemistry**, [*s. l.*], v. 155, n. November 2019, p. 374–383, 2020. Disponível em: https://doi.org/10.1016/j.plaphy.2020.06.013.

KOPKA, Joachim *et al.* GMD@CSB.DB: The Golm metabolome database. **Bioinformatics**, [*s. l.*], v. 21, n. 8, p. 1635–1638, 2005.

LEE, Sichul; MASCLAUX-DAUBRESSE, Celine. Current understanding of leaf senescence in rice. **International Journal of Molecular Sciences**, [*s. l.*], v. 22, n. 9, p. 1–19, 2021.

LI, Xiaobai *et al.* Unraveling the complex trait of harvest index with association mapping in rice (Oryza sativa L.). **PLoS ONE**, [*s. l.*], v. 7, n. 1, p. 1–10, 2012.

LICHTENTHALER, HARTMUT K.; WELLBURN, ALAN R. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents . **Biochemical Society Transactions**, [*s. l.*], v. 11, n. 5, p. 591–592, 1983.

LIMA-MELO, Yugo *et al.* Mitochondrial GPX1 silencing triggers differential photosynthesis impairment in response to salinity in rice plants. **Journal of integrative plant biology**, [*s. l.*], v. 58, n. 8, p. 737–748, 2016.

LISEC, Jan *et al.* Gas chromatography mass spectrometry-based metabolite profiling in plants. **Nature Protocols**, [*s. l.*], v. 1, n. 1, p. 387–396, 2006.

MACHADO, Eduardo C.; SILVEIRA, Joaquim A. G.. Trocas de C02, acúmulo de fitomassa e remobilização de reservas durante o crescimento de panículas de duas cultivares de arroz. **Revista Brasileira de Fisiologia vegetal***,* 2(1), 63-70, 1990.

MCKERSIE, B D; TOMES, D T. Predicting the Vigor and. [*s. l.*], v. 343, p. 337–343, 1981.

POUCET, Théo *et al.* Ammonium supply induces differential metabolic adaptive responses in tomato according to leaf phenological stage. **Journal of Experimental Botany**, [*s. l.*], v. 72, n. 8, p. 3185–3199, 2021.

SAITO, Hiroki *et al.* Two Novel QTLs for the Harvest Index that Contribute to High-Yield Production in Rice (Oryza sativa L.). **Rice**, [*s. l.*], v. 14, n. 1, p. 1–11, 2021.

SILVEIRA, Joaquim A.G.; CARVALHO, Fabricio E.L. Proteomics, photosynthesis and salt resistance in crops: An integrative view. **Journal of Proteomics**, [*s. l.*], v. 143, p. 24–35, 2016a. Disponível em: http://dx.doi.org/10.1016/j.jprot.2016.03.013.

SILVEIRA, Joaquim A. G., CASTRO, P. R. C., DECHEN, A. R. Partição de nitrogênio e distribuição da matéria seca em três cultivares de caupi. **Pesq. agropec. bras.***,* 24(9): 1153-1160, 1989.

SILVEIRA, Joaquim Albenisio *et al.* Proline accumulation and glutamine synthetase activity are increased by salt-induced proteolysis in cashew leaves. **Journal of Plant Physiology**, [*s. l.*], v. 160, n. 2, p. 115–123, 2003.

SILVEIRA, Joaquim A. G., MACHADO, Eduardo C. Mobilização de nitrogênio e de carboidratos durante o desenvolvimento de panículas de duas cultivares de arroz. **Revista**

Brasileira de Fisiologia vegetal, 2(2), 37-46, 1990.

SILVEIRA, Joaquim A.G.; SOUSA, Rachel H.V. Looking for a systemic concept and physiological diagnosis of a plant stress state. **Theoretical and Experimental Plant Physiology**, [*s. l.*], n. 0123456789, 2024. Disponível em: https://doi.org/10.1007/s40626-024-00318-3.

SOUSA, Rikaely T. *et al.* Ammonium overaccumulation in senescent leaves as a novel exclusion mechanism to avoid toxicity in photosynthetically active rice leaves. **Environmental and Experimental Botany**, [*s. l.*], v. 186, n. February, p. 104452, 2021. Disponível em: https://doi.org/10.1016/j.envexpbot.2021.104452.

TSUGAWA, Hiroshi *et al.* MS-DIAL: Data-independent MS/MS deconvolution for comprehensive metabolome analysis. **Nature Methods**, [*s. l.*], v. 12, n. 6, p. 523–526, 2015.

VIÉGAS, Ricardo A. *et al.* Plant growth, accumulation and solute partitioning of four forest species under salt stress. **Revista Brasileira de Engenharia Agrícola e Ambiental**, [*s. l.*], v. 7, n. 2, p. 258–262, 2003.

VIEGAS, R. A; SILVEIRA, Joaquim A.G. AMMONIA ASSIMILATION AND PROLINE ACCUMULATION IN YOUNG CASHEW PLANTS. [*s. l.*], n. August, 1999.

VOIGT, Eduardo Luiz *et al.* Source-sink regulation of cotyledonary reserve mobilization during cashew (Anacardium occidentale) seedling establishment under NaCl salinity. **Journal of Plant Physiology**, [*s. l.*], v. 166, n. 1, p. 80–89, 2009.

WEI, Huanhe *et al.* Sink-source relationship during rice grain filling is associated with grain nitrogen concentration. **Field Crops Research**, [*s. l.*], v. 215, n. May 2017, p. 23–38, 2018. Disponível em: https://doi.org/10.1016/j.fcr.2017.09.029.

YANG, Jianchang; ZHANG, Jianhua. Crop management techniques to enhance harvest index in rice. **Journal of Experimental Botany**, [*s. l.*], v. 61, n. 12, p. 3177–3189, 2010.

YEMM, E. W.; COCKING, E. C.; RICKETTS, R. E. The determination of amino-acids with ninhydrin. **The Analyst**, [*s. l.*], v. 80, n. 948, p. 209–214, 1955.

ZHANG, Jing *et al.* Response of grain-filling rate and grain quality of mid-season indica rice to nitrogen application. **Journal of Integrative Agriculture**, [*s. l.*], v. 20, n. 6, p. 1465–1473, 2021. Disponível em: http://dx.doi.org/10.1016/S2095-3119(20)63311-1.

5 CHAPTER 3: HEAT COMBINED WITH SALINITY STIMULATE ANTIOXIDANT DEFENSE BUT INDUCE SEVERE IMPAIRMENT IN PHOTOSYNTHESIS OF RICE PLANTS

Article submitted to Plant and soil Journal

(Impact factor: 4.9. Qualis: A1)

REGULAR ARTICLE

Heat combined with salinity stimulate antioxidant defense but induce severe impairment in photosynthesis of rice plants

Kellyane R. Mendes[1#], Aurenivia Bonifacio[2#], Marcio O. Martins[1], Rachel H. Sousa[3], Matheus V. Monteiro[1], Joaquim A. G. Silveira[1*]

[1]LabPlant, Department of Biochemistry and Molecular Biology, Federal University of Ceará, Fortaleza, Ceará 60451-970, Brazil

[2]Laboratory of Plant Physiology and Biochemistry, Department of Biology, Federal University of Piaui, Teresina, Piaui, Brazil

[3]Center of Agricultural Sciences and Biodiversity, Federal University of Cariri, Crato, CE, Brazil

Corresponding author

Joaquim A. G. Silveira*. Department of Biochemistry and Molecular Biology, Federal University of Ceará, Laboratory of Plant Metabolism (LABPLANT), Av. Humberto Monte, s/n, CP 6004, CEP 60451-970, Fortaleza, Ceará, Brazil. Tel: +55 85 3366 9821. E-mail: silveira@ufc.br

[#] These authors have contributed equally to manuscript

ABSTRACT

Aims To test the hypothesis if high temperature could aggravate salt stress through stimulation of Na^+ accumulation, impairment in photosynthesis and generation of oxidative stress in rice leaves.

Methods Rice plants were previously exposed to 0 and 100 mM NaCl for eight days at 27 °C and afterwards two groups were transferred to high temperature (42 °C) for 10 hours (heat and heat + salt) whereas two others remained at 27 °C (control and single salt).

Results Heat combined with salinity greatly stimulated Na^+ accumulation in leaves causing intense decrease in K^+/Na^+ ratios, inducing significant osmotic and ionic alterations. Stomata were closed intensely causing drastic impairment in CO_2 assimilation and decrease in water use efficiency. In contrast, the PSII activity was much lesser affected, corroborated by low increase in closed reaction center fraction of PSII and slight decrease in electron transport rates. Unexpectedly, that stress combination partially favored oxidative protection as indicated by reduction in the levels of H_2O_2 and lipid peroxidation associated with reduction in the contents of reduced ascorbate and glutathione. These favorable antioxidant responses were accompanied by increases in the activities of ascorbate peroxidases, superoxide dismutases, glutathione peroxidases, and phenol peroxidases whereas catalases and glycolate oxidases decreased. These antioxidant responses were not enough to mitigate overall physiological damages caused by combined stress as indicated by drastic increase in membrane damage.

Conclusions High temperature drastically aggravates negative effects caused by salt stress on the photosynthetic efficiency, especially CO_2 assimilation, despite that combination has partially favored the antioxidant defense.

Keywords - Combined stress. Heat. Photosynthesis. Oxidative metabolism. Salinity. *Oryza sativa*

ABBREVIATIONS

APX - Ascorbate peroxidases

ASA - Reduced ascorbate

CAT - Catalases

Ci – Intercellular CO_2 concentration

E - Transpiration rate

ETR - Apparent electron transport rate of PSII

ETR/P_N - Ratios between apparent electron transport rate of PSII and CO_2 assimilation

Fo - Initial fluorescence of PSII

Fv/Fm - Maximum potential quantum yield of PSII

GO - Glycolate oxidases

GPOD - Guaiacol peroxidases

GPX - Glutathione peroxidases

gs - Stomatal conductance

GSH - Reduced glutathione

NPQ - Non-photochemical quenching

P_N/Ci - Instantaneous carboxylation efficiency

qP - Photochemical quenching

1-qP – Fraction of closed PSII reaction centers

PSII – Photosystem II

RWC - Relative water content

SOD - Superoxide dismutases

WUE - Water use efficiency

ΦPSII - Effective quantum yield of PSII

INTRODUCTION

Rice and several other plant species are commonly cultivated in tropical regions subjected to high temperatures, excess radiation, and salinity, especially in paddy soils (Fahad et al., 2019; Radha, 2023). In a future scenario of intensification in global climate changes, these adverse combined conditions tend to be intensely aggravated (Mukhopadhyay et al., 2021; Zandalinas et al., 2021) and possible solution such as utilization of tolerant cultivars are scarce to date. In parallel or before to plant breeding programs, is urgent to develop integrated studies of plant physiology to understand responses and defense mechanisms against combined stress factors such as salinity and heat (Nahar et al., 2022; Zandalinas and Mittler, 2022). In fact, several studies have evidenced that isolated environmental factors when act in interaction with other can induce effects completely different of those caused by single stress factors (Ferreira-Silva et al., 2011; Georgieva and Vassileva, 2023; Hussain et al., 2019).

In general, salinity and heat display negative interaction inducing more adverse effects of salinity on growth and photosynthesis in several plant species (Suzuki et al., 2016). Nevertheless, this fact is not true to thermotolerant species like *Anacardium occidentale* where high temperatures are capable to mitigate adverse effects caused by salt stress (Ferreira-Silva et al., 2011). In that study, the authors demonstrated that heat favored intensely the antioxidant protection stimulating the utilization of non-enzymatic antioxidants ascorbate (ASA) and glutathione (GSH) and inducing activities of antioxidant enzymes such as catalases (CAT), ascorbate peroxidases (APX) and superoxide dismutase isoforms (SOD). Actually, the interactive effects caused simultaneously by salt stress and heat on photosynthesis and redox metabolism are scarcely known yet, especially in crops like rice.

Salinity initially induces osmotic effects and ABA synthesis in roots followed by stomata closure and ionic effects by disturbances in K^+/Na^+ homeostasis, provoking direct and indirect effects on the functioning of the photosynthetic apparatus (Farooq et al. 2021; Georgieva and Vassileva, 2023; Munns and Tester, 2008; Negrão et al. 2017; Rodrigues et al., 2013). However, little is known how heat and its interaction with salinity might affect these and other physiological processes including photosynthesis and redox metabolism. Imbalance in K^+/Na^+ homeostasis is directly associated with expression/activities of K^+ - Na^+ transporters, initially in roots and xylem (Assaha et al. 2017; Rubio et al., 2020). Some works have evidenced that single heat is capable to stimulate the Na^+ transport from roots to leaves, inducing decreases in K^+/Na^+ ratios in cytosol. If the plant species displays a limited capacity to storage Na^+ in vacuoles, the progressive reduction in that ratios is capable to generate ionic stress provoked by Na^+-induced K^+-deficiency (Keisham et al., 2018; Rubio et al., 2020).

Excess Na^+ (and other salt ions) might affect photosynthesis by osmotic and ionic effects. The direct mechanisms induced on photosynthetic activity by accumulation of salt ions in chloroplasts and the physiological role of heat on them are scarcely known (Nahar et al., 2023). It's commonly accepted that the most important causes of salt stress on photosynthesis impairment are osmotic by stomatal closure and restriction in Rubisco carboxylation (Hameed at al., 2021; Munns and Tester, 2008; Rodrigues et al., 2013). Thus, has been amply evidenced that salt-induced osmotic effects are more prominent on the CO_2 assimilation phase rather than on photochemical reactions (Hameed et al., 2021). In these circumstances, salt stress should progressively generate an imbalance between energy input/output, inducing excess energy in chloroplast (Silveira and Carvalho, 2016).

That above condition could favor the advent of another stress type: oxidative stress generated by overaccumulation of reactive oxygen species (ROS). Therefore, the interaction among heat and salinity might generate some stress types which could affect plants growth by several mechanisms. In this context, the performance of photosynthesis and antioxidant metabolism is particularly important because serious disturbance in the first process could generate oxidative stress. These processes involving salinity → Na^+ accumulation → photosynthesis impairment → ROS accumulation in leaves should generate a vicious cycle to progressively aggravate salt stress. How does that vicious cycle can operate in presence of combined stress of high temperature and salinity? Could high temperature favor Na^+ accumulation in detrimental of K^+, stimulating osmotic and ionic adverse effects on photosynthesis? In these circumstances, the antioxidant metabolism should be enough to mitigate or aggravate the heat and salt combined stress?

This work was performed to test that hypothesis raised previously employing a moderately salt and heat sensitive rice cultivar. Our results clearly evidence that high temperature was capable to intensely stimulate Na^+ accumulation in leaves, drastically decreasing K^+/Na^+ ratios. These responses caused strong stomatal closure, causing almost complete cessation in CO_2 assimilation. Remarkably, plants exposed to single heat displayed increased stomatal conductance and transpiration and caused no effect on photosynthesis. The combinate heat and salinity stress caused negative effects more intensely on the carboxylation phase (energy output) as compared with PSII activity and electron transport rates (energy input). This imbalance must have generated a great excess energy in chloroplasts. Unexpectedly, this energy accumulation did not aggravate oxidative stress as revealed by decreased H_2O_2 and lipid peroxidation levels associated with decrease in the contents of reduced ASA and GSH and increases in the activities of SOD, APX, GPX and GPOD enzymes. The significance these results for combined abiotic stresses in a scenario of global changes and heating is discussed.

MATERIAL AND METHODS

Plant growth and treatments conditions

Rice seeds (Oryza sativa spp. Japonica), from a salt sensitive cultivar (Nipponbare), 10-days after germination, were transferred to 2-L pots containing a ¼ diluted Hoagland-Arnon's nutritive solution (Hoagland and Arnon, 1950). The seedlings were grown initially in a greenhouse (average of maximum photosynthetically active radiation – PAR of 800 µmol m^{-2} s^{1}; average temperature of 27 °C; 12-h photoperiod; and average relative humidity of 68%). 31-days old plants were transferred to a growth chamber at 27 °C/24 °C temperatures (day/night), PAR of 600 µmol m^{-2} s^{-1} and 12h-photoperiod to acclimation for 2 days. Afterwards, a group of plants (salt treatment) received NaCl supplied in nutrient solution in two steps of 50 mM per day. After 8 days of salt treatment, the chamber temperature was gradually elevated to 42 °C in a rate of 4 °C/h, from 27 °C (6:00 a.m. – after turning on the lights), reaching 4 hours of heat acclimatization. At this time, plants were separated in four groups (treatments): nutrient solution + 27 °C (control); 100 mM NaCl dissolved in nutrient solution + 27 °C (salt stress); nutrient solution + 42 °C (heat treatment), and 100 mM NaCl + 42 °C (salt stress + heat) for a total of ten hours exposure. At the end of the experiments, fresh and dry mass were measured, and leaf samples (20 cm of leaf central portion from non-senescent mature leaves) were frozen in liquid N_2 and stored at -80 °C for biochemical determinations.

Leaf gas exchange and chlorophyll a fluorescence measurements

Gas exchange parameters were measured by using a portable infrared gas analyzer system (LI-6400XT, LI- COR, Lincoln, NE, USA) in mature leaves. PPFD and temperature inside the measurement chamber were kept at 1000 µmol m^{-2} s^{-1} and 28 °C, respectively. In all measurements, the amount of blue light was set up to 10% of the PPFD to maximize stomatal aperture (FLEXAS *et al.*, 2008), the CO_2 partial pressure was 40 Pa and the leaf-to-air vapor pressure difference was 1.85 ± 0.14 kPa. Measurements were recorded when the total coefficient of variation was lower than 5% and temporal stability was achieved (on average, 3 min after the beginning of each step). *In vivo* chlorophyll *a* fluorescence was measured using a fluorometer (LI-6400-40, LI-COR, Lincoln, NE, USA) coupled with the IRGA. The actinic light utilized for measuring the chlorophyll *a* fluorescence was 1000 μmol m^{-2} s^{-1} of PPFD. The fluorescence parameters were measured using the saturation pulse method (Schreiber, 2004) in leaves previously exposed to natural light conditions. The intensity and duration of the saturation light pulse were 8000 μmol m^{-2} s^{-1} and 0.7 s, respectively. The effective quantum yield of PSII was calculated [ΦPSII = (Fm'−Fs)/Fm'], where Fm' represents the maximum fluorescence and Fs the steady state fluorescence under actinic light conditions. The following fluorescence parameters were obtained: the maximum quantum yield of photosystem II (PSII) [$F_v/F_m = (F_m - F_o)/F_m$], the electron transport rate (ETR) [ETR = $\Delta F/F_m'$ x PPFD x 0.5 x 0.84)], the excitation capture efficiency of PSII open centers [$F_v'/F_m' = (F_m' - F_o')/F_m'$], the effective quantum yield of PSII [$\Delta F/F_m' = (F_m' - F_s)/F_m'$] and the photochemical (qP) and non-photochemical quenching coefficient [NPQ = $(F_m - F_m')/F_m'$], where F_m and F_o are, respectively, maximum and minimum fluorescence of dark-adapted leaves; F_m' and F_s are, respectively, maximum and steady state fluorescence in the light-adapted state and F_o' is minimum fluorescence after far-red illumination of the previously light-exposed leaves. The ratio ETR/P_N was calculated to estimate the use of electrons in other processes not related to photosynthetic CO_2 assimilation rate (Ribeiro et al., 2009).

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, lipid peroxidation and electrolyte leakage in leaves

Hydrogen peroxide content was detected by the titanium tetrachloride method in accordance with Brennan and Frenkel (1977). Fresh leaf samples immediately harvested were macerated with liquid N_2 in presence of 5% (w/v) TCA, centrifuged at 12,000 *g* (4 °C) and the supernatant was immediately used for H_2O_2 determination. The measurement at 415 nm was performed after reaction of the titanium reagent with H_2O_2 and formation of the hydroperoxide-titanium complex. H_2O_2 content was calculated at from a standard curve and the results were expressed as μmol H_2O_2 g^{-1} FW. Lipid peroxidation was determined by measuring the thiobarbituric acid-reactive substances (TBARS), according to Cakmak and Horst (1991), with minor modifications as described previously (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} FW. Membrane damage (MD) was measured by electrolyte leakage as described previously by Blum and Ebercon (1981). Twenty leaf segments (2.0 cm length) were placed in test tubes containing 20 mL deionized water. The tubes were incubated in a shaking water bath at 25 °C for 6 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 × 100.

Reduced ascorbate and glutathione determinations

Reduced ascorbate content (ASA) was assayed according to Kampfenkel et al. (1995). Fresh leaf samples were homogenized in 5% (w/v) TCA, centrifuged at 12,000 *g* (4 °C) for 20 min and the supernatant was then used. The assay is based on the reduction of Fe^{3+} to Fe^{2+} by ascorbate and the detection by spectrophotometry of the Fe^{2+} complex with 2,2`-bipirydyl and read at 525 nm. The reduced ascorbate content was expressed as μmol AsA g^{-1} FW. The reduced glutathione (GSH) content was assayed as described by Griffith (1980). Fresh leaf samples were homogenized in 5% (w/v) TCA, centrifuged at 12,000 *g* (4 °C) for 20 min and the supernatant was then used. The assay mixture was prepared by adding extract, DTNB (5,5-dithio-bis-(2-nitrobenzoic acid)) and 150 mM phosphate potassium buffer. The mixture was stabilized at 30 °C for 10 min. Then the absorbance was read at 412 nm in the spectrophotometer and the GSH content was expressed as μmol GSH g^{-1} FW.

Enzymatic extraction

Leaf samples (0.5 g FW) were ground to fine powder in presence of liquid N_2 in a mortar and pestle and extracted in 3 mL of ice-cold 100 mM K-phosphate buffer pH 6.8 for 5 min, containing 0.1 mM EDTA and 1 mM ascorbate to avoid APX denaturation. After filtration through cheesecloth, the homogenate was centrifuged at 4 °C at 15,000 *g* for 15 min and the obtained extract was used for determination of the enzymatic activities. The protein content of crude enzyme extracts was estimated according to Bradford (1976), using BSA as standard.

Enzyme activity assays

Ascorbate peroxidase (APX) activity was measured following ascorbate oxidation by the decrease in absorbance at 290 nm (Nakano and Asada, 1981). APX activity was assayed in a reaction mixture containing 0.5 mM ascorbate and 0.1 mM EDTA dissolved in 100 mM K-phosphate buffer (pH 7.0) and enzyme extract. The reaction was started by addition of 30 mM H_2O_2. The enzyme activity was measured by the decrease in absorbance at 290 nm at 25 °C over 300 s. APX activity was estimated utilizing the molar extinction coefficient of AsA (2.8 mM^{-1} cm^{-1}) and expressed as µmol H_2O_2 mg $protein^{-1}$ min^{-1}.

Glutathione peroxidase (GPX) activity was measured by the method of Awasthi et al. (1975) with cumene hydroperoxide as a substrate. Aliquots (0.1 mL) of the enzyme extract were mixed with a reaction mixture consisting of 4 mM GSH, 0.2 mM NADPH, 0.05 U of GR (type II from wheat; Sigma) and 0.5 mM substrate in phosphate buffer (0.1 M; pH 7.0). The GPX activity was determined by the decrease of NADPH absorption at 340 nm. The nonspecific NADPH decrease was corrected by using additional measurements without substrate. The GPX activity was estimated utilizing the molar extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}) and expressed as µmol NADPH mg $protein^{-1}$ min^{-1}.

SOD (Superoxide dismutase) activity was determined by inhibiting blue formazan production via nitroblue tetrazolium (NBT) photoreduction. The reaction was started by illumination (30 W fluorescent lamp) at 25 °C for 6 min, and the absorbance was measured at 540 nm (Giannopolitis and Ries 1977). One SOD activity unit (UA) was defined as the amount of enzyme required to inhibit 50% of NBT photoreduction, and the activity was expressed as UA mg protein−1 min−1 (Beauchamp and Fridovich 1971).

Catalase (CAT) activity was measured following the oxidation of H_2O_2 at 240 nm. CAT was determined after the reaction of the enzymatic extract in the presence of 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM H_2O_2. The reaction took place at 30 °C, with monitoring of the absorbance at 240 nm over 300 s (Havir and Mchale, 1987). CAT activity was calculated using the molar extinction coefficient of H_2O_2 (36 mM^{-1} cm^{-1}) and expressed as µmol H_2O_2 mg $protein^{-1}$ min^{-1}.

GPOD activity (Guaiacol peroxidase) was assayed by measuring the rate of pyrogallol oxidation at 430 nm by the method of Amako et al. (1994) with minor modifications. The GPOD assay mixture contained 50 mM phosphate buffer (pH 7.0), 20 mM pyrogallol as an electron donor, 0.1 mM hydrogen peroxide and enzyme extract. In order to avoid APX interference, two determinations were carried out in parallel as described above for APX activity assay. GPOD activity was estimated utilizing the molar extinction coefficient of pyrogallol (2.47 mM^{-1} cm^{-1}) and expressed as mmol pyrogallol mg $protein^{-1}$ min^{-1}.

Glycolate oxidase (GO) activity was assayed by the formation of glyoxylate-phenylhydrazone complex at 324 nm (Baker and Tolbert, 1966). The GO assay mixture contained 100 mM phosphate buffer (pH 8.3), 40 mM glycolic acid, 100 mM L-cysteine and 100 mM phenylhydrazine. The reaction was started with the addition of the 1 mM FMN and the absorbance was monitored over 300 s. GO activity was calculated using the molar extinction coefficient of the glyoxylate-phenylhydrazone complex (17 mM^{-1} cm^{-1}) and expressed as ηmol H_2O_2 produced mg $protein^{-1}$ min^{-1}.

Statistical analysis

The experiment was arranged in a completely randomized design, with four independent replicates, each consisting of one pot containing three plants. Data were analyzed by ANOVA and means were compared by the Tukey´s test at the 0.05 level of confidence.

Next, multivariate statistical analysis was performed. Data was analyzed by partial least squares discriminant analysis (PLS-DA) using the Metaboanalyst platform (Chong et al. 2018). Beyond differentiating sample groups, PLS-DA also provides variable importance in projection (VIP) scores of the main parameters responsible for the discrimination found between the two major components of the PLS-DA. Parameters with VIP scores higher than 1.0 are considered most greatly representatives of the PLS-DA. The data was normalized by log transformation and mean centering options in the Metaboanalyst® 5.0 platform.

RESULTS

High temperature strongly stimulates Na^+ transport towards leaves significantly decreasing K^+/Na^+ ratios and aggravating osmotic and ionic effects

Rice plants previously exposed to 100 mM NaCl for eight days followed by exposure to high temperature (42 °C) for ten hours (a short-term heat shock) displayed immense increase in the Na^+ accumulation in leaves – by approx. 100% (Fig. 1A). In these plants the leaf K^+ content was not altered by heat treatment (Fig. 1B) and consequently the K^+/Na^+ ratios decreased by near to two-fold, reaching values so low as 0.32, whereas control plants showed values of 6.75 (Fig. 1C). Interestingly, single heat treatment only stimulated leaf K^+ accumulation in not salt-treated plants in parallel to significant raise in stomatal conductance and transpiration rates (Table 1). The drastic disturbances caused by high temperature on ionic homeostasis and Na^+ accumulation in leaves induced strong restriction in stomatal conductance, transpiration rates and decreased intercellular CO_2 concentration (Ci), accompanied by reduction in leaf water status as indicated by significant reduction in relative water content – RWC (Table 1). Single salt stress induced reduction of approx. 40% in shoot dry mass accumulation, but high temperature did not affect that trait possibly due to low time of exposure (10 hours).

Heat and salt interaction strongly affect CO_2 assimilation but reduced PSII activity and electron transport in a lower intensity

High temperature induced drastic reduction in photosynthetic CO_2 assimilation of salt stressed rice, decreasing the values from 5.45 to 1.09 umol m^{-2} s^{-1}, representing a decrease of six-fold (Fig. 2A). This strong decrease was accompanied by a proportional decrease in P_N/Ci ratios (Fig. 2B), suggesting that the decrease in CO_2 assimilation could have been associated with reduction in Rubisco carboxylation rates. However, the stomatal closure induced by heat and salt stress interaction was almost complete reaching values near to zero (0.03) compared with the g_s control of 0.88 mol m^{-2} s^{-1} (Table 1). This result can indicate that stomatal restrictions could have been preponderant to impairment in CO_2 assimilation, but the ionic effects caused by excess Na^+ accumulation on photosynthesis does not should be discarded. Anyway, the current results evidence that drastic effects caused by the negative interaction between heat and salinity on CO_2 assimilation could have involved both osmotic and ionic effects.

Remarkably, the severe negative effects caused by high temperature on CO_2 assimilation and gas exchange parameters of salt stressed plants were not observed on the PSII activity related parameters (Fig. 3 and Table S1). In fact, the combined salt + heat treatment induced only slight decrease on potential quantum yield of PSII (Fv/Fm), a parameter associated with PSII integrity. In addition, the actual quantum yield of PSII (ΦPSII), a parameter related to functional PSII activity, and its derivative electron transport rate of PSII (ETR) were also decreased in a lesser extension. The same trend was observed for other photochemical parameters 1-qP, NPQ and Fo (Table S1), strongly suggesting that integrity and photochemical activity of PSII were less affected by high temperature + salt combined stress. Because of this unbalance between CO_2 assimilation and PSII activity performances, the values of ETR/P_N ratios raised approx. 4-fold in combined stress (Fig. 3B). This strongly increased values might indicate occurrence of excess energy in chloroplasts associated with imbalance between input and output of photochemical energy in PSII.

Provable energy excess in chloroplasts did not enhance ROS accumulation due to stimulation of antioxidant defense by heat in salt treated plants

Although high temperature has drastically intensified salt stress and caused impairment in CO_2 assimilation in parallel to strong imbalance between input and output of energy in chloroplasts, it did not stimulate ROS overaccumulation on leaves as expected, as indicated by decreased levels of H_2O_2 and lipid peroxidation, compared to salt stress alone (Fig. 4). However, the combined effects of high temperature and salt stress strongly caused increase in leaf membrane damage from 37% to 65%, evidencing intense physiological stress possibly caused essentially by salt (osmotic + ionic effects) and not by oxidative stress. The heat-induced oxidative stress attenuation also was evidenced by significant decreases in the contents of both glutathione (GSH) and ascorbate (ASA) in their reduced states (Table 3). Indeed, these non-enzymatic antioxidants are commonly and intensely utilized as reducing to reduce and minimize ROS overaccumulation, especially H_2O_2.

Corroborating the stimulation of antioxidant defense induced by heat in salt stressed plants, the activities of some important antioxidant enzymes were stimulated or maintained at elevated levels in that treatment. In fact, the activities of ascorbate peroxidases (APX), phenol peroxidases (GPOD) and superoxide dismutases (SOD) were stimulated whereas the activities of glutathione peroxidases (GPX), glycolate oxidases (GO) were maintained in suitable levels, whereas catalase activity (CAT) was the unique to be decreased drastically in rice leaves exposed to high temperature and salinity (Figure 5 and Table S2). Thus, despite the heat treatment has been favorable to avoid ROS overaccumulation in salt stressed plants, it was not enough to avoid intensification of salt stress mainly an almost complete impairment in CO_2 assimilation followed by intense imbalance in energy input by PSII reactions and energy output by CO_2 assimilation.

Multivariate and grouping analyzes revelated that high temperature + salt stress

represented a unique combination in comparison with effects of individual factors

To verify if high temperature combined with salt stress displays unique responses in comparison with these isolated factors, we performed a multivariate and group discrimination analyzes employing photosynthetic, ionic homeostasis and redox metabolism variables. The partial least squares discriminant analysis (PLS-DA) employing photosynthetic, ionic homeostasis and stress indicators (physiological variables) clearly evidenced that the four treatment groups (control, heat, salt, and salt + heat) displayed contrasting spatial localizations after the multivariate analysis (Fig. S1 A). The group arrangement clearly indicated that heat + salt treatment occupied a localization fully contrasting in relation to the other treatments and, interestingly, single salt presented a position more near to control than heat stress. Moreover, the localization of heat + salt combination strongly placed to the fourth quadrant at the right extreme whereas single heat was placed on the second quadrant (left) and isolated salt stress at begin of the third quadrant.

The variable importance in projection (VIP) analysis indicated that gs, K^+/Na^+, Na^+, E, P_N, P_N/Ci and ETR/P_N were the most important physiological variables to PLS-DA results. In addition, the heat map localized at right of the graphic, represents the response (decreased or raised) of each specific variable within each treatment. Except for Na^+, ETR/P_N, NPQ and 1-qP, the combination heat + salt significantly decreased all other variables in relation to control and another treatment (Fig. S1 B). When the PLS-DA analysis was performed employing all redox metabolism variables the result was like that obtained employing physiological variables, but the discrimination between the treatments was less evident. Indeed, in this case, the heat + salt treatment also exhibited a very contrasting localization compared with control and single heat, but the differences between isolated salt and heat + salt were lower than those observed employing physiological indicators (Fig. S1 A and Fig. S2 A). In this analysis, the most important redox variables to PLS-DA discrimination were GSH, ASA, APX, SOD, H_2O_2, and GPOD. The activities of virtually all antioxidant enzymes, except CAT, displayed increased values in heat + salt treatment (Fig. S2 B).

DISCUSSION

High temperature drastically aggravates salt stress provably by stimulating Na^+ transport towards leaves

Studies with multiple stressors in plants have revealed that stress combination effects largely differ from the plant response to isolated stressors, and these differences are intensified when stress factors exhibit antagonistic interactions (Nahar et al., 2023). In the present study, we initially evaluated if a salt-sensitive rice cultivar displays negative or positive interaction among previously induced salt stress followed by short-term exposure to high temperature. Initially, we investigated how this interaction could occur at the level of Na^+ and K^+ transport and accumulation towards leaves, affecting the K^+/Na^+ homeostasis and inducing ionic stress. Secondly and consequently, how heat could aggravate the osmotic effects caused by Na^+ accumulation in leaves as indicated by induction of stomatal closure and impairment in transpiration.

Plant responses to multiple stresses of high temperature and salinity differs among species in accordance with the level of salinity and heat tolerance/sensitivity. For instance, in wheat and Arabidopsis the damaging effects of salt stress and heat applied individually were exacerbated by the combination of these stressors (Keles and Oncel 2002; Suzuki et al., 2016). In tomato plants, however, the combination of salinity and high temperature enhanced the protection against the deleterious effects caused on some aspects of plant metabolism in comparison with isolated salinity (Rivero et al. 2014). Similarly, in *Anacardium occidentale* (cashew nuts), adapted species to semiarid regions, displayed a positive interaction after combination of salinity followed by high temperature (Ferreira-Silva et al., 2011). In contrast, a study conducted by Suzuki et al. (2016) reported that this stress combination had a significant negative impact on Arabidopsis in terms of growth, chlorophyll content, and plant survival.

Rice is a sensitive species to salinity despite the salt sensitivity widely vary among cultivars (Nahar et al., 2022). Studies have shown that salinity displaying electrical conductivity around 1.9 dSm^{-1} (roughly equivalent to 20 mM NaCl) for long-term exposure can decrease grain yield in rice. Moreover, salinity equivalent to 30 mM NaCl can impact the yield of most cultivated rice genotypes (Hoang et al., 2016). Concerning high temperatures, rice is also a heat sensitive species, but the level of heat stress sensibility depends on other factors such as genotype, development stage, and temperature intensity (You et al., 2023). For example, a recent study carried out with rice exposed to the combined stress of salinity and high temperature has shown that moderate heat (30 °C) did not intensify the salt stress damages (Nahar et al., 2022). In contrast, when a high temperature (42 °C) was applied even for a short-term exposure as that supplied in this current study the results reveal that the salt stress and photosynthesis were drastically affected by the interaction salt stress x heat. By which mechanisms the combination of salinity followed by high temperature can result in negative or positive interaction for rice performance?

The most important mechanisms underlying to Na^+ accumulation in leaves and consequently the intensity of osmotic and ionic adverse effects is understanding its transport from roots towards shoot. Several Na^+-transporters (SOS1, NHX1, HKT2;1, HKT1;5) (Keisham et al., 2018), including H^+-PPases and H^+-ATPases (Zhang et al., 2006, Mariamma et al., 1997), are involved with Na^+ transport in plants in general and rice in particular (Keisham et al., 2018; Nahar et al., 2022). These transporters act in an integrate way to regulate the levels of uptake, short- and long-distance transport, determining in last instance, the Na^+ amounts accumulated in determined parts of plants (roots, stem, leaves) and cellular compartments such as apoplast, cytosol, vacuoles, and other organelles. These transporters act also in the transport in direction root – xylem – leaves contributing immensely to overall Na^+ accumulation in rice leaves.

The Na^+-transporters can act in direction of the inclusion and exclusion of Na^+ ions and the balance all activities will determine the intensity of accumulation in a specific local of the cell as cytosol for instance. Unfortunately, much less is known on the effects of high temperature in short-term on the expression and activities of those several Na^+-transporters in plant species in general and more scarcely in rice plants (Keisham et al., 2018). Anyway, in a very simplified manner, the Na^+ accumulation in rice leaves might represent a net balance among all transporters in a determined physiological-environmental circumstance. In our current study, as has been amply noticed in literature, salt stress was accompanied by high Na^+ accumulation followed by decreased stomatal conductance in rice leaves, evidencing osmotic and ionic effects (Silveira and Carvalho, 2016).

Interestingly, the presence of heat for only ten hours is enough to increase in twice as much the Na^+ content in leaves, clearly evidencing a great stimulation in Na^+ transport from root medium towards leaves. In parallel, these plants exposed to salt stress followed by heat also display an intense increase in membrane damage and a great decrease in leaf K^+/Na^+ ratios, evidencing breakdown of cytosolic ionic homeostasis (Farooq et al., 2021). Altogether, these data evidence intense action of osmotic and ionic causes as induced by high temperature, possibly via induction of Na^+ transporters since leaf K^+ accumulation is not affected by heat in plants previously exposed to NaCl-salinity.

These outcomes tightly indicate that high temperature strongly aggravates the salt stress in rice plants, associated with higher Na^+ transport and accumulation in rice leaves.

Indeed, high temperatures can alter the membrane fluidity, impairing its selectivity and leading to the disruption of membrane-bound ion transporters related to Na^+ transport, such as H^+-PPase and H^+-ATPase (Zhang et al., 2006, Mariamma et al., 1997). It is also known that high H^+-ATPase activity is crucial for salt tolerance and ionic balance (Morsomme and Boutry, 2000). Considering that the time of heat exposure was only of 10 hours, it is possible to propose that this rise in Na^+ accumulation induced by high temperature could has been related to increase in activity of transporters involved in long-distance Na^+ transport from root to shoot as is widely known (Farooq et al., 2021).

Na^+ accumulation in plant cells can induce several direct and indirect causes to metabolism disturbances (Hauser and Horie, 2010). The cytosol of plant cells typically presents concentrations of K^+ and Na^+ ranging around 100–200 mM and 1–10 mM, respectively (Farooq et al., 2021). In addition to toxicity directly caused by Na^+ ions, the Na^+ over-accumulation in the cytosol also can induce K^+ efflux to apoplast stimulating a deficiency in this nutrient since it is a crucial ion which participates in several metabolic processes. K^+ is a co-factor of more than 50 enzymes and it plays roles in the regulation of cytosolic pH, protein synthesis, and stomatal movement (Wu, 2018; Liu et al., 2021). Moreover, K^+-deficiency and Na^+ overaccumulation are also involved in some metabolic processes associated with photosynthetic efficiency (Pan et al., 2020). Unfortunately, the direct involvement of these ions on photosynthesis in salinity conditions is scarcely known yet.

CO_2 assimilation is extremely affected in combination of salt stress followed by high temperature generating unbalance and excess energy in PSII

Remarkably, in this study high temperature supplied on previously salt stressed rice plants induces very contrasting effects on photosystem II activity (energy input) and CO_2 assimilation rates (energy output). Possibly, these differential effects were induced firstly by osmotic causes induced by the great Na^+ accumulation in roots and leaves, causing stomatal closure, followed by impairment in CO_2 and transpiration (Silveira and Carvalho, 2016). Further, the intense Na^+ accumulation in leaves and possibly inside chloroplasts induced by heat shock caused ionic effects associated with K^+/Na^+ homeostasis breakdown. Indeed, the stomatal conductance in that circumstance was reduced to values near to zero. However, it’s very intriguing that in that condition all PSII activity and integrity parameters show slight changes in plants exposed to salinity followed by heat. Salt accumulation can impact negatively both photosynthetic processes, the carbon assimilation and photochemical reaction (Wungrampha et al., 2018; Pan et al., 2020).

In contrast to gas exchange processes, the activity and integrity of photosystem II was much less affected by the combination salt + heat suggesting that the osmotic effects were more preponderant than ionic causes, since some evidences have suggested that excess Na^+ and imbalances in K^+/Na^+ ratios inside chloroplasts induce damages on photosystem II reactions and structure (Ahmadizadeh et al. 2016; Nongpiur et al. 2016). In fact, in this current study ΦPSII and Fv/Fm were much less affected by salt + heat combination in comparison with CO_2 assimilation, associated with minor alterations in 1-qP and ETR, indicating the capacity of capture and transfer of energy via PSII (input) was much less affected than CO_2 assimilation (output). This imbalanced condition induces a strong increase in ETR/P_N ratios, a robust indicator of high availability of non-utilized electrons in chloroplasts by the Calvin-Benson cycle.

That condition of excess energy in photosystems might lead to a high energy pressure on thylakoid membranes favoring ROS overaccumulation in PSII and PSI (Lima-Melo et al., 2019; Lima-Neto et al., 2014; Foyer, 2018; Ahmadizadeh et al. 2016; Nongpiur et al. 2016). Several studies have shown that PSII activity can be inhibited by very high Na^+ accumulation and, consequently, the electron transport chain could be disrupted (Oukarroum et al., 2015; Belkhodja et al. 1994). The intense increase in ETR/P_N ratios accompanied by increases in 1-qP and NPQ parameters in leaves of combined salt stress followed by high temperature, strongly suggest a condition of excess energy in chloroplasts (Lima-Neto et al., 2014; Lima-Melo et al., 2019). This physiological condition is highly favorable to overaccumulation of ROS at levels of PSII and PSI, which can cause intense oxidative stress in virtually all parts of the leaves after continued exposure to combined stress conditions.

Excess energy generated in response to heat combined with salt stress doesn't aggravate oxidative stress due triggering of antioxidant defense

The effects of high temperature and salt stress combination on aggravating or attenuating of physiological and oxidative stress associated with photosynthesis impairment are complex, controversial and the underlying mechanisms are scarcely understood (Sousa et al., 2023). As this combination involves interactive processes, the resulting effects are in fact unpredictable and probabilistic. Some experimental results have evidenced that combination of heat and salt stress can display antagonist and negative interaction on photosynthesis, accompanied by generation of oxidative stress (Suzuki et al., 2016). In opposition, in dependence of salinity type and heat intensity, some works have shown that this interaction can positive, especially depending upon plant species types (Ferreira-Silva et al., 2011; Nahar et al., 2023; Rivero et al., 2014).

An imbalance between energy capture/transference by PSII and activities of consumer centers such as CO_2 assimilation by Calvin-Benson cycle is widely known as generator of excess energy in chloroplasts (Hameed et al., 2021). The more immediate consequence this energy unbalance is over accumulation of ROS at PSII and PSI levels, which can be further generalized to chloroplasts and other organelles. However, plant species are capable to trigger down-regulation in energy capture and transport associated with PSII photoinhibition, excess energy dissipation and triggering of effective antioxidant systems (Bilal et al., 2023; Foyer 2018; Pan et al., 2020). Thus, the excess energy produced inside chloroplasts provoked by imbalance between input and output of energy and electron fluxes might generate or not ROS overaccumulation and oxidative stress (Sousa et al., 2023).

Anacardium occidentale, a thermotolerant species adapted to semi-arid conditions, displays an effective antioxidant defense system in response to salinity followed by high temperature (Ferreira-Silva et al., 2011). In this species, the triggering of antioxidant response occurs in parallel to a down regulation in PSII activity associated with intense non-photochemical energy dissipation and dynamic PSII photo-inhibition, maintaining a relative equilibrium between PSII and CO_2 assimilation activities, avoiding occurrence of drastic physiological stress, as revealed by absence of significant oxidative stress and membrane damages. In these circumstances, that plant species displays an effective antioxidant response especially represented by utilization of non-enzymatic antioxidant reducing ASA and GSH, accompanied by strong stimulation in the activities of SOD, APX and CAT, inducing an effective protection as revealed by intense reduction in lipid peroxidation and H_2O_2 levels (Ferreira-Silva et al., 2011).

In contrast, *Jatropha curcas*, a plant species highly drought tolerant but relatively salt sensitive (Silva et al., 2010; Silva et al., 2011; Lima-Neto et al., 2014), when exposed to salinity followed by heat shock displays a negative interaction. In fact, that combination strongly aggravate the salinity effects on CO_2 assimilation inducing an almost complete stomatal closure and negatively affecting K^+/Na^+ homeostasis, exacerbating salt stress (Silva et al., 2012). These responses are accompanied by increase in lipid peroxidation and membrane damages, whereas the activities of some antioxidant enzymes such as SOD, APX and CAT are slightly altered and accompanied by decrease in ASA redox state. These adverse oxidative effects caused by salt stress in *Jatropha curcas* are associated with excess energy generated in chloroplasts by imbalances between the photochemical phase and strong decrease in CO_2 assimilation (Lima-Neto et al., 2014).

Interestingly, recent work with rice exposed to salinity and mild temperature evidenced that this specific condition of combination triggered a positive interaction in contrast to our current results employing a high temperature. Considering other published works involving different species, plant age, salt, and heat doses, among other factors, (Bilal et al., 2023; Ferreira-Silva et al., 2011; Rivero et al., 2014; Suzuki et al., 2016), it's possible to infer that plant responses to salt and heat combination is strongly dependent on these conditions. In another terms, as these factors strongly interact among their and the emergent propriety resulting from these interactions are complex and unpredictable, it is virtually impossible predict if such interactions could be positive or negative. As plants are self-organized complex dynamic systems, their stress states are dependent on homeostasis balance in response to interaction plant – stressful factors.

SUMMARY AND CONCLUSION

Interaction between high temperature and salt stress displays distinct responses on different physiological processes in rice plants (summarized by Fig. 6). In a temporal scale, heat intensely stimulates Na^+ transport towards leaves, stimulating its accumulation followed by great intensification in osmotic and drastic ionic effects, as revealed intense decrease in K^+/Na^+ ratios. In parallel, the combined stress induces intense stomatal closure associated with an almost complete impairment in CO_2 assimilation. Interestingly, energy capture and transport (input) from PSII is less affected in combined stress as revealed by photochemical parameters Fv/Fm, ΦPSII and 1-qP which are altered in a minor extension compared to CO_2 assimilation by Calvin-Benson cycle. This unbalanced condition tends to progressively generate excess energy in chloroplasts creating metabolic and physiological disturbances including ROS overaccumulation. Remarkably, rice leaves exposed to that adverse condition are capable to trigger an antioxidant system represented by ASA and GSH in its reduced forms associated with increased activities of SOD, APX, GPX, and GPOD activities. This antioxidant defense is capable to decrease H_2O_2 levels and lipid peroxidation as compared to single salt stress. However, that antioxidant response is not enough to avoid physiological disturbances, as indicated by drastic increase in leaf membrane damage. In conclusion, high temperature supplied in short-term for salt stressed rice causes dangerous effects essentially associated with intensification of osmotic stress and K^+/Na^+ homeostasis breakdown associated with impairment in CO_2 assimilation.

Statements & Declarations

Funding

This work was supported by the National Institute of Science and Technology in Plant Physiology under Stress Conditions (INCT Plant Stress Physiology – Grant: 406455/2022–8), INCT Plant Stress Biotech (465480/2014-4) and the National Council for Scientific and Technological Development (CNPq, Grant No. 404817/2021–1).

Conflict of interest

The authors declare no conflict of interest.

FIGURES, TABLES AND CAPTIONS

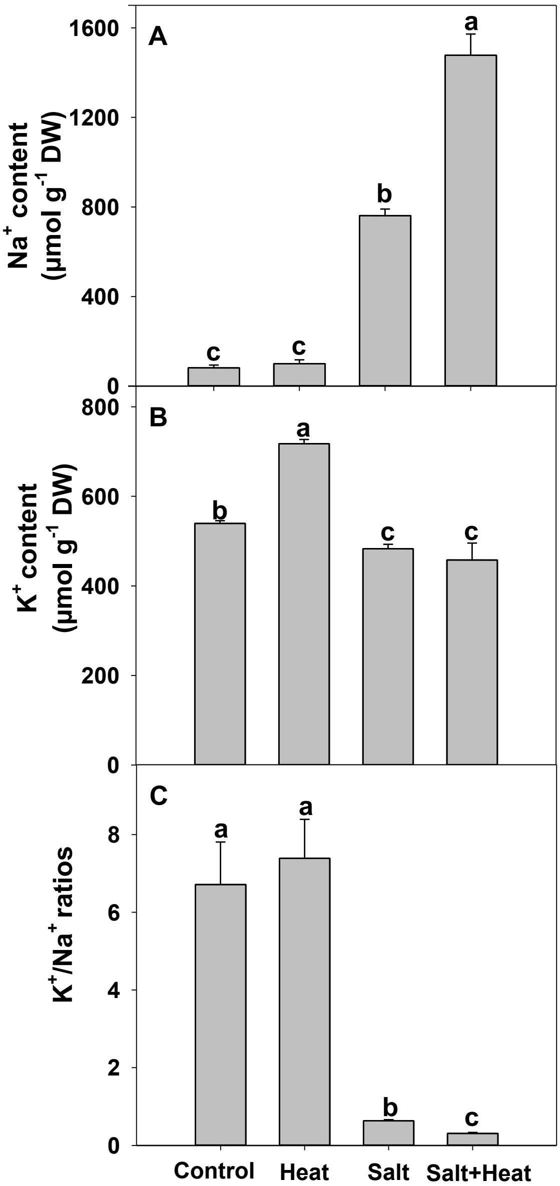


Figure 1. High temperature aggravates breakdown of K^+/Na^+ homeostasis in salt stressed plants. Effects of multiple treatments (control, heat, salt, and salt + heat) on Na^+, and K^+ accumulation and changes in K^+/Na^+ ratios in leaves of 31-day old rice plants. (A) Na^+ content, (B) K^+ content, and (C) K^+/Na^+ ratios. Plants were previously exposed to 0 and 100 mM NaCl salinity for 8 days at 27 °C and afterwards exposed to 27 °C or 42 °C for 10 hours, as described in M&M. Different lower-case letters represent significant differences among treatments at $p<0.05$ indicated by the Tuckey's test.

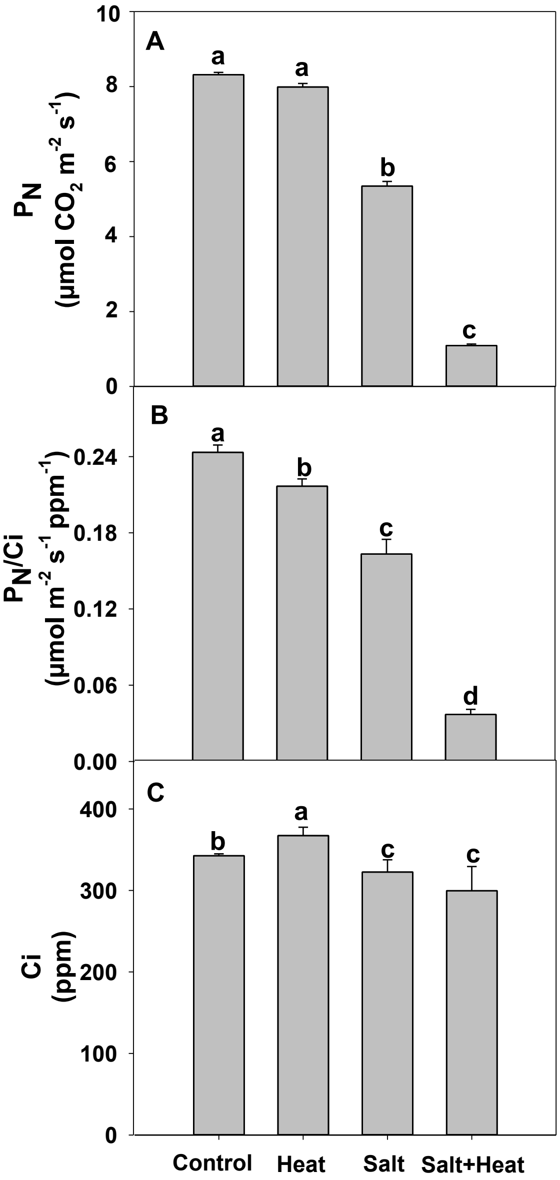


Figure 2. High temperature induces drastic impairment in CO_2 assimilation of salt stressed plants. Differential effects of control, single heat, isolated salt, and combined heat + salt treatments on (A) Net photosynthesis (P_N), (B) instantaneous carboxylation efficiency (P_N/Ci), and (C) intercellular CO_2 concentration (Ci) in leaves of 31-day old rice plants. Different lower-case letters represent significant differences among treatments at $p<0.05$ indicated by Tuckey's test.

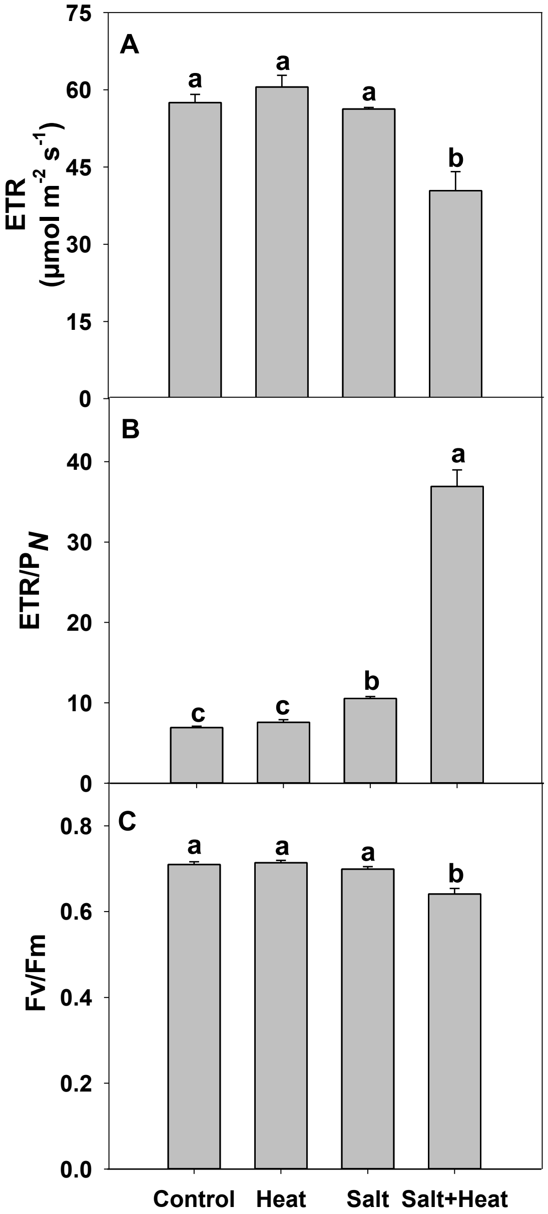


Figure 3. High temperature induces slight decrease in PSII activity and integrity in salt stressed plants. Effects of multiple treatments (control, heat, salt, and salt + heat) in 31-day old rice plants. (A) electron transport rate of PSII (ETR), (B) ratios between electron transport rate of PSII and CO_2 assimilation rate (ETR/P_N), and (C) maximum quantum yield of PSII (Fv/Fm). Different lower-case letters represent significant differences at $p<0.05$ by the Tuckey's test.

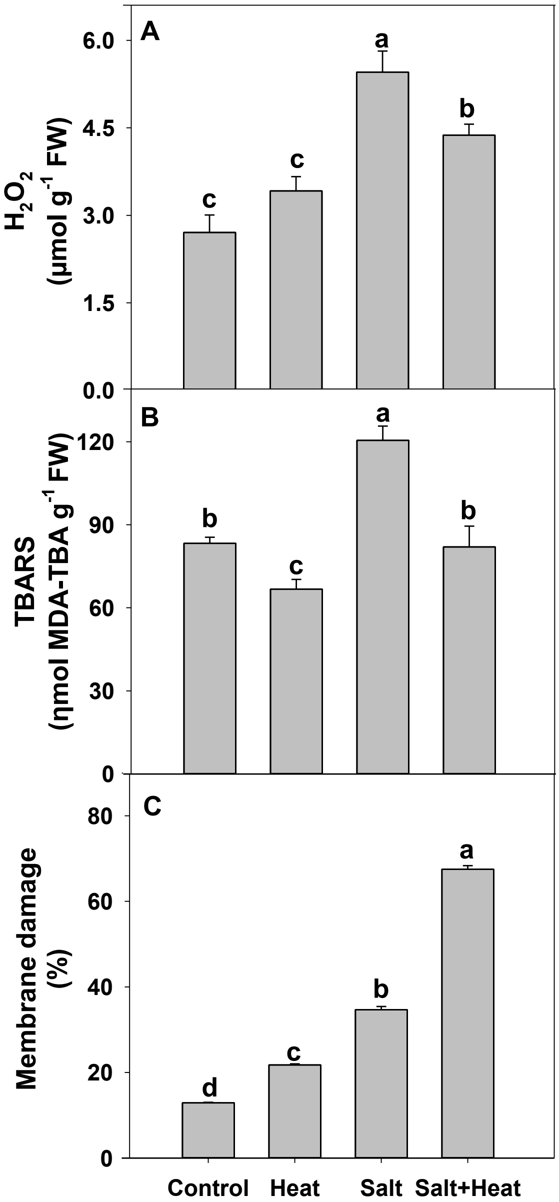


Figure 4. High temperature combined with salt decreases oxidative stress indicators but strongly increases membrane damage. Effects of multiple treatments (control, heat, salt, and salt + heat) on oxidative stress indicators of 31-day old rice plants. Changes in the contents of (A) hydrogen peroxide, (B) lipid peroxidation, and (C) membrane damage. Different lower-case letters represent significant differences at $p<0.05$ by the Tuckey's test.

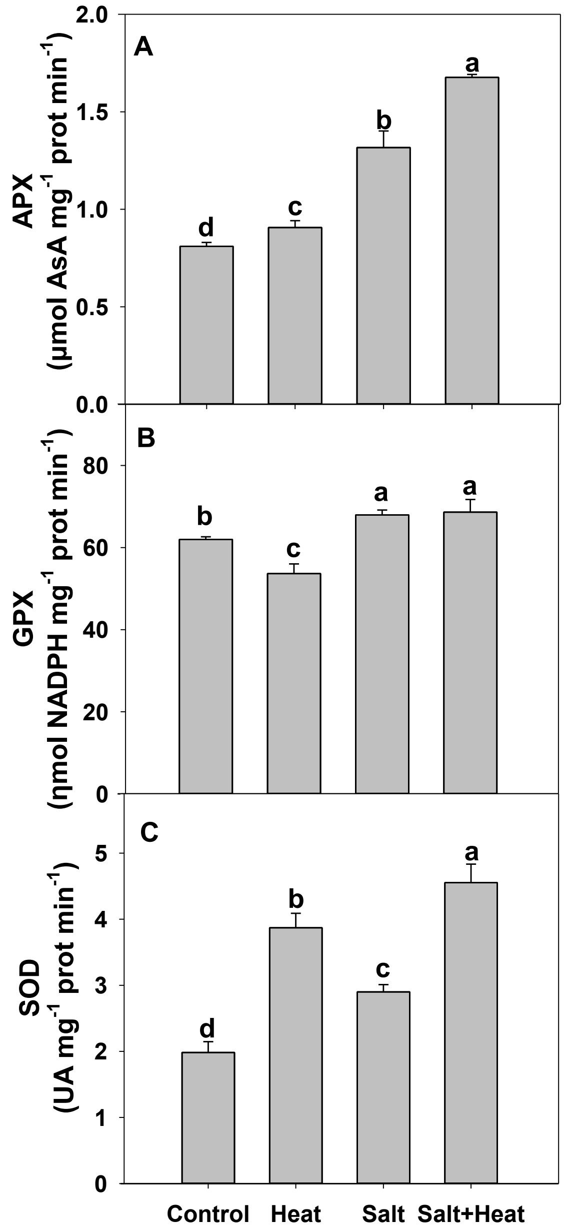


Figure 5. High temperature stimulates activities of antioxidant enzymes in salt stressed plants. Effects of multiple treatments (control, heat, salt, and salt + heat) on activities of (A) ascorbate peroxidase, (B) glutathione peroxidase, and (C) superoxide dismutase in leaves of 31-day old rice plants Different lower-case letters represent significant differences among treatments at $p<0.05$ by the Tuckey's test.

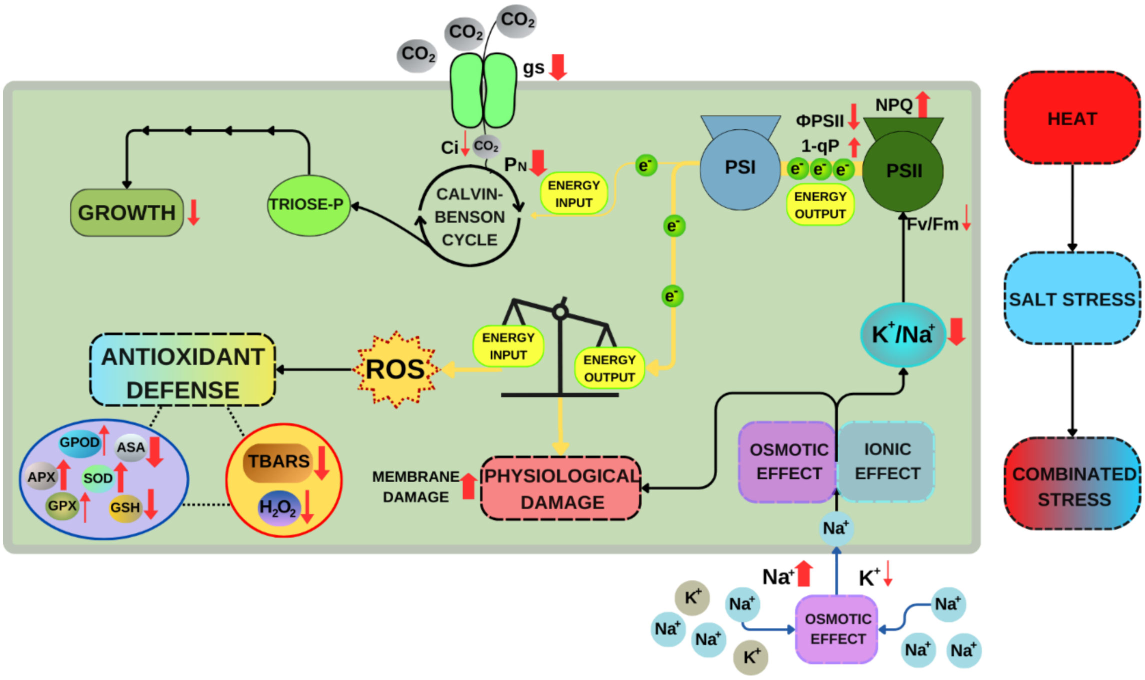


Figure 6. Heat combined with salt stress induce several disturbances on ionic homeostasis, photosynthesis, and redox metabolism. Scheme representing effects of the interaction between heat and salinity in 31-day old rice plants. In a chronological sequence, high temperature increases Na$^+$ transport towards leaves, inducing osmotic effects followed by alterations in ionic homeostasis represented by intense decrease in K$^+$/Na$^+$ ratios. These osmotic effects induce fast and intense stomatal closure reducing the intercellular CO2 concentrations and causing drastic impairment in Rubisco carboxylation. As the PSII activity is lesser reduced by combination of heat and salt, indicated by slight decrease in ETR and low increase in 1-qP values, compared with CO2 assimilation, it's generated an energy imbalance inside chloroplasts (energy input > energy output). That imbalance tends to stimulate ROS production but, unexpectedly, the interaction heat – salinity was capable to stimulate antioxidant defenses indicated by reduction in the levels of H2O2 and lipid peroxidation associated with consumption of reduced ASA and GSH. This antioxidant action was accompanied by increases in the activities of SOD, APX, GPX, and GPOD enzymes. The red arrows indicate intensities of changes (increase/decrease) in specific physiological processes and their width are in scale.

Table 1. Changes related to gas exchange, water use efficiency, and biomass accumulation in in 31-day old rice plants exposed to multiple treatments (control, heat, salt, and salt + heat). E, transpiration rate; gs, stomatal conductance; WUE, water use efficiency; RWC, relative water content, and shoot dry weight.

Treatments	**E** **(mmol H_2O m^{-2} s^{-1})**	**gs** **(mol H_2O m^{-2} s^{-1})**	**WUE** **(µmol CO_2/ mmol H_2O)**	**RWC** **(%)**	**Shoot weight** **(g DW plant)**
Control	4.93 **b**	0.88 **a**	1.69 **a**	92.00 **a**	9.86 **a**
Heat	6.16 **a**	1.86 **b**	1.29 **b**	90.50 **a**	10.28 **a**
Salt	2.83 **c**	0.20 **c**	1.77 **a**	91.00 **a**	6.05 **b**
Salt + heat	0.66 **d**	0.03 **d**	1.67 **a**	71.00 **b**	6.33 **b**

Letters represent significant differences among treatments at a confidence level of 0.05. The data are presented as the average from four replicates and compared by the Tukey's test ($p<0.05$).

SUPPLENTARY MATERIAL

Table S1. Changes related to photosystem II activity in leaves of 31-day old rice plants exposed to multiple treatments (control, heat, salt, and salt + heat).
Φ PSII, effective quantum yield of PSII; qP, photochemical quenching; NPQ, non-photochemical quenching; and Fo, initial fluorescence of PSII.

Treatments	**ΦPSII**	**1-qP**	**NPQ**	**F$_0$**
Control	0.34 **a**	0.32 **b**	0.44 **a**	388.33 **ab**
Heat	0.36 **a**	0.28 **b**	0.39 **b**	376.01 **b**
Salt	0.34 **a**	0.33 **b**	0.37 **c**	391.50 **a**
Salt + heat	0.26 **b**	0.44 **a**	0.59 **d**	383.52 **ab**

Different letters represent significant differences among treatments at a confidence level of 0.05. The data are presented as average from four replicates compared by the Tukey's test.

Table S2. Activities of catalases (CAT), phenol peroxidases (GPOD), and glycolate oxidases (GO) and contents of reduced ascorbate (ASA) and glutathione (GSH) non-enzymatic antioxidant in leaves of 31-day old rice plants exposed to multiple treatments (control, heat, salt, and salt + heat).

Treatments	**CAT**	**GPOD**	**GO**	**GSH**	**ASA**
	(µmol H_2O_2 mg^{-1} prot min^{-1})	**(µmol pyrogallol mg^{-1} prot min^{-1})**	**(ηmol H_2O_2 mg^{-1} prot min^{-1})**	**(µmol g^{-1} FW)**	
Control	54.00 **a**	4.83 **c**	62.41 **c**	1.29 **a**	5.70 **b**
Heat	47.00 **b**	4.43 **c**	77.53 **a**	1.08 **b**	7.09 **a**
Salt	51.33 **a**	6.90 **b**	79.95 **a**	0.64 **c**	4.48 **c**
Salt + heat	36.67 **c**	7.73 **a**	71.85 **b**	0.49 **d**	2.52 **d**

Different letters represent significant differences among treatments at a confidence level of 0.05. The data are averages of four replicates and compared by the Tukey's test.

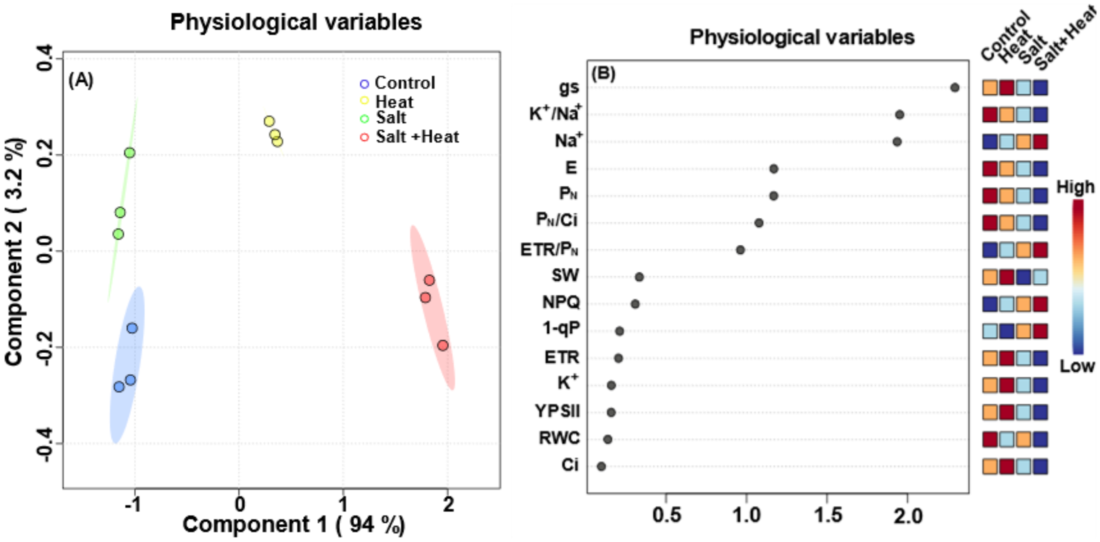


Figure S1. Group discrimination analysis reveals that combination of heat and salt display strong and unique interaction. (A) Partial least squares discriminant analysis (PLS-DA) for group discrimination and (B) variable importance in projection (VIP) scores using physiological (photosynthetic and ionic homeostasis) data in leaves of 31-day old rice plants under multiple treatments (control, heat, salt, and salt + heat). The heat map localized at right side of (B) represents changes (decreased or increased) of specific variables within each treatment. These analyses were carried out using Metaboanalyst.

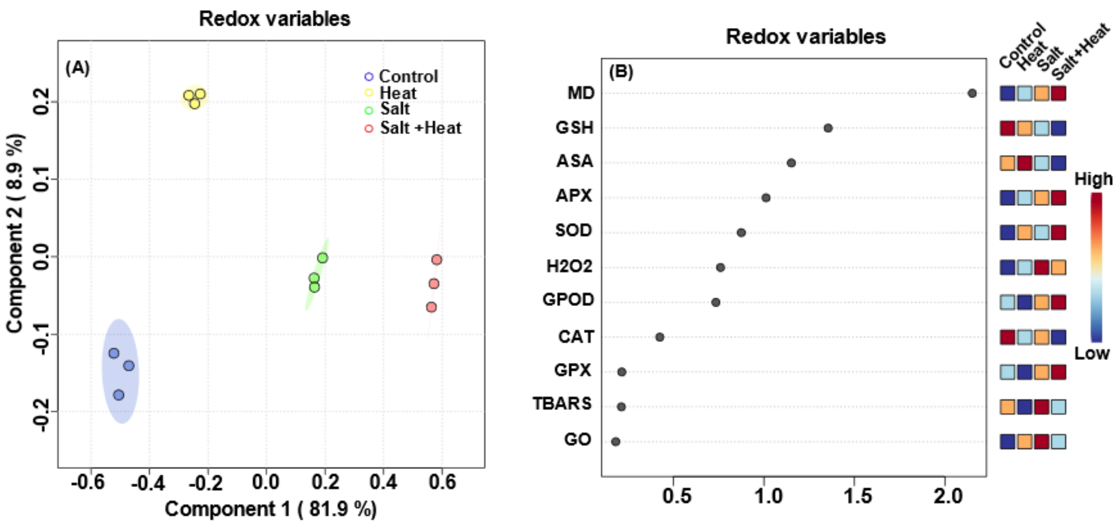


Figure S2. Group discrimination analysis reveals that combination of heat and salt display using redox metabolism variables are less distinct in comparison with isolated salt. (A) Partial least squares discriminant analysis (PLS-DA) for group discrimination and (B) variable importance in projection (VIP) scores using redox metabolism data in leaves of 31-day old rice plants under multiple treatments (control, heat, salt, and salt + heat). The heat map localized at right side of (B) represents changes (decreased or increased) of specific variables within each treatment. These analyses were carried out using Metaboanalyst.

REFERENCES

Ahmadizadeh M, Vispo NA, Calapit-Palao CDO, Pangaan ID, Viña CD, Singh RK (2016). Reproductive stage salinity tolerance in rice: a complex trait to phenotype. Indian Journal of Plant Physiology, 21: 528-536. https://doi.org/10.1007/s40502-016-0268-6

Amako K, Chen GX, Asada K. (1994) Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. Plant Cell Physiology 35: 497-504. https://doi.org/10.1093/oxfordjournals.pcp.a078621

Assaha DV, Ueda A, Saneoka H, Al-Yahyai R, Yaish MW (2017). The role of Na^+ and K^+ transporters in salt stress adaptation in glycophytes. Frontiers in physiology, 8: 509. https://doi.org/10.3389/fphys.2017.00509

Awasthi YC, Beutler E, Srivastava SK (1975) Purification and properties of human erythrocyte Glutathione Peroxidase. The Journal of Biological Chemistry 250: 5144-5149.

Baker AL, Tolbert NE (1966) Glycolate oxidase (ferredoxin-containing form). Methods in Enzymology 9: 339-340. https://doi.org/10.1016/0076-6879(66)09069-4

Beauchamp C, Fridovich I (1971) Superoxide dismutase: Improved assay applicable to acrylamide gels. Analytical Biochemistry 44: 2762-2787. https://doi.org/10.1016/0003-2697(71)90370-8

Belkhodja R, Morales F, Abadia A, Gomez-Aparisi J, Abadia J (1994). Chlorophyll fluorescence as a possible tool for salinity tolerance screening in barley (*Hordeum vulgare* L.). Plant Physiology, 104 (2): 667-673. https://doi.org/10.1104/pp.104.2.667

Bilal S, Shahzad R, Asaf S, Imran M, Al-Harrasi A, Lee IJ (2023). Efficacy of endophytic SB10 and glycine betaine duo in alleviating phytotoxic impact of combined heat and salinity in Glycine max L. via regulation of redox homeostasis and physiological and molecular responses. Environmental Pollution, 316: 120658. https://doi.org/10.1016/j.envpol.2022.120658

Blum A, Ebercon A (1981) Cell membrane stability as a measure of drought and heat tolerance in wheat. Crop Science 21: 43-47. https://doi.org/10.2135/cropsci1981.0011183X002100010013x

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 722: 248-254. https://doi.org/10.1006/abio.1976.9999

Brennan T, Frenkel C (1977) Involvement of hydrogen peroxide in the regulation of senescence in pear. Plant Physiology 59: 411-416. https://doi.org/10.1104/pp.59.3.411

Cakmak I, Horst WJ (1991) Effect of aluminum on lipid-peroxidation, superoxide-dismutase, catalase and peroxidase-activities in root-tips of soybean (*Glycine max*). Physiologia Plantarum 83: 463-468. https://doi.org/10.1111/j.1399-3054.1991.tb00121.x

Cavalcanti FR, Oliveira JTA, Martins-Miranda AS, Viegas RA, Silveira JAG (2004)

Superoxide dismutase, catalase and peroxidase activities do not confer protection against oxidative damage in salt-stressed cowpea leaves. New Phytologist 163: 563-571. https://doi.org/10.1111/j.1469-8137.2004.01139.x

Chong J, Soufan O, Li C, Caraus I, Li S, Bourque G, Wishart DS, Xia J (2018) MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. Nucleic Acids Res 46: 486– 494. https://doi.org/10.1093/nar/gky310

Fahad S, Adnan M, Noor M, Arif M, Alam M, Khan IA, Ullah H, Wahid F, Mian IA, Jamal Y, Basir A, Hassan S, Saud S, Amanullah, Riaz M, Wu C, Khan MA, Wang D (2019). Major constraints for global rice production. In Advances in rice research for abiotic stress tolerance. p. 1-22. Woodhead Publishing. https://doi.org/10.1016/B978-0-12-814332-2.00001-0

Farooq M, Park JR, Jang YH, Kim EG, Kim KM (2021). Rice cultivars under salt stress Show differential expression of genes related to the regulation of Na^+/K^+ balance. Frontiers in Plant Science, 12: 680131. https://doi.org/10.3389/fpls.2021.680131

Flexas J, Ribas-Carbo M, Diaz-Espejo A, Galmés J, Medrano H (2008). Mesophyll conductance to CO2: current knowledge and future prospects. Plant, cell & environment, 31(5): 602-621.

Ferreira-Silva SL, Voigt EL, Silva EN, Maia JM, Fontenele AV, Silveira JAG (2011). High temperature positively modulates oxidative protection in salt-stressed cashew plants. Environmental and Experimental Botany, 74: 162-170. https://doi.org/10.1016/j.envexpbot.2011.05.015

Foyer CH (2018). Reactive oxygen species, oxidative signaling and the regulation of photosynthesis. Environmental and experimental botany, 154: 134-142. https://doi.org/10.1016/j.envexpbot.2018.05.003

Georgieva M, Vassileva V (2023). Stress Management in Plants: Examining Provisional and Unique Dose-Dependent Responses. International Journal of Molecular Sciences 24 (6): 5105. https://doi.org/10.3390/ijms24065105

Giannopolitis CN, Ries SK (1977) Superoxide dismutases: I. Occurrence in Higher Plants. Plant Physiology 59: 309-314. https://doi.org/10.1104/pp.59.2.309

Griffith OW (1980) Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Analytical Biochemistry 106: 207-212. https://doi.org/10.1016/0003-2697(80)90139-6

Hameed A, Ahmed MZ, Hussain T, Aziz I, Ahmad N, Gul B, Nielsen BL (2021). Effects of salinity stress on chloroplast structure and function. Cells. 10 (8): 2023. https://doi.org/10.3390/cells10082023

Hauser F, Horie T. (2010) A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high $K(^+)/Na(^+)$ ratio in leaves during salinity stress. Plant Cell Environment, 33(4): 552-65. https://doi.org/10.1111/j.1365-3040.2009.02056.x

Havir EA, McHale NA (1987) Biochemical and Developmental Characterization of Multiple

Forms of Catalase in Tobacco-Leaves. Plant Physiology 84, 450-455. https://doi.org/10.1104/pp.84.2.450

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. California Agricultural Experiment Station, Berkeley. 32p.

Hoang TML, Tran TN, Nguyen TKT, Williams B, Wurm P, Bellairs S, Mundree S (2016). Improvement of salinity stress tolerance in rice: challenges and opportunities. Agronomy, 6(4): 54. https://doi.org/10.3390/agronomy6040054

Hussain HA, Men S, Hussain S, Chen Y, Ali S, Zhang S, Zhang K, Li Y, Xu Q, Liao C, Wang LC (2019). Interactive effects of drought and heat stresses on morpho-physiological attributes, yield, nutrient uptake and oxidative status in maize hybrids. Scientific Reports 9: 3890. https://doi.org/10.1038/s41598-019-40362-7

Kampfenkel K, Montagu MV, Inzé R (1995) Extraction and determination of ascorbate and dehydroascorbate from plant tissue. Analytical Biochemistry 225: 165-167. https://doi.org/10.1006/abio.1995.1127

Keisham M, Mukherjee S, Bhatla SC (2018). Mechanisms of sodium transport in plants-progresses and challenges. International journal of molecular sciences, 19(3): 647. https://doi.org/10.3390/ijms19030647.

Keles Y, Oncel I (2002). Response of antioxidative defence system to temperature and water stress combinations in wheat seedlings. Plant Science, 163 (4): 783-790. https://doi.org/10.1016/S0168-9452(02)00213-3

Lima-Melo Y, Alencar, VT, Lobo AK, Sousa RH, Tikkanen M, Aro EM, Silveira JAG, Gollan, PJ (2019). Photoinhibition of photosystem I provides oxidative protection during imbalanced photosynthetic electron transport in Arabidopsis thaliana. Frontiers in plant science, 10: 916. https://doi.org/10.3389/fpls.2019.00916

Lima-Neto MC, Lobo AK, Martins MO, Fontenele AV, Silveira JAG (2014). Dissipation of excess photosynthetic energy contributes to salinity tolerance: a comparative study of salt-tolerant Ricinus communis and salt-sensitive Jatropha curcas. Journal of plant physiology, 171(1): 23-30. http://dx.doi.org/10.1016/j.jplph.2013.09.002

Liu J, Li G, Chen L, Gu J, Wu H, Li Z (2021). Cerium oxide nanoparticles improve cotton salt tolerance by enabling better ability to maintain cytosolic K^+/Na^+ ratio. Journal Nanobiotechnology.;19(1):1-16. https://doi.org/10.1186/s12951-021-00892-7

Mariamma M, Muthukumar B, Gnanam A (1997). Thermotolerance and effect of heat shock on the stability of the ATPase enzyme in rice. Journal of plant physiology, 150 (6): 739-742.https://doi.org/10.1016/S0176-1617(97)80292-1

Morsomme P, Boutry M (2000). The plant plasma membrane H^+-ATPase: structure, function and regulation. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1465(1-2): 1-16. https://doi.org/10.1016/s0005-2736(00)00128-0

Mukhopadhyay R, Sarkar B, Jat HS, Sharma PC, Bolan, NS (2021). Soil salinity under climate change: Challenges for sustainable agriculture and food security. Journal of Environmental Management, 280: 111736. https://doi.org/10.1016/j.jenvman.2020.111736

Munns R, Tester M (2008). Mechanisms of salinity tolerance. Annual Review of Plant Biology, 59: 651-681. https://doi.org/10.1146/annurev.arplant.59.032607.092911

Nahar L, Aycan M, Hanamata S, Baslam M, Mitsui T (2022). Impact of Single and Combined Salinity and High-Temperature Stresses on Agro-Physiological, Biochemical, and Transcriptional Responses in Rice and Stress-Release. Plants, 11 (4): 501. https://doi.org/10.3390/plants11040501

Nahar L, Aycan M, Lopes Hornai EM, Baslam M, Mitsui T (2023). Tolerance with High Yield Potential Is Provided by Lower Na+ Ion Accumulation and Higher Photosynthetic Activity in Tolerant YNU31-2-4 Rice Genotype under Salinity and Multiple Heat and Salinity Stress. Plants, 12(9): 1910. https://doi.org/10.3390/plants12091910

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiology 22: 1068-1072. https://doi.org/10.1093/oxfordjournals.pcp.a076232

Negrão S, Schmöckel SM, Tester M (2017). Evaluating physiological responses of plants to salinity stress. Annals of botany, 119: 1-11. https://doi.org/10.1093/aob/mcw191

Nongpiur CR, Singla-Pareek LS, Pareek A (2016). Genomics approaches for improving salinity stress tolerance in crop plants. Current genomics, 17(4): 343-357. https://doi.org/10.2174/1389202917666160331202517

Oukarroum A, Bussotti F, Goltsev V, Kalaji HM (2015). Correlation between reactive oxygen species production and photochemistry of photosystems I and II in Lemna gibba L. plants under salt stress. Environmental and Experimental Botany, 109: 80-88. http://dx.doi.org/10.1016/j.envexpbot.2014.08.005

Pan T, Liu M, Kreslavski VD, Zharmukhamedov SK, Nie C, Yu M, Kuznetsov VV, Allakhverdiev SI, Shabala, S. (2020). Non-stomatal limitation of photosynthesis by soil salinity. Critical Reviews in Environmental Science and Technology, 51(8): 791-825. https://doi.org/10.1080/10643389.2020.1735231

Radha B, Sunitha NC, Sah RP, Md Azharudheen TP, Krishna GK, Umesh DK, Thomas S, Anilkumar C, Upadhyay S, Kumar A, Manikanta CLN, Behera S, Marndi BC, Siddique KH (2023). Physiological and molecular implications of multiple abiotic stresses on yield and quality of rice. Frontiers in Plant Science, 13: 996514. https://doi.org/10.3389/fpls.2022.996514

Ribeiro RV, Machado EC, Santos MG, Oliveira RF (2009) Photosynthesis and water relations of well-watered orange plants as affected by winter and summer conditions. Photosynthetica 47(2): 215-222. https://doi.org/10.1007/s11099-009-0035-2

Rivero RM, Mestre TC, Mittler RON, Rubio F, Garcia-Sanchez F, Martinez V (2014). The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. Plant, cell & environment, 37 (5): 1059-1073. https://doi.org/10.1111/pce.12199

Rodrigues CRF, Silva EN, Ferreira-Silva SL, Voigt EL, Viégas RA, Silveira JAG (2013). High K^+ supply avoids Na^+ toxicity and improves photosynthesis by allowing favorable K^+: Na^+ ratios through the inhibition of Na^+ uptake and transport to the shoots of *Jatropha curcas* plants. Journal of Plant Nutrition and Soil Science, 176(2): 157-164.

https://doi.org/10.1002/jpln.201200230

Rosa SB, Caverzan A, Teixeira FK, Lazzarotto F, Silveira JAG, Ferreira-Silva SL, Abreu-Neto J, Margis R, Margis-Pinheiro M (2010) Cytosolic APx knockdown indicates an ambiguous redox responses in rice. Phytochemistry 71: 548-558. https://doi.org/10.1016/j.phytochem.2010.01.003

Rubio F, Nieves-Cordones M, Horie T, Shabala S (2020) Doing 'business as usual' comes with a cost: evaluating energy cost of maintaining plant intracellular K^+ homeostasis under saline conditions. New Phytologist 225 (3):1097-1104. https://doi.org/10.1111/nph.15852

Schreiber U (2004). Pulse-amplitude-modulation (PAM) fluorometry and saturation pulse method: an overview. In: Papageorgiou, G.C., Govindjee (Eds.), Advances in photosynthesis and respiration: Chlorophyll a Fluorescence, a signature of photosynthesis. Springer, Dordrecht, 19: 279-319. https://doi.org/10.1007/978-1-4020-3218-9_11

Silva EN, Ferreira-Silva SL, Fontenelle AV, Ribeiro RV, Viégas RA, Silveira JAG (2010) Photosynthetic changes and protective mechanisms against oxidative damage subjected to isolated and combined drought and heat stresses in *Jatropha curcas* plants. Journal of Plant Physiology 167: 1157-1164. https://doi.org/10.1016/j.jplph.2010.03.005

Silva EN, Ribeiro RV, Ferreira-Silva SL, Viégas RA, Silveira JAG (2011). Salt stress induced damages on the photosynthesis of physic nut young plants. Scientia Agricola, 68: 62-68. https://doi.org/10.1590/S0103-90162011000100010

Silva EN, Vieira SA, Ribeiro RV, Ponte LF, Ferreira-Silva SL, Silveira JAG (2012). Contrasting physiological responses of *Jatropha curcas* plants to single and combined stresses of salinity and heat. Journal of Plant Growth Regulation, 32: 159-169. https://doi.org/10.1007/s00344-012-9287-3

Silveira JAG, Carvalho FE (2016). Proteomics, photosynthesis and salt resistance in crops: An integrative view. Journal of Proteomics, 143: 24-35. https://doi.org/10.1016/j.jprot.2016.03.013

Sousa RH, Carvalho FE, Daloso DM, Lima-Melo Y, Margis-Pinheiro M, Komatsu S, Silveira, JAG (2023). Impairment in photosynthesis induced by CAT inhibition depends on the intensity of photorespiration and peroxisomal APX expression in rice. Plant Physiology and Biochemistry, 203: 108066. https://doi.org/10.1016/j.plaphy.2023.108066

Suzuki N, Bassil E, Hamilton JS, Inupakutika MA, Zandalinas SI, Tripatia D, Luo Y, Dion E, Fukui G, Kumazaki A, Nakano R, Rivero RM, Verbeck GF, Azad RK, Blumwald E, Mittler R (2016). ABA is required for plant acclimation to a combination of salt and heat stress. PLoS One, 11 (1):1-21. https://doi.org/10.1371/journal.pone.0147625

Wu H (2018). Plant salt tolerance and Na+ sensing and transport. The Crop Journal, 6(3), 215-225. https://doi.org/10.1016/j.cj.2018.01.003 2214-5141

Wungrampha S, Joshi R, Singla-Pareek SL, Pareek A (2018). Photosynthesis and salinity: are these mutually exclusive? Photosynthetica, 56: 366-381. https://doi.org/10.1007/s11099-017-0763-7

You C, Xu P, He Y, et al. (2023). The application of mixed nitrogen increases photosynthetic and antioxidant capacity in rice (Oryza sativa) under heat stress at flowering stage. Acta

Physiol Plant.;45(8):1-11. https://doi.org/10.1007/s11738-023-03578-9

Zandalinas SI, Mittler R (2022). Plant responses to multifactorial stress combination. New Phytologist, 234 (4):1161-1167. https://doi.org/10.1111/nph.18087

Zandalinas SI, Sengupta S, Fritschi FB, Azad RK, Nechushtai R, Mittler R (2021). The impact of multifactorial stress combination on plant growth and survival. New Phytologist, 230(3): 1034-1048.m https://doi.org/10.1111/nph.17232

Zhang S, Malmersjö S, Li J, Ando H, Aizman O, Uhlén P, Mikoshiba A, Aperia A (2006). Distinct role of the N-terminal tail of the Na, K-ATPase catalytic subunit as a signal transducer. Journal of Biological Chemistry, 281(31): 21954-21962. https://doi.org/10.1074/jbc.M601578200

6 CONCLUSION AND PERSPECTIVES

As rice (Oryza sativa L.) is a staple food, consumed by a large part of the world's population, studies are important that include the assessment of the impacts of stress factors, which harm the productivity of this crop. Rice is mainly grown in tropical climates, where water scarcity, high temperatures, salinity and nutrient deficiencies can significantly reduce productivity. Most current studies and research ignore important factors such as the multiplicity of stress factors to which plants are exposed, as well as the changes that occur within the plant in different organs or collection times. We seek to bring a more comprehensive study, considering the systemic responses and complexity of plants, to get closer to the reality in the field.

We observed that the intensity of saline stress is altered temporally (plant stage) and also spatially, where plant parts respond differently to salinity, and in general, younger leaves had a more altered metabolism due to saline stress. Salinity also drastically affects N metabolism, as indicated by strong changes in GS activity, nitrogen fractions, and metabolomic profiles. Regarding the interaction between high temperature and saline stress, rice plants present distinct responses in different physiological processes, where on a temporal scale, heat intensely stimulates the transport of Na^+ towards the leaves, stimulating its accumulation followed by a great intensification of flows osmotic and drastic ionic. changes, as it revealed an intense decrease in the K^+/Na^+ ratios. Rice leaves exposed to this adverse condition are able to trigger an antioxidant system and decrease H_2O_2 levels and lipid peroxidation compared to single salt stress. However, this antioxidant response is not sufficient to prevent physiological disturbances, as indicated by the drastic increase in leaf membrane damage. In conclusion, the high temperature provided in the short term to rice under saline stress causes dangerous effects essentially associated with the intensification of osmotic stress and the breakdown of ionic homeostasis associated with impaired photosynthesis.

We hope that this work can awaken the need for a more systemic and complex view in plant studies. Furthermore, in the future, additional studies involving next-generation sequencing platforms and high-throughput phenotyping, considering the complexities presented here, will be carried out. Such studies could help identify new candidate genes responsible for regulating grain development in situations of combined stress, as well as consider stages and organs of the plant more susceptible to stress, for the development of more resistant crops and/or to facilitate the more appropriate management of the crop, considering space-time changes and climate variations.

REFERENCES

AHMADI, Fatemeh *et al.* Cell-type-specific H+-ATPase activity and antioxidant enzymes improve the Echinacea purpurea L. Moench tolerance to salinity stress at different NO3-/NH4+ ratios. **Industrial Crops and Products**, [*s. l.*], v. 186, n. June, p. 115199, 2022. Disponível em: https://doi.org/10.1016/j.indcrop.2022.115199.

ALENCAR, V. T.C.B. *et al.* High ammonium supply impairs photosynthetic efficiency in rice exposed to excess light. **Photosynthesis Research**, [*s. l.*], v. 140, n. 3, p. 321–335, 2019. Disponível em: http://dx.doi.org/10.1007/s11120-019-00614-z.

ARIF, Yamshi *et al.* **Salinity induced physiological and biochemical changes in plants: An omic approach towards salt stress tolerance**. [*S. l.*]: Elsevier Masson SAS, 2020.

AULER, Priscila Ariane *et al.* Stress memory of physiological, biochemical and metabolomic responses in two different rice genotypes under drought stress: The scale matters. **Plant Science**, [*s. l.*], v. 311, n. July, 2021.

BAETHGEN, W. E.; ALLEY, M. M. A manual colorimetric procedure for measuring ammonium nitrogen in soil and plant kjeldahl digests. **Communications in Soil Science and Plant Analysis**, [*s. l.*], v. 20, n. 9–10, p. 961–969, 1989.

BILAL, Saqib *et al.* Efficacy of endophytic SB10 and glycine betaine duo in alleviating phytotoxic impact of combined heat and salinity in Glycine max L. via regulation of redox homeostasis and physiological and molecular responses. **Environmental Pollution**, [*s. l.*], v. 316, n. July 2022, 2023.

BLUM, A.; EBERCON, A. Cell membrane stability as a measure of drought and heat tolerance in wheat. **Crop Science**, [*s. l.*], v. 21, n. 1, p. 43–47, 1981.

BRADFORD, Marion M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye biding. **Analytical Biochemistry**, [*s. l.*], v. 72, p. 248–254, 1976.

CAVALCANTI, Fabio Rossi *et al.* Superoxide dismutase, catalase and peroxidase activities do not confer protection against oxidative damage in salt-stressed cowpea leaves. **New Phytologist**, [*s. l.*], v. 163, n. 3, p. 563–571, 2004.

CHONG, Jasmine; WISHART, David S.; XIA, Jianguo. Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. **Current Protocols in Bioinformatics**, [*s. l.*], v. 68, n. 1, p. 1–128, 2019.

COUNCE, Paul A.; KEISLING, Terry C.; MITCHELL, Andrew J. A uniform, objectives, and adaptive system for expressing rice development. **Crop Science**, [*s. l.*], v. 40, n. 2, p. 436–443, 2000.

DA ROCHA, Iza Marineves Almeida *et al.* Exogenous ornithine is an effective precursor and the δ-ornithine amino transferase pathway contributes to proline accumulation under high N recycling in salt-stressed cashew leaves. **Journal of Plant Physiology**, [*s. l.*], v. 169, n. 1, p. 41–49, 2012. Disponível em: http://dx.doi.org/10.1016/j.jplph.2011.08.001.

FEDURAEV, P. *et al.* Phenylalanine and Tyrosine as Exogenous Precursors of Wheat (Triticum aestivum L.) Secondary Metabolism through PAL-Associated Pathways. **Plants**, [*s. l.*], v. 9, n. 4, p. 476, 2020.

FELKER, Peter. Microdetermination of Nitrogen in Seed Protein Extracts with the Salicylate-Dichloroisocyanurate Color Reaction. **Analytical Chemistry**, [*s. l.*], v. 49, n. 7, p. 1080, 1977.

FERREIRA-SILVA, Sérgio Luiz *et al.* High temperature positively modulates oxidative protection in salt-stressed cashew plants. **Environmental and Experimental Botany**, [*s. l.*], v. 74, n. 1, p. 162–170, 2011.

FLEXAS, Jaume *et al.* Mesophyll conductance to CO2: Current knowledge and future prospects. **Plant, Cell and Environment**, [*s. l.*], v. 31, n. 5, p. 602–621, 2008.

FRANCINI, Alessandra; SEBASTIANI, Luca. Abiotic stress effects on performance of horticultural crops. **Horticulturae**, [*s. l.*], v. 5, n. 4, 2019.

GALVIZ, Yutcelia; SOUZA, Gustavo M.; LÜTTGE, Ulrich. The biological concept of stress revisited: relations of stress and memory of plants as a matter of space–time. **Theoretical and Experimental Plant Physiology**, [*s. l.*], v. 34, n. 2, p. 239–264, 2022. Disponível em: https://doi.org/10.1007/s40626-022-00245-1.

GEORGIEVA, Mariyana; VASSILEVA, Valya. **Stress Management in Plants: Examining Provisional and Unique Dose-Dependent Responses**. [*S. l.*]: MDPI, 2023.

GUILHERME, Eliezer A. *et al.* Nitrogen-utilization efficiency during early deficiency after a luxury consumption is improved by sustaining nitrate reductase activity and photosynthesis in cotton plants. **Plant and Soil**, [*s. l.*], v. 443, n. 1–2, p. 185–198, 2019.

HIREL, Bertrand; GADAL, Pierre. Glutamine Synthetase in Rice. **Plant Physiology**, [*s. l.*], v. 66, n. 4, p. 619–623, 1980.

HOAGLAND, D. R.; ARNON, D. I. Preparing the nutrient solution. **The Water-Culture Method for Growing Plants without Soil**, [*s. l.*], v. 347, p. 29–31, 1950.

HUANG, Jie *et al.* Effects of nitric oxide on nitrogen metabolism and the salt resistance of rice (Oryza sativa L.) seedlings with different salt tolerances. **Plant Physiology and Biochemistry**, [*s. l.*], v. 155, n. November 2019, p. 374–383, 2020. Disponível em: https://doi.org/10.1016/j.plaphy.2020.06.013.

JAHAN, Eisrat; SHARWOOD, Robert Edward; TISSUE, David T. Effects of leaf age during drought and recovery on photosynthesis, mesophyll conductance and leaf anatomy in wheat leaves. **Frontiers in Plant Science**, [*s. l.*], v. 14, n. June, p. 1–15, 2023.

JIAO, Peipei *et al.* Short-term transcriptomic responses of Populus euphratica roots and leaves to drought stress. **Journal of Forestry Research**, [*s. l.*], v. 32, n. 2, p. 841–853, 2021. Disponível em: https://doi.org/10.1007/s11676-020-01123-9.

KAISER, Elias *et al.* Dynamic photosynthesis in different environmental conditions. **Journal of Experimental Botany**, [*s. l.*], v. 66, n. 9, p. 2415–2426, 2015.

KHORASANINEJAD, Sarah; ZARE, Farshad; HEMMATI, Khodayar. Effects of silicon on some phytochemical traits of purple coneflower (Echinacea purpurea L.) under salinity. **Scientia Horticulturae**, [*s. l.*], v. 264, n. 3, 2020.

KILASI, Newton Lwiyiso *et al.* Heat stress tolerance in rice (oryza sativa L.): Identification of quantitative trait loci and candidate genes for seedling growth under heat stress. **Frontiers in Plant Science**, [*s. l.*], v. 871, n. November, p. 1–11, 2018.

KOPKA, Joachim *et al.* GMD@CSB.DB: The Golm metabolome database. **Bioinformatics**, [*s. l.*], v. 21, n. 8, p. 1635–1638, 2005.

LEE, Sichul; MASCLAUX-DAUBRESSE, Celine. Current understanding of leaf senescence in rice. **International Journal of Molecular Sciences**, [*s. l.*], v. 22, n. 9, p. 1–19, 2021.

LI, Xiaobai *et al.* Unraveling the complex trait of harvest index with association mapping in rice (Oryza sativa L.). **PLoS ONE**, [*s. l.*], v. 7, n. 1, p. 1–10, 2012.

LIANG, Wenji *et al.* Plant salt-tolerance mechanism: A review. **Biochemical and Biophysical Research Communications**, [*s. l.*], v. 495, n. 1, p. 286–291, 2018. Disponível em: https://doi.org/10.1016/j.bbrc.2017.11.043.

LICHTENTHALER, HARTMUT K.; WELLBURN, ALAN R. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents . **Biochemical Society Transactions**, [*s. l.*], v. 11, n. 5, p. 591–592, 1983.

LIMA-MELO, Yugo *et al.* Mitochondrial GPX1 silencing triggers differential photosynthesis impairment in response to salinity in rice plants. **Journal of integrative plant biology**, [*s. l.*], v. 58, n. 8, p. 737–748, 2016.

LISEC, Jan *et al.* Gas chromatography mass spectrometry-based metabolite profiling in plants. **Nature Protocols**, [*s. l.*], v. 1, n. 1, p. 387–396, 2006.

LIU, Jiahao *et al.* Cerium oxide nanoparticles improve cotton salt tolerance by enabling better ability to maintain cytosolic K+/Na+ ratio. **Journal of Nanobiotechnology**, [*s. l.*], v. 19, n. 1, p. 1–16, 2021.

LÜTTGE, Ulrich. Integrative emergence in contrast to separating modularity in plant biology: views on systems biology with information, signals and memory at scalar levels from molecules to the biosphere. **Theoretical and Experimental Plant Physiology**, [*s. l.*], v. 33, n. 1, p. 1–13, 2021.

MACHADO, Eduardo C.; SILVEIRA, Joaquim A. G.. Trocas de C02, acúmulo de fitomassa e remobilização de reservas durante o crescimento de panículas de duas cultivares de arroz. **Revista Brasileira de Fisiologia vegetal***,* 2(1), 63-70, 1990.

MCKERSIE, B D; TOMES, D T. Predicting the Vigor and. [*s. l.*], v. 343, p. 337–343, 1981.

NAHAR, Lutfun *et al.* Impact of Single and Combined Salinity and High-Temperature Stresses on Agro-Physiological, Biochemical, and Transcriptional Responses in Rice and Stress-Release. **Plants**, [*s. l.*], v. 11, n. 4, 2022.

NAHAR, Lutfun *et al.* Tolerance with High Yield Potential Is Provided by Lower Na+ Ion Accumulation and Higher Photosynthetic Activity in Tolerant YNU31-2-4 Rice Genotype under Salinity and Multiple Heat and Salinity Stress. **Plants**, [*s. l.*], v. 12, n. 9, p. 1–25, 2023.

NETO, Milton C.Lima *et al.* Understanding photosynthesis in a spatial–temporal multiscale: The need for a systemic view. **Theoretical and Experimental Plant Physiology**, [*s. l.*], v. 33, n. 2, p. 113–124, 2021. Disponível em: https://doi.org/10.1007/s40626-021-00199-w.

NIKOLIĆ, Nebojša *et al.* Effects of the salinity-temperature interaction on seed germination and early seedling development: a comparative study of crop and weed species. **BMC Plant Biology**, [*s. l.*], v. 23, n. 1, p. 1–14, 2023.

POUCET, Théo *et al.* Ammonium supply induces differential metabolic adaptive responses in tomato according to leaf phenological stage. **Journal of Experimental Botany**, [*s. l.*], v. 72, n. 8, p. 3185–3199, 2021.

PRASERTSUK, Sirapat; WIJITKOSUM, Saowanee. Innovative use of rice husk biochar for rice cultivation in salt-affected soils with alternated wetting and drying irrigation. **Engineering Journal**, [*s. l.*], v. 25, n. 9, p. 19–32, 2021.

PRITTESH, Patel *et al.* Amelioration effect of salt-tolerant plant growth-promoting bacteria on growth and physiological properties of rice (Oryza sativa) under salt-stressed conditions. **Archives of Microbiology**, [*s. l.*], v. 202, n. 9, p. 2419–2428, 2020. Disponível em: https://doi.org/10.1007/s00203-020-01962-4.

PYUNG, Ok Lim; HONG, Gil Nam. Aging and senescence of the leaf organ. **Journal of Plant Biology**, [*s. l.*], v. 50, n. 3, p. 291–300, 2007.

RADHA, Beena *et al.* **Physiological and molecular implications of multiple abiotic stresses on yield and quality of rice**. [*S. l.*]: Frontiers Media S.A., 2023.

RILLIG, Matthias C. *et al.* The role of multiple global change factors in driving soil functions and microbial biodiversity. **Science**, [*s. l.*], v. 366, n. 6467, p. 886–890, 2019.

RIVERO, Rosa M. *et al.* The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. **Plant, Cell and Environment**, [*s. l.*], v. 37, n. 5, p. 1059–1073, 2014.

RUBIO, Francisco *et al.* Doing ‘business as usual’ comes with a cost: evaluating energy cost of maintaining plant intracellular K+ homeostasis under saline conditions. **New Phytologist**, [*s. l.*], v. 225, n. 3, p. 1097–1104, 2020.

SAHIN, Ustun *et al.* Effects of individual and combined effects of salinity and drought on physiological, nutritional and biochemical properties of cabbage (Brassica oleracea var. capitata). **Scientia Horticulturae**, [*s. l.*], v. 240, n. June, p. 196–204, 2018. Disponível em: https://doi.org/10.1016/j.scienta.2018.06.016.

SAITO, Hiroki *et al.* Two Novel QTLs for the Harvest Index that Contribute to High-Yield Production in Rice (Oryza sativa L.). **Rice**, [*s. l.*], v. 14, n. 1, p. 1–11, 2021.

SCHREIBER, Ulrich. **Pulse-Amplitude-Modulation (PAM) Fluorometry and Saturation Pulse Method: An Overview**. [*S. l.: s. n.*], 2007.

SENEWEERA, Saman *et al.* New insight into photosynthetic acclimation to elevated CO2: The role of leaf nitrogen and ribulose-1,5-bisphosphate carboxylase/oxygenase content in rice leaves. **Environmental and Experimental Botany**, [*s. l.*], v. 71, n. 2, p. 128–136, 2011. Disponível em: http://dx.doi.org/10.1016/j.envexpbot.2010.11.002.

SILVEIRA, Joaquim A.G. *et al.* Mecanismos biomoleculares envolvidos com a resistência ao estresse salino em plantas. **Manejo da salinidade na agricultura: Estudos básicos e aplicados**, [*s. l.*], v. 1, p. 161–18, 2010.

SILVEIRA, Joaquim A.G.; CARVALHO, Fabricio E.L. Proteomics, photosynthesis and salt resistance in crops: An integrative view. **Journal of Proteomics**, [*s. l.*], v. 143, p. 24–35, 2016a. Disponível em: http://dx.doi.org/10.1016/j.jprot.2016.03.013.

SILVEIRA, Joaquim A. G., CASTRO, P. R. C., DECHEN, A. R. Partição de nitrogênio e distribuição da matéria seca em três cultivares de caupi. **Pesq. agropec. bras.**, 24(9): 1153-1160, 1989.

SILVEIRA, Joaquim Albenisio *et al.* Proline accumulation and glutamine synthetase activity are increased by salt-induced proteolysis in cashew leaves. **Journal of Plant Physiology**, [*s. l.*], v. 160, n. 2, p. 115–123, 2003.

SILVEIRA, Joaquim A. G., MACHADO, Eduardo C. Mobilização de nitrogênio e de carboidratos durante o desenvolvimento de panículas de duas cultivares de arroz. **Revista Brasileira de Fisiologia vegetal**, 2(2), 37-46, 1990.

SILVEIRA, Joaquim A.G.; SOUSA, Rachel H.V. Looking for a systemic concept and physiological diagnosis of a plant stress state. **Theoretical and Experimental Plant Physiology**, [*s. l.*], n. 0123456789, 2024. Disponível em: https://doi.org/10.1007/s40626-024-00318-3.

SOUSA, Rikaely T. *et al.* Ammonium overaccumulation in senescent leaves as a novel exclusion mechanism to avoid toxicity in photosynthetically active rice leaves. **Environmental and Experimental Botany**, [*s. l.*], v. 186, n. February, p. 104452, 2021. Disponível em: https://doi.org/10.1016/j.envexpbot.2021.104452.

SOUZA, Gustavo M.; BERTOLLI, Suzana C.; LÜTTGE, Ulrich. Hierarchy and Information in a System Approach to Plant Biology: Explaining the Irreducibility in Plant Ecophysiology. [*s. l.*], p. 167–186, 2016.

SUZUKI, Nobuhiro *et al.* ABA is required for plant acclimation to a combination of salt and heat stress. **PLoS ONE**, [*s. l.*], v. 11, n. 1, p. 1–21, 2016.

TSUGAWA, Hiroshi *et al.* MS-DIAL: Data-independent MS/MS deconvolution for comprehensive metabolome analysis. **Nature Methods**, [*s. l.*], v. 12, n. 6, p. 523–526, 2015.

VIÉGAS, Ricardo A. *et al.* Plant growth, accumulation and solute partitioning of four forest species under salt stress. **Revista Brasileira de Engenharia Agrícola e Ambiental**, [*s. l.*], v. 7, n. 2, p. 258–262, 2003.

VIEGAS, R. A; SILVEIRA, Joaquim A.G. AMMONIA ASSIMILATION AND PROLINE ACCUMULATION IN YOUNG CASHEW PLANTS. [*s. l.*], n. August, 1999.

VOIGT, Eduardo Luiz *et al.* Source-sink regulation of cotyledonary reserve mobilization during cashew (Anacardium occidentale) seedling establishment under NaCl salinity. **Journal of Plant Physiology**, [*s. l.*], v. 166, n. 1, p. 80–89, 2009.

WEI, Huanhe *et al.* Sink-source relationship during rice grain filling is associated with grain nitrogen concentration. **Field Crops Research**, [*s. l.*], v. 215, n. May 2017, p. 23–38, 2018. Disponível em: https://doi.org/10.1016/j.fcr.2017.09.029.

WILHELM, Christian; WIRTH, Christian. Physiodiversity - New tools allow physiologist to embrace biodiversity and reconstruct the evolution of "physiologies"? **Journal of Plant Physiology**, [*s. l.*], v. 172, p. 1–3, 2015.

YANG, Jianchang; ZHANG, Jianhua. Crop management techniques to enhance harvest index in rice. **Journal of Experimental Botany**, [*s. l.*], v. 61, n. 12, p. 3177–3189, 2010.

YEMM, E. W.; COCKING, E. C.; RICKETTS, R. E. The determination of amino-acids with ninhydrin. **The Analyst**, [*s. l.*], v. 80, n. 948, p. 209–214, 1955.

ZHANG, Huiming *et al.* Abiotic stress responses in plants. **Nature Reviews Genetics**, [*s. l.*], v. 23, n. 2, p. 104–119, 2022.

ZHANG, Jing *et al.* Response of grain-filling rate and grain quality of mid-season indica rice to nitrogen application. **Journal of Integrative Agriculture**, [*s. l.*], v. 20, n. 6, p. 1465–1473, 2021. Disponível em: http://dx.doi.org/10.1016/S2095-3119(20)63311-1.

ZHU, Guanglong *et al.* Optimum nitrogen management enhances growth, antioxidant ability and yield performance of rice in saline soil of coastal area of China. **Chilean Journal of Agricultural Research**, [*s. l.*], v. 80, n. 4, p. 629–639, 2020.

APPENDIX A - LIST OF FIGURES

Chapter 1 Interaction between spatiotemporal changes and heat affecting salt stress intensity in rice plants: a review

Chapter 2 Salt stress intensity is dependent on spatio-temporal-scale variations throughout reproductive phase in rice

Chapter 3 Heat combined with salinity stimulate antioxidant defense but induce severe impairment in photosynthesis of rice

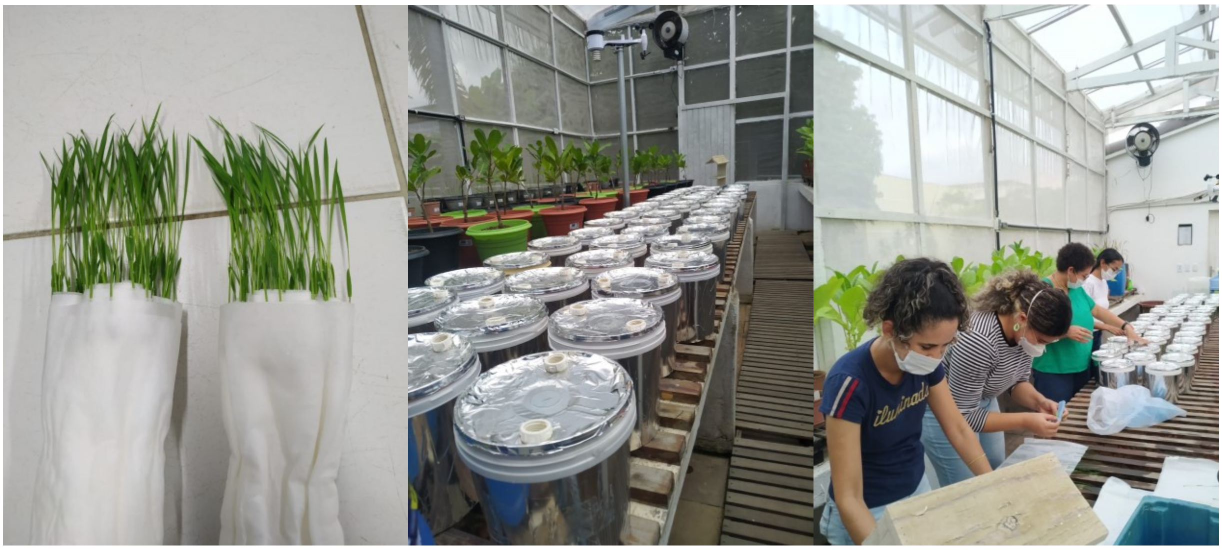
APPENDIX B - LIST OF TABLES

Chapter 2 Salt stress intensity is dependent on spatio-temporal-scale variations throughout reproductive phase in rice

Chapter 3 Heat combined with salinity stimulate antioxidant defense but induce severe impairment in photosynthesis of rice

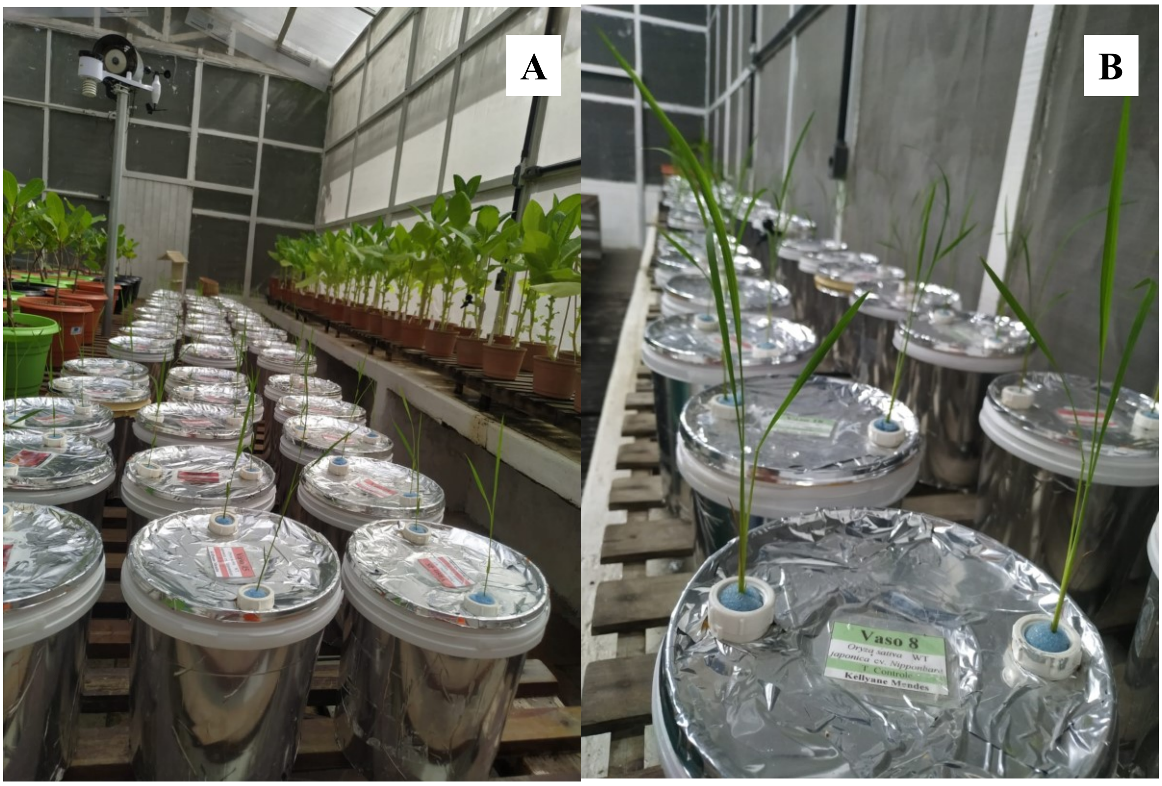
APPENDIX C - ORIGINAL PHOTOS OF EXPERIMENTS

Figure 1. Transplanting rice plant seedlings and setting up the experiment.



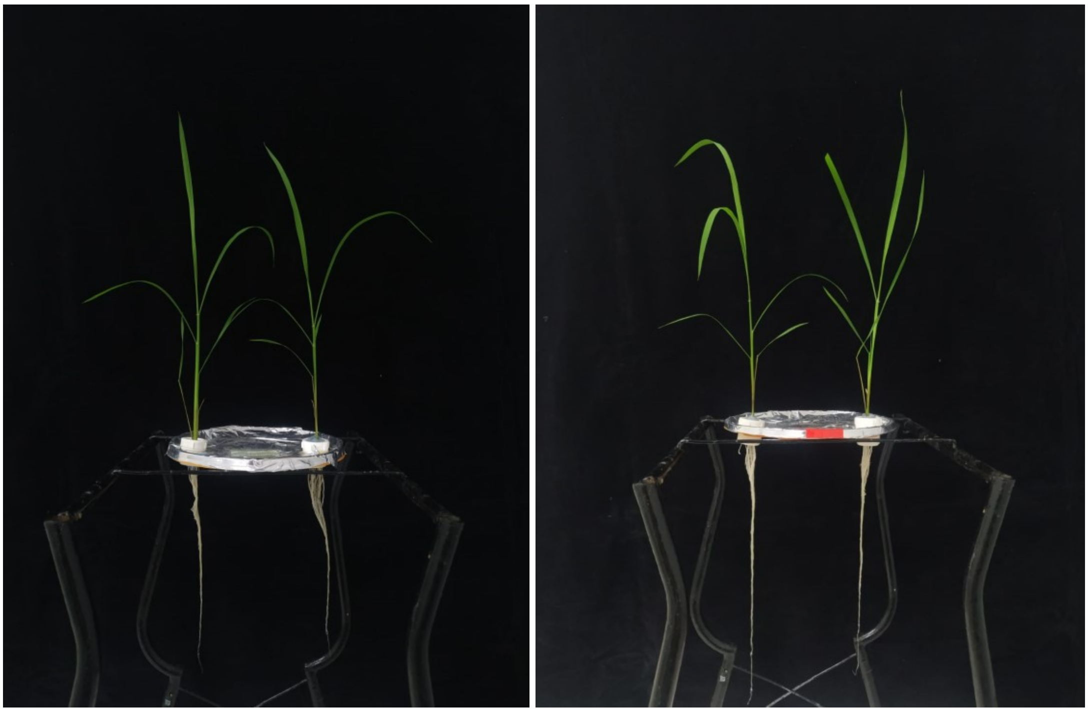
Source: elaborated by the author.

Figure 2 . Establishment of rice plant seedlings. (A) Three days after transplanting and (B) seven days after transplanting.



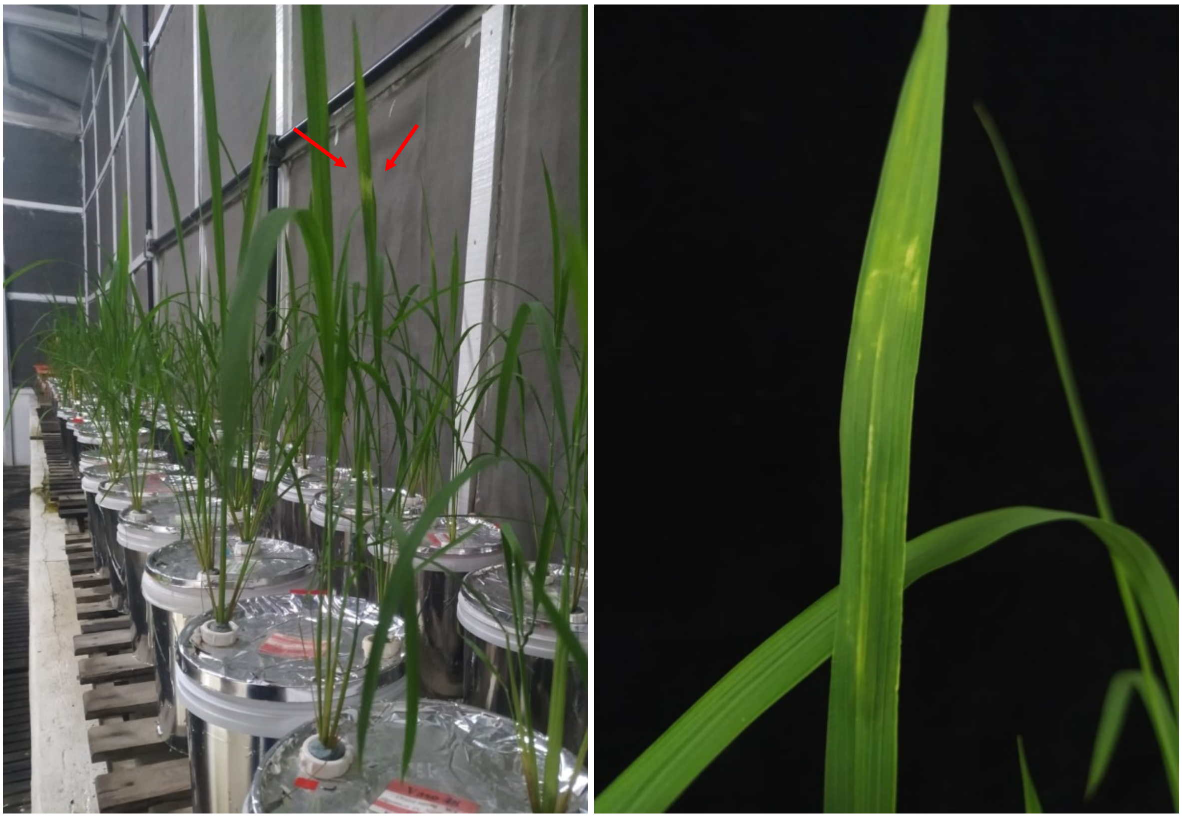
Source: elaborated by the author.

Figure 3. Rice plants reached stage V4 and treatment began (14 days after transplanting), with 25 mM NaCl.



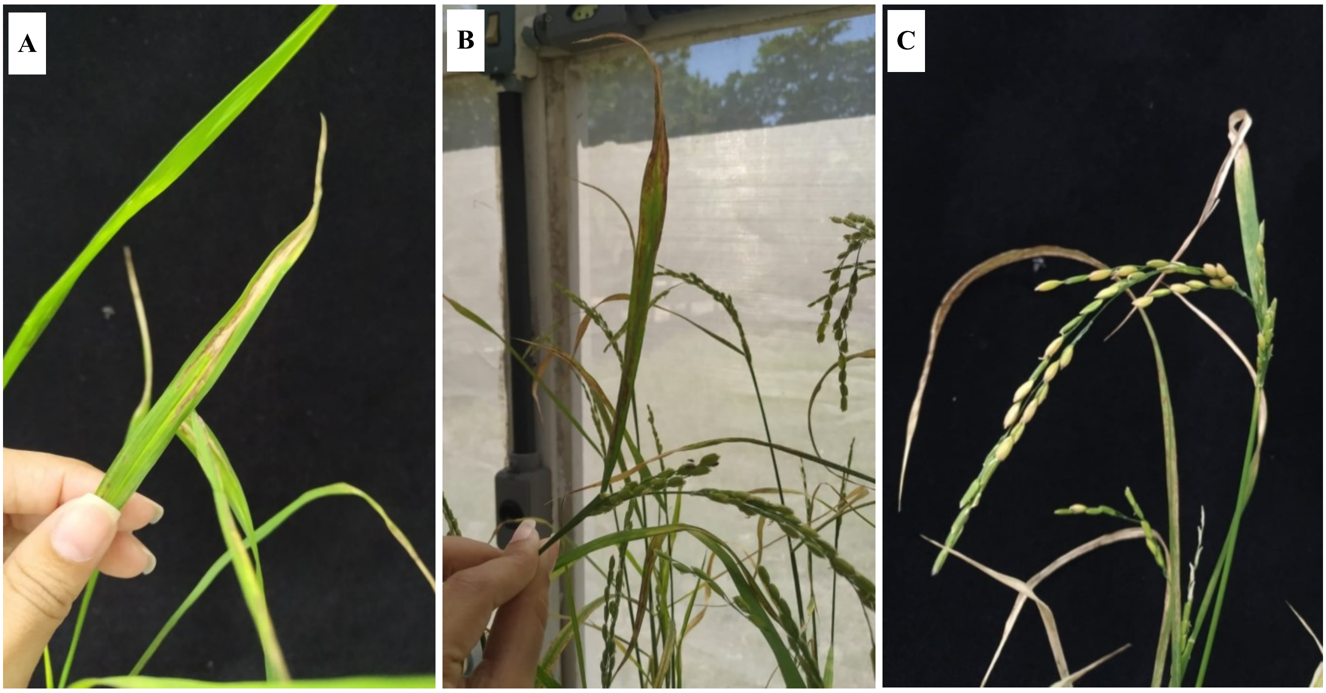
Source: elaborated by the author.

Figure 4. Initial symptoms of salinity in rice plants, observed one week after the start of treatment



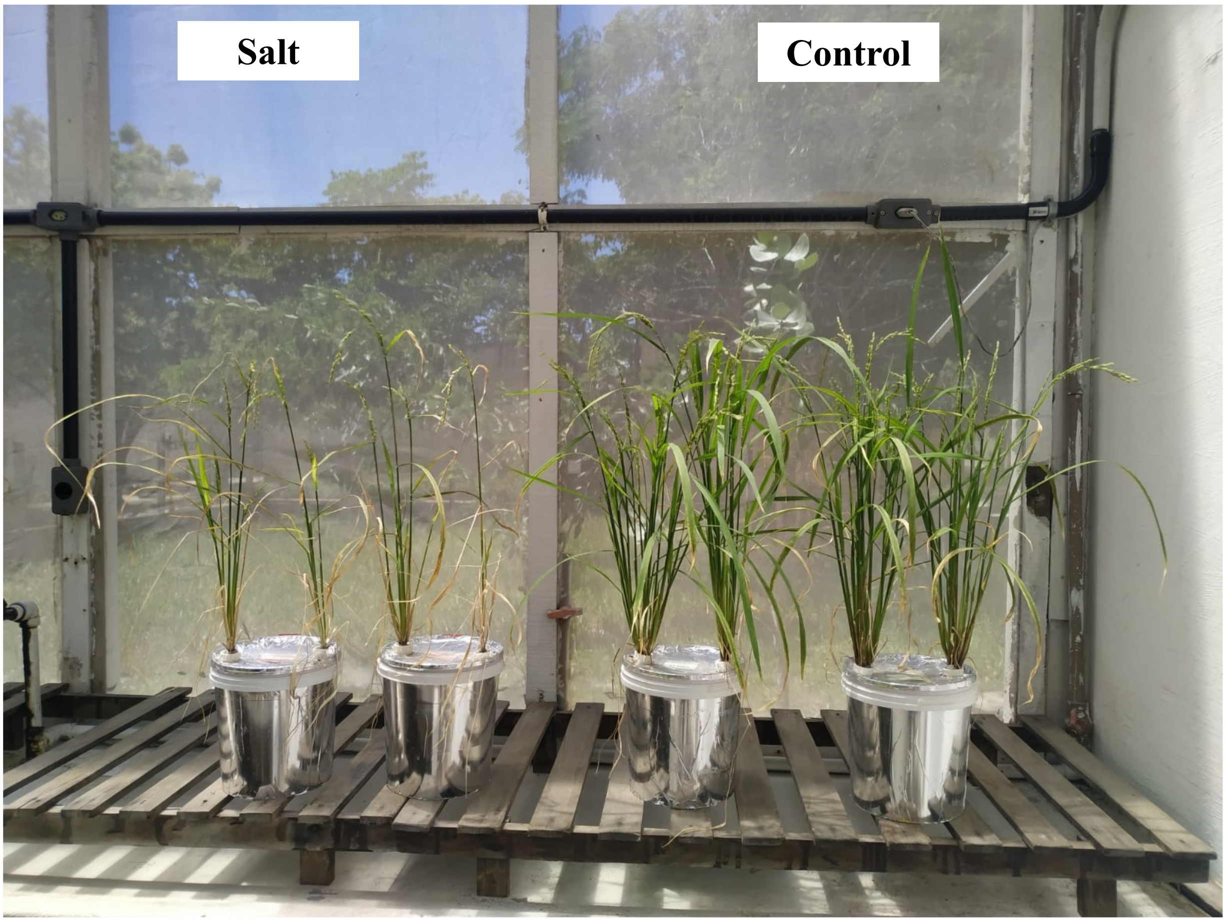
Source: elaborated by the author.

Figure 5. Symptoms of the effect of salinity on the flag leaf of rice plants. (A) 39 days after transplanting, (B) 52 days and (C) 66 days. Stages R3, R5 and R7 respectively.



Source: elaborated by the author.

Figure 6. Rice plants 56 days after transplanting, and 42 days under salinity**.**



Source: elaborated by the author.

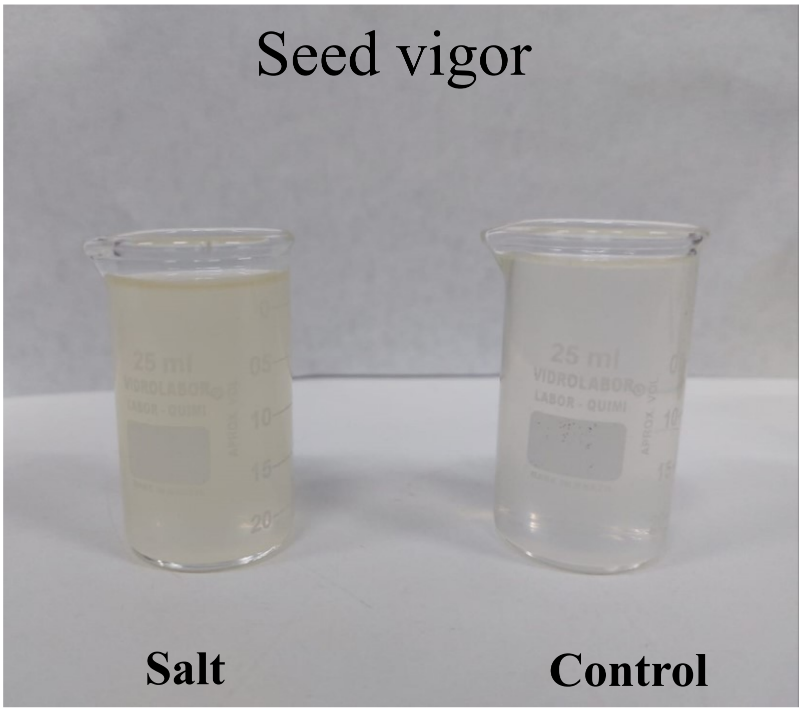
Figure 7 . Panicles per plant, collected at stage R9, at the end of seed maturation of rice plants, under control and salt treatments

Source: elaborated by the author.

Figure 8. Germination test, carried out at the end of the experiment with rice plant seeds produced under the control and salt treatments.

Source: elaborated by the author.

Figure 9. Electrolyte leakage (seed vigor) test, carried out at the end of the experiment with rice plant seeds produced under the control and salt treatments.



Source: elaborated by the author.

APPENDIX D - PARTICIPATION IN SCIENTIFIC EVENTS AND COURSES

CERTIFICADO

A Sociedade Brasileira de Fisiologia Vegetal, inscrita no CNPJ n° 80.919.590/0001-90 DECLARA que ***KELLYANE DA ROCHA MENDES****, CPF n° 009.187.792-00 participou do* ***METABOLISMO VEGETAL: ASPECTOS TEÓRICOS E PRÁTICOS (DA COLETA DE MATERIAL ÀS ANÁLISES METABOLÔMICAS E DE REDES METABÓLICAS).****, ocorrido em ONLINE no período de 11/07/2023 18:00 até 03/08/2023 20:00 , com carga horária de 16h.*

Fortaleza, 22 de Agosto de 2023.

Danilo M. Veloso

Danilo M. Veloso
Presidente da SBFV

Certificado emitido em 22/08/2023 11:58:46

Verifique sua autenticidade em
https://portal.sbfv.org.br/certificado/verificador/bedfe93a-950a-4147-b5ad-bd8298b83f6b

---

CERTIFICADO

A Sociedade Brasileira de Fisiologia Vegetal, inscrita no CNPJ n° 80.919.590/0001-90 DECLARA que ***KELLYANE DA ROCHA MENDES****, CPF n° 009.187.792-00 participou do* ***CAFÉ CIENTÍFICO: EXPLORANDO A FISIOLOGIA VEGETAL****, ocorrido em ON-LINE* 03/08/2023

Rio Claro, 06 de Abril de 2024.

Danilo M. Veloso

Danilo de Menezes Daloso
Presidente da SBFV

Certificado emitido em 06/04/2024 09:51:21

Verifique sua autenticidade em
https://portal.sbfv.org.br/certificado/verificador/eae02934-088b-4745-83cd-cc4cfc8e627d

CERTIFICADO

A Sociedade Brasileira de Fisiologia Vegetal, inscrita no CNPJ n° 80.919.590/0001-90 DECLARA que ***KELLYANE DA ROCHA MENDES****, CPF n° 009.187.792-00 participou do* ***O CICLO VIRTUOSO DA CIÊNCIA E DESENVOLVIMENTO.****, ocorrido em ON-LINE*

Rio Claro, 06 de Abril de 2024.

Danilo M. Daloso

Danilo de Menezes Daloso
Presidente da SBFV

Certificado emitido em 06/04/2024 09:51:46

Verifique sua autenticidade em
https://portal.sbfv.org.br/certificado/verificador/08e1a7fd-4a34-4ffa-b262-abf68d87c8f3

CERTIFICADO

A Sociedade Brasileira de Fisiologia Vegetal, inscrita no CNPJ n° 80.919.590/0001-90 DECLARA que ***KELLYANE DA ROCHA MENDES****, CPF n° 009.187.792-00 participou do* ***V SIMPÓSIO DE ECOFISIOLOGIA SISTÊMICA (V SES -2023)****, ocorrido em FORTALEZA/CE , 05 a 08 de dezembro de 2023*

Rio Claro, 06 de Abril de 2024.

Danilo M. Daloso

Danilo de Menezes Daloso
Presidente da SBFV

Certificado emitido em 06/04/2024 09:52:03

Verifique sua autenticidade em
https://portal.sbfv.org.br/certificado/verificador/1557d6b6-8b65-4c97-888e-432465230f73

CERTIFICADO

Certificamos que **KELLYANE DA ROCHA MENDES** participou como ouvinte da palestra: "*On the role of stomatal movements for yield and stress tolerance improvement in the climate change scenario*", promovida pelo Programa de Pós-Graduação em Agronomia/Fitotecnia da Universidade Federal do Ceará, com carga horária de **2h** no dia 13 de abril de 2023.

Rosilene Oliveira Mesquita

Professora Rosilene Oliveira Mesquita
Coordenadora do programa de pós-graduação em Agronomia/Fitotecnia

Cândida H. P. de Magalhães

Professora Cândida Hermínia Campos de Magalhães
Vice-coordenadora do programa de pós-graduação em Agronomia/Fitotecnia

---

Verifique o código de autenticidade **3944122.3308514.4.6.0551446669721558** em https://www.even3.com.br//documento

SES 2020

Simpósio de Ecofisiologia Sistêmica
Virtual, 4 a 6 de novembro

Certificamos que **Kellyane da Rocha Mendes** participou do Simpósio de Ecofisiologia Sistêmica 2020, realizado de 04/11/2020 a 06/11/2020, na modalidade *on-line*, contabilizando carga horária total de 10 horas.

Pelotas, 06 de novembro de 2020.

Realização:

LABPlant

LACEV

LCroP

Laboratório de
Ecofisiologia Vegetal
UNESP-Botucatu

Apoio:

Sociedade Brasileira de Fisiologia Vegetal

Verifique o código de autenticidade **3949730.3308514.4.6.050369669721558** em https://www.even3.com.br//documentos

CERTIFICATE

I BSP
1st Brazilian Symposium on Photosynthesis

I hereby confirm that Kellyane da Rocha Mendes, participated in the 1st Brazilian Symposium on Photosynthesis: Perspectives to improve photosynthetic efficiency in a climate change scenario, an online event held from 27th to 30th of October 2020.

São Vicente, SP, Brasil, 30th of October, 2020.

Prof. Dr. Milton Costa Lima Neto
Chair

Verifique o código de autenticidade **3344867.3308514.4.6.344867330851446** em https://www.even3.com.br//documentos

III Ciclo de Palestras do PPG em Fisiologia Vegetal

Múltiplas facetas da Fisiologia Vegetal no mercado de trabalho

CERTIFICADO

Certificamos que **Kellyane da Rocha Mendes** participou com êxito do "III Ciclo de Palestras do Programa de Pós-Graduação em Fisiologia Vegetal", realizado de forma on-line nos dias 25 e 26 de março de 2021, contabilizando carga horária total de 6 horas.

Viçosa - MG, 30 de março de 2021.

Prof. Wagner L. Araújo
Departamento de Biologia Vegetal
Universidade Federal de Viçosa
36570-000 Viçosa, MG

Professor Wagner L. Araújo
Coordenador Comissào Organizadora

PÓS-GRADUAÇÃO FISIOLOGIA VEGETAL

UFV
Universidade Federal de Viçosa

Verify the authenticity code **3290697.3308514.868643.6.07297344285618989149** at https://www.even3.com.br//documentos

XVIII BRAZILIAN CONGRESS OF PLANT PHYSIOLOGY

I IBERO-LATINAMERICAN CONGRESS OF PLANT BIOLOGY

6-9 September, 2022
Centro de Eventos da PUCRS, Porto Alegre, RS - Brazil

CERTIFICATE

We hereby certify that **Kellyane da Rocha Mendes**, participated in the quality of **Presenter of the study Nitrate alleviates ammonium toxicity and favor salinity acclimation by improving oxidative protection and CO2 assimilation in Jatropha curcas**, at the event XVIII Brazilian Congress of Plant Physiology and I Ibero-latinoamerican Congress of Plant Biology, in the **Poster** Modalitie.

September 6th, 2022

Gustavo Maia Souza
XVIII BCPP and I ILCBP President

Paulo Eduardo Ribeiro Marchiori
SBFV President

Promotion:

Diamond sponsor:

Ruby sponsor:

https://www.even3.com.br/documentos/imprimir?i=3290697.3308514.868643.6.07297344285618989149&cc=B50AF10A-0BC8-4EB6-A44B-CCB550CC380C

VII REUNIÃO NORDESTINA DE CIÊNCIA DO SOLO

Certificamos que, **Kellyane da Rocha Mendes**; cpf 009187792-00 participou com sucesso do evento VII REUNIÃO NORDESTINA DE CIÊNCIA DO SOLO, realizada de 05 a 09 de dezembro de 2022.

Mossoró, 12/12/2022

Carolina Malala Martins Souza.
Carolina Malala Martins Souza
Coordenadora da VII RNCS

Welka Preston Leite Batista da Costa Alves
Welka Preston Leite Batista da Costa
Coordenadora Científica da VII RNCS

APPENDIX E - PUBLISHED ABSTRACTS (AUTHOR AND CO-AUTHOR)

VII REUNIÃO NORDESTINA DE CIÊNCIA DO SOLO
Saúde do Solo, Sustentabilidade e Avanços na Agricultura do Nordeste
05 a 09 de Dezembro de 2022
UFC

HEAT AGGRAVATES SALINITY EFFECTS ON THE PHOTOSYNTHESIS EVEN STIMULATING ANTIOXIDANT DEFENSE IN RICE

Kellyane da Rocha Mendes[1]; Aurenivia Bonifacio[2]; Joaquim Albenísio Gomes da Silveira[1]
[1]Universidade Federal do Ceará; [2]Universidade Federal do Piauí.
Kellyane.mendes@hotmail.com

In the field, plants are exposed to multiple stresses, such as salinity, drought, intense light, heat/cold and others, resulting in different physiological responses. The combination of stresses can change the plant's metabolism differently compared to a single stress. The objective of this work was to evaluate the action of enzymes and antioxidant compounds to prevent photosynthetic damage under a combination of thermal and saline stresses. To evaluate these effects, 30-day-old rice plants were submitted to the following treatments: Control (without NaCl and at 27 °C), heat stress (without NaCl and at 42 °C), salt stress (with NaCl 100 mM and at 27 °C) and combined salt+heat stress (with NaCl 100 mM and at 42 °C). The control and salt stress treatments lasted 8 days and the heat and combined stress treatments lasted 6 hours. At the end of the experimental period, gas exchange, chlorophyll fluorescence and electrolyte leakage were measured and leaves were collected for biochemical determinations. Isolated heat and salt stresses were not enough to cause damage to the photochemical apparatus and heat stress only modified the stomatal opening. In the combined heat and salt stress, photosynthetic restriction was accompanied by stomatal closure, changes in transpiration parameters and chlorophyll fluorescence in plants exposed to salt+heat stress. The results indicate that photosynthetic processes were affected at the level of CO2 assimilation and quantum efficiency. These responses seem to be associated with increased sodium concentrations in leaves intensified by high temperature. Electrolyte leakage, TBARS and H2O2 content were high in the salt + heat treatment, but in isolated heat stress TBARS was decreased. Reduced ascorbate and glutathione were also reduced in plants exposed to the combination of salt and heat. All enzymes evaluated here were modulated differently according to treatment and exposure to stresses, single and combined. Taken together, the data indicate a more intense impairment of photosynthesis in rice plants in the combination of salt and heat, but the effective modulation of the antioxidant system was effective in establishing a new redox homeostasis and providing tolerance to abiotic stress.

Keywords: Photosynthesis, photochemical efficiency, salinity, multiple stresses

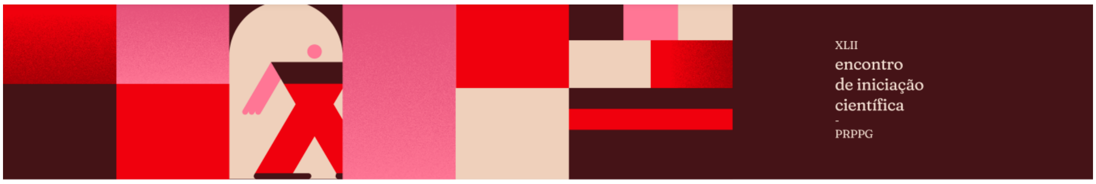
Nitrate alleviates ammonium toxicity and favor salinity acclimation by improving oxidative protection and CO_2 assimilation in *Jatropha curcas*

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

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.



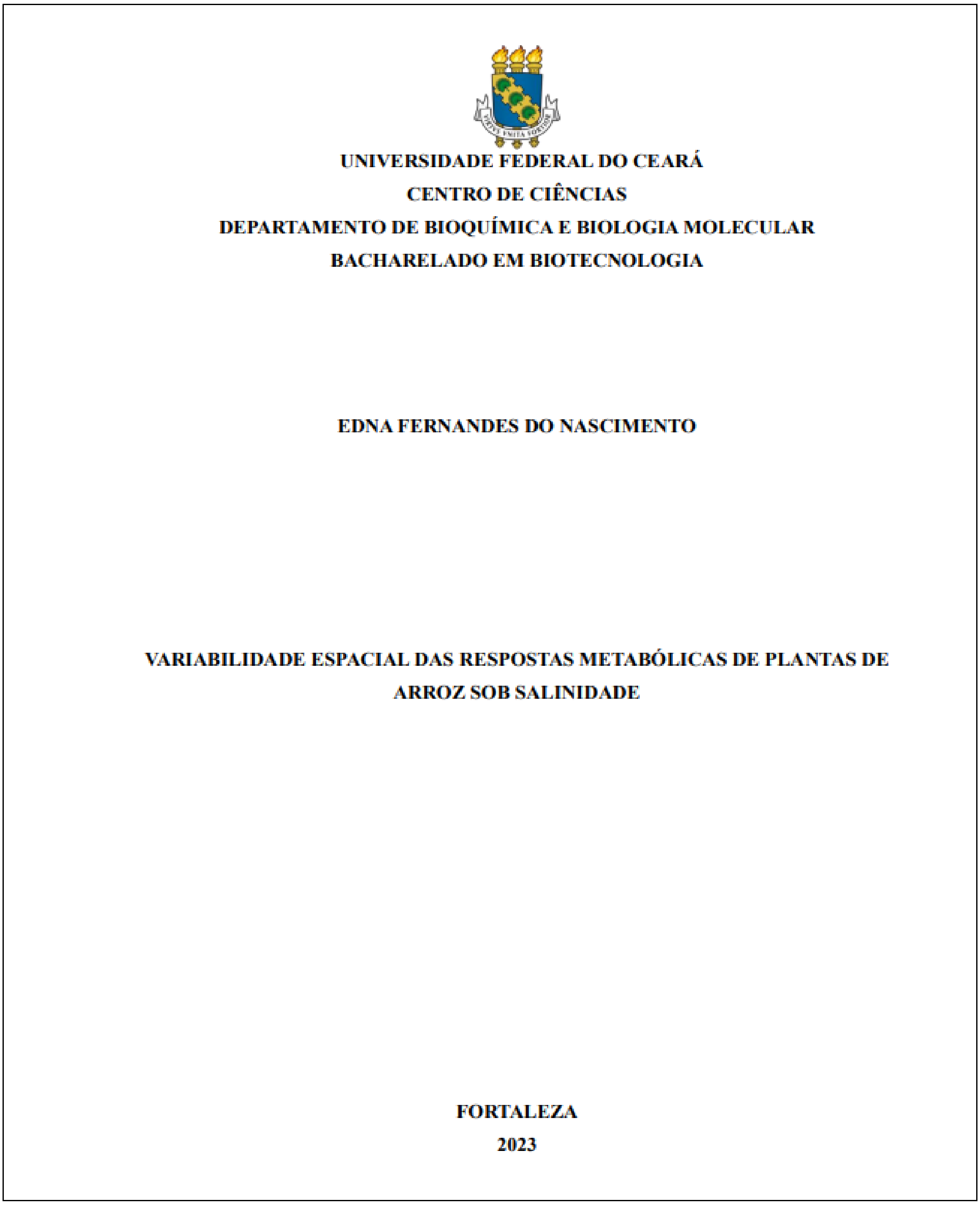
EFEITOS FISIOLÓGICOS E BIOQUÍMICOS DO USO DA TECNOLOGIA DE BIOMODULADORES EM PLANTAS DE FEIJÃO-CAUPI.

Ana Caroline Pinto Martins, Matheus Vasconcelos Monteiro, Kellyane da Rocha Mendes, Magna Pereira dos Santos, Francisco Bruno Silva Freire, Edna Fernandes do Nascimento, Humaira Bahadar.

O uso de bioestimulantes e biomoduladores se apresenta como uma estratégia promissora para incrementar a produtividade e/ou resiliência de plantas agrícolas. Neste sentido, o objetivo deste trabalho foi avaliar o efeito da aplicação de biomoduladores em processos bioquímicos e fisiológicos de plantas de feijão-caupi (*Vigna unguiculata* (L.) Walp.). Essa espécie é cultivada no Brasil desde do século XVI, sendo a segunda leguminosa (*Fabaceae*) mais cultivada no país. Os tratamentos consistiram na aplicação foliar de um biomodulador fornecido pela empresa Fertsan no estágio V3 da planta, comparados a um grupo de controle que recebeu a aplicação de água. Foram realizadas avaliações de parâmetros fisiológicos como: condutância estomática, taxa de fotossíntese, concentração intercelular de CO_2, taxa de transpiração, eficiência quântica do transporte fotossintético de elétrons, proporção de abertura dos centros de reações, taxa de transporte de elétrons, massas secas da parte aérea e da raiz. Além disso, foram realizadas avaliações bioquímicas como a atividade das enzimas catalase, peroxidade do ascorbato e dismutase do superóxido. Os resultados obtidos foram submetidos à análise de variância e comparados por meio do teste de t de Student, com nível de significância de 5% ($P < 0.05$). A aplicação do biomodulador não alterou os parâmetros fisiológicos e a atividade das enzimas analisadas, em comparação com o grupo de controle. Embora esses resultados sugiram que o biomodulador não afeta o processo fotossintético e a atividade de enzimas do sistema antioxidante da planta, cabe ressaltar que o mesmo se mostrou eficiente em alterar o crescimento de plantas cultivadas em campo, sugerindo que o biomodulador pode manifestar efeitos positivos a longo prazo. Diante disso, novos experimentos serão realizados com um tempo de exposição ao biomodulador mais prolongado, como forma de melhor avaliar o impacto da sua aplicação no crescimento de feijão-caupi.

Palavras-chave: Biomodulador, parâmetros fisiológicos, parâmetros bioquímicos, feijão-caupi.

APPENDIX F - CO-SUPERVISION OF GRADUATION WORK



UNIVERSIDADE FEDERAL DO CEARÁ
CENTRO DE CIÊNCIAS
DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR
BACHARELADO EM BIOTECNOLOGIA

EDNA FERNANDES DO NASCIMENTO

VARIABILIDADE ESPACIAL DAS RESPOSTAS METABÓLICAS DE PLANTAS DE ARROZ SOB SALINIDADE

FORTALEZA
2023

APENNDIX G - MANUSCRIPT SUBMITTED AS CO-AUTHOR

Plant Physiology and Biochemistry
Abscisic acid-mediated guard cell metabolism regulation
--Manuscript Draft--

Manuscript Number:	PLAPHY-D-23-03840
Article Type:	Research Paper
Section/Category:	drought stress; general stress (UV-B stress, waterlogging stress)
Keywords:	13C-labelling analysis; glycolysis; metabolic regulation; metabolomics; TCA cycle; water deficit
Corresponding Author:	Danilo M. Daloso Federal University of Ceara Fortaleza, CE BRAZIL
First Author:	Priscila Ariane Auler, Ph.D
Order of Authors:	Priscila Ariane Auler, Ph.D
	Moaciria Lemos
	Nicole P. Porto
	Kellyane R. Mendes
	Raissa S.C. Bret
	Danilo M. Daloso
Abstract:	Abscisic acid (ABA) is crucial for plant water deficit (WD) acclimation. However, it remains unclear how the interplay between ABA and guard cell (GC) primary metabolism aids plant WD acclimation. Here, we investigated how ABA regulates GC metabolism and how this influences plant WD acclimation using tomato wild type (WT) and the ABA-deficient sitiens mutant. ABA deficiency altered the level of sugars and organic acids in GCs of tomato plants under either irrigated or WD conditions and the dynamic of accumulation/degradation of these compounds in GCs during the dark-to-light transition. WD-induced metabolic changes were more pronounced in sitiens than WT GCs. Results from a 13C-glucose labelling experiment using isolated GCs indicate that ABA is required for the glycolytic fluxes toward malate and acts as a negative regulator of a putative sucrose substrate cycle. The expression of key ABA-biosynthetic genes was higher in WT than sitiens GCs after two cycles of WD. Additionally, the intrinsic leaf water use efficiency increased only in WT after the second WD cycle, compared to sitiens. Our results collectively highlight that ABA deficiency disrupts the homeostasis of GC primary metabolism and the WD memory, negatively affecting the plant's ability to acclimate recurring WD periods.
Suggested Reviewers:	Boon Leong Lim The University of Hong Kong School of Biological Sciences bllim@hku.hk
	Fabia Barbosa da Silva Goiano Federal Institute fabiabarbosabiologa@gmail.com
	Vicent Arbona Institute of Molecular and Cell Biology of Plants arbona@uji.es