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FRANCISCO DALTON BARRETO DE OLIVEIRA

INDUÇÃO DE TOLERÂNCIA AO ESTRESSE SALINO EM PLÂNTULAS DE ARROZ POR MEIO DO PRÉ-TRATAMENTO QUÍMICO DE SEMENTES COM TUNICAMICINA

FORTALEZA
2024

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Tese apresentada ao Programa de Pós-Graduação em Bioquímica da Universidade Federal do Ceará, como requisito parcial à obtenção do título de doutor em Bioquímica. Área de concentração: Bioquímica vegetal.

Orientador: Prof. Dr. Humberto Henrique de Carvalho.

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"A adversidade desperta em nós capacidades que, em circunstâncias favoráveis, teriam ficado adormecidas"

Horácio (65 a. C – 8 a.C.)

RESUMO

A salinidade é um fator abiótico que representa um grave problema para as plantas, afetando processos bioquímicos e fisiológicos em culturas de interesse e, consequentemente, diminuindo sua biomassa e produtividade. O aumento do processo de salinização em áreas agricultáveis ao redor do mundo torna esse problema ainda mais preocupante. Sabe-se que o estresse salino induz o surgimento de estresses secundários, como o oxidativo (superprodução de EROs, espécies reativas de oxigênio) e o estresse do retículo endoplasmático (RE), caracterizado por um acúmulo de proteínas mal dobradas no lúmen do RE. Ambos podem acarretar morte celular programada se não mediadas a tempo, e assim, estratégias que sejam capazes de contorná-los se mostram cada vez mais necessárias. O condicionamento fisiológico de sementes com agentes químicos é uma boa alternativa que pode induzir a tolerância de plantas a estresses, uma vez que ativa mecanismos de defesa na semente, como a ativação de enzimas antioxidantes e metabólitos responsivos a estresses, como a salinidade, em culturas de interesse, como o arroz (*Oryza sativa* L.). Portanto, esse trabalho teve como objetivo testar a hipótese de que a tunicamicina (TM), um antibiótico natural e estressor específico do RE, é capaz de aliviar a toxicidade do sal durante o estabelecimento de plântulas de arroz, cv. SCSBRS 113, através da ativação de um eficiente sistema antioxidante e da modulação positiva de metabólitos primários e responsivos ao estresse salino. Esta tese de doutorado foi dividida em dois capítulos: O primeiro é uma fundamentação teórica acerca dos principais tópicos deste trabalho e o segundo capítulo, na forma de um artigo científico, descreve dois grandes experimentos: o primeiro compreendeu três *screenings*, a fim de se determinar as melhores concentrações de NaCl e TM para as análises posteriores, do segundo experimento, com concentrações crescentes de NaCl (0, 100, 150, 200, e 250 mM de NaCl,) e com concentrações fixas de TM (ou 0 ou 0,50 µg. mL^{-1}, *screenings* I e II, respectivamente), e o terceiro *screening* foi executado com concentrações crescentes de TM (0; 0,125; 0,250; 0,375 e 0,500 µg. mL^{-1} de TM) e uma fixa de NaCl (150 mM). O segundo grande experimento abrange as análises posteriores com quatro tratamentos, escolhidos a partir dos resultados dos *screenings*, uma série de ensaios como os conteúdos de pigmentos fotossintéticos (clorofilas *a* e *b*, clorofilas totais e carotenoides), de íons inorgânicos (K^+, Na^+, e Cl^-), e EROS (H_2O_2 e $\bullet O_2^-$), bem como acúmulo de Na^+ no citosol por microscopia confocal, vazamento de eletrólitos, potencial osmótico, peroxidação lipídica, atividades de enzimas antioxidantes (SOD, CAT, APX e GPOD) e o metaboloma de partes aéreas e raízes de plântulas de arroz com 10 DAT (dias após tratamento). Os resultados mostraram que o NaCl a

150 mM permitiu o estabelecimento da plântula, enquanto 0,25 $\mu g.mL^{-1}$ de TM foi capaz de aliviar os efeitos da salinidade nos parâmetros de germinação e biomassa. Além do mais, plântulas condicionadas com TM, comparadas às não-condicionadas, sob estresse salino não só exibiram valores de massa seca, comprimento, pigmentos fotossintéticos, potencial osmótico, de conteúdo dos íons K^+ e de atividades de enzimas antioxidantes aumentados, como também mostraram redução no vazamento de eletrólitos, redução do conteúdo dos íons Na^+ e Cl^-, assim como diminuição do acúmulo de Na^+ nos tecidos, dos níveis de EROs e da peroxidação lipídica. Análises de metabolômica revelaram 40 metabólitos identificados, sendo 15 deles aminoácidos, 10 ácidos orgânicos, 11 carboidratos e 4 na categoria "outros". O *priming* com TM, na parte aérea também modulou positivamente metabólitos de defesa à salinidade, como os osmoprotetores beta-alanina e maltose, enquanto que em raízes houve um maior acúmulo de ácido fosfórico e aminoácidos, como N-acetilserina e O-acetilserina, responsáveis pelos metabolismos de ferro e enxofre na célula, além da síntese de energia, e também dos açúcares maltose, sorbitol e rafinose, que não só fornecem energia para a célula, como também atuam como osmoprotetores, o que pode ter contribuído para redução dos efeitos da salinidade. Como conclusão, o condicionamento de sementes de arroz com TM aliviou o estresse severo de NaCl em plântulas de arroz, principalmente ao ativar enzimas antioxidantes e modular positivamente metabólitos primários e de defesa, que ajudaram a gerar energia, reduzir os teores de íons Na^+, e assim, provando ser uma estratégia promissora para mitigar o estresse salino. Este trabalho fornece perspectivas que podem levar a análises posteriores a fim se melhor compreender os mecanismos moleculares ativados por TM que levam à aclimatação ao estresse salino.

Palavras-chave: pré-tratamento; tunicamicina; antioxidantes; *Oryza sativa* L.; perfis metabólicos.

ABSTRACT

Salinity is an abiotic fator that represents a major problem for plants, affecting several biochemical and physiological processes in crops of interest, therefore decreasing their biomasses and yield, and the increase of salinization processes in arable lands makes it a concerning issue. It is also known that salt stress induces secondary stresses, such as the oxidative one (characterized by an overproduction of reactive oxygen species) and the endoplasmic reticulum (ER) one, which is represented by an accumulation of unfolded or misfolded proteins in the ER lumen. Both can lead to programmed cell death, so strategies that are able to overcome them prove to be necessary. Chemical seed priming is a good alternative to lead to plant tolerance to abiotic stresses, for it activates defense mechanisms in the seed, such as activation of antioxidante enzymes and positive modulation of stress-responsive metabolites in crops of importance, such as rice (*Oryza sativa* L.). Based on that, this work had as goal to test the hypothesis that tunicamycin (TM), a natural antibiotic and a specific ER stressor, is capable of alleviating salt toxicity during the establishment of rice seedlings, cv. SCSBRS 113, through activation of an efficient antioxidant system, Na^+ exclusion of the cell and positively modulating primary and salt-responsive metabolites. This PhD thesis was divided in two chapters: the first one was a theorical foundation about the main topics of this work. The second one, in the form of a paper, to be submitted, describes two experiments, both with completely randomized designs. The first one comprised three screenings, in order to determine the best NaCl and TM concentrations for further analyses, with increasing NaCl concentrations (0, 100, 150, 200, and 250 mM NaCl) and fixed TM concentrations (either 0 or 0.50 µg . mL^{-1}, screenings I and II, respectively), and the third screening was performed with increasing TM concentrations (0, 0.125, 0.250, 0.375 and 0.500 µg. mL^{-1} TM) and one NaCl concentration (150 mM); the second experiment comprised further analyses with four treatments, a set of assays such as contents of photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophyll and carotenoids), inorganic ions (K^+, Na^+, and Cl^-), and ROS (H_2O_2 and $\bullet O_2^-$) as well as Na^+ accumulation in tissues by confocal microscopy, electrolyte leakage, osmotic potential, lipid peroxidation, antioxidant enzyme activities (such as SOD, CAT, APX and GPOD) and metabolomics of shots and roots of 10 DAT (days after treatment) rice seedlings, being chosen from the screenings. Results showed that 150 mM NaCl allowed seedling establishment while 0.25 µg.mL TM was able to alleviate salinity effects in germination and biomass parameters. Moreover, TM primed seedlings, compared to unprimed ones under salinity, not only displayed increased dry mass, length,

photosynthetic pigments and K^+ contents, osmotic potential and antioxidant enzyme activities, but also showed decreased electrolyte leakage, Na^+ and Cl^- contents, ROS levels and lipid peroxidation. Metabolomics analyses revealed 40 identified metabolites, being 15 of them amino acids, 10 organic acids, 11 carbohydrates, and 4 others. TM-priming, in shoots, also modulated positively salt-defensive metabolites, such as the osmoprotectants beta-alanine and maltose, whereas in roots it had a higher accumulation of phosphoric acid, the amino acids o-acetylserine and N-acetylserine, responsible for Fe and S metabolisms in the cell such as energy synthesis, and also the sugars maltose, raffinose and sorbitol, which not only provide energy to the cell but also act as osmoprotectants, which might have led to a mitigation of salt effects. In conclusion, TM seed priming alleviated severe salt stress in rice seedlings by mainly activating antioxidant enzymes and also positively modulating defense metabolites that helped generate energy, sending Na^+ ions out of the cell, therefore proving to be an efficient strategy to mitigate salt stress. This work provides insights that can lead to further analyses in order to better comprehend the molecular mechanisms activated by TM that lead to salt acclimation.

Keywords: seed priming; tunicamycin; antioxidants; *Oryza sativa* L; metabolic profiles.

LISTA DE ABREVIATURAS E SIGLAS

APX	Ascorbato – peroxidase (enzima)
AsA	Ascorbato
bZIP	Zíper de Leucina Básica
CAT	Catalase (enzima)
GPOD	Guaiacol – peroxidase (enzima)
GSH	Glutationa (reduzida)
IRE1	*Inositol Requiring Enzyme 1*
IVEPA/ SESI	Índice de velocidade de emissão de parte aérea / *Shoot emission speed index*
IVG/ GSI	Índice de velocidade de germinação / *Germination speed index*
MDA	Malondialdeído
PCA	*Principal Component Analysis*
PSI	Fotossistema I
PSII	Fotossistema II
SOD	Superóxido – dismutase (enzima)
TCA	tricarboxylic acid cycle
TMEPA/	Tempo médio de emissão de parte aérea / *mean shoot emission time*
TMG/ MGT	Tempo médio de germinação / *mean germination time*
UPR	*Unfolded protein response*

SUMÁRIO

1 INTRODUÇÃO

As plantas, geralmente estão submetidas a diversos fatores bióticos, como vírus e fungos, e abióticos, como déficit hídrico e a salinidade (SINGH; SINGLA-PAREEK; PAREEK, 2021). Dentre estes, a salinidade se destaca com um dos fatores que mais limita a produção das culturas, especialmente em áreas com baixa pluviosidade, como o semiárido nordestino (ABDENNOUR et al., 2021). Além disso, várias áreas agricultáveis no mundo estão presenciando uma rápida salinização dos solos, o que pode ameaçar os estoques de alimentos a nível global (QIU et al., 2023; RENGASAMY, 2006). De fato, os principais fatores que contribuem para a salinização dos solos são os de causas naturais, como o clima, que pode aumentar a evapotranspição e favorecer a irregularidade de chuvas ou os provocados pela atividade humana (fatores antropogênicos), como práticas agrícolas inadequadas tais como sistemas de irrigação precários que distribuem a água de modo não uniforme, uso de água de má qualidade, além de drenagem insuficiente (AKÇA et al., 2020; PESSOA et al., 2022; ZHOU et al., 2013).

Sabe-se que a salinidade inibe vários processos bioquímicos e fisiológicos essenciais para o desenvolvimento da planta, como a germinação, a fotossíntese e a abertura estomática (CHELE et al., 2021). Isso se deve aos dois estágios de manifestação do estresse salino: o osmótico, que reduz a absorção de água pelas raízes, levando a um desbalanço hídrico, e o iônico, que leva aos principais fenômenos moleculares afetados no organismo vegetal, como a fotossíntese (CHELE et al., 2021; YAN et al., 2018), além de promover o surgimento de estresses secundários (YAN et al., 2018).

Dentre os estresses secundários induzidos pela salinidade, pode-se citar o estresse oxidativo, que se caracteriza por uma superprodução de espécies reativas de oxigênio (EROs), e ocorre especialmente nos peroxissomos, na parede celular, na membrana plasmática e nas organelas energéticas cloroplastos e mitocôndrias (JANKU; LUHOVÁ; PETRIVALSKÝ, 2019). Um outro tipo de estresse secundário diz respeito ao estresse do retículo endoplasmático (RE), que se caracteriza por um acúmulo de proteínas mal dobradas e que, se não tratado a tempo, pode levar à morte celular programada (KIM; MOCHIDA; SHINOZAKI, 2022). De fato, tais eventos ocorrem no RE como consequência dos estresses de seca, salinidade e altas temperaturas, dentre outros (REYES-IMPELLIZZERI; MORENO, 2021). Para lidar com o estresse do RE, um mecanismo molecular protetor denominado resposta a proteínas mal dobradas (do inglês, *unfolded protein response* - UPR) tem sido amplamente descrito em *Arabidopis thaliana* e mais recentemente em outras culturas como o arroz (ZHOU et al., 2022), a soja (PAN et al., 2023) e o milho (LI et al., 2020). Além disso, o acúmulo de

proteínas no RE levam à ativação dede fatores de transcrição controlando eventos subsequentes como diminuição de EROS (CAO et al., 2022).

Vários agentes químicos têm sido usados para induzir diretamente o estresse do RE, destacando-se dentre eles o ditiotreitol (DTT) e a tunicamicina (TM) (MANGHWAR; LI, 2022). A TM atua inibindo a enzima N-acetilglicosamina-fosfotransferase (GPT), situada na membrana do RE (ASTANI et al., 2022). Pelo fato de esta enzima se situar na membrana do RE e realizar o primeiro passo de N-glicosilação de proteínas, estudos mostram que este composto induz o estresse do RE de forma específica (HÄWEKER et al., 2010; MANGHWAR; LI, 2022). De fato, a rapidez da RE é essencial para a sobrevivência da célula pois os efeitos são agravados com o aumento do tempo de exposição ao estresse, promovendo diminuição da expressão de genes da via UPR, redução de vias metabólicas relacionadas ao metabolismo primário e consequentemente diminuição do crescimento da plântula (LIMA et al., 2022), de modo que concentrações menores de TM (0.5 $ug.L^{-1}$) promovem uma manutenção da biomassa, comprimento da plântula, e expressão de genes UPR, enquanto que concentrações maiores impactam negativamente o metabolismo (CAVALCANTE et al., 2023). Torna-se evidente a dupla função do RE em ativar mecanismos de sobrevivência ou morte celular em respostas aos estresses, como o salino (CARVALHO et al., 2014; SIMONI et al., 2022). Com base nisso, estratégias e abordagens para se entender esses fenômenos moleculares que ativam principalmente os mecanismos de sobrevivência se tornam necessários.

Dentre as estratégias empregadas para geração de tolerância nas culturas, o condicionamento fisiológico, sobretudo em sementes, também chamado de *priming*, é uma boa alternativa para impulsionar a tolerância a estresses pelas plantas, uma vez que envolve a ativação de mecanismos reparatórios da semente, como a síntese *de novo* de DNA e proteínas, colaborando para a garantia da germinação e desenvolvimento da plântula (DHANYA THOMAS; DINAKAR; PUTHUR, 2020). Os métodos de *priming* são classificados de acordo com a natureza do agente condicionante: o *priming* químico, utiliza compostos químicos como hormônios e moléculas sinalizadoras (ZULFIQAR et al., 2022). Esta abordagem apresenta maior oportunidade de uso mais eficiente de condicionamento fisiológico em estudos moleculares e fisiológicos em plantas e gerenciamento de estresses em culturas, por ser uma estratégia de fácil controle e aplicabilidade (SAVVIDES et al., 2016).

Além de detecção das melhorias morfofisiológicas e bioquímicas, os efeitos do condicionamento fisiológico podem ser evidenciados ou elucidados por abordagens mais robustas, como a metabolômica (TUGIZIMANA et al., 2018). Esta envolve o estudo

quantitativo e qualitativo dos metabólitos (primários e secundários), seus níveis e interações nas mais diversas condições. Com o conhecimento dos perfis metabólicos em diferentes níveis de estresse, pode-se perceber como o metabolismo varia de uma condição para a outra (RAJKUMARI et al., 2023). Assim, a metabolômica pode ajudar a identificar mecanismos-chave envolvidos na aclimatação a estresses em culturas de importância socioconômica, como o arroz.

O arroz (*Oryza sativa* L.) é uma espécie gramínea de elevada importância econômica. É o terceiro cereal mais produzido no mundo, constituindo-se da alimentação básica de aproximadamente dois terços da população mundial da qual é uma importante fonte de carboidratos, proteínas e vitaminas (FUKAGAWA; ZISKA, 2019). A importância do arroz para o país também é notória, pois o Brasil é o principal mercado do grão fora da Ásia (ZANIN; BACCHI; ALMEIDA, 2019). A cultura representa também não só a base alimentar da população como também é uma fonte de renda para muitos brasileiros, sendo a agricultura familiar responsável por 34% da produção nacional de arroz (SARAIVA et al., 2013). Diante do exposto, o presente trabalho teve como objetivo principal avaliar, por meio de abordagens bioquímicas, fisiológicas e moleculares, se o *priming* de sementes de arroz pelo tratamento com TM alivia os efeitos do estresse salino, durante o crescimento inicial das plântulas de arroz e quais os mecanismos envolvidos.

2 HIPÓTESE

O condicionamento fisiológico de sementes de arroz com TM induz uma maior tolerância das plântulas à salinidade na fase inicial de crescimento, por meio da ativação do sistema antioxidante e modulação metabólica, induzindo o acúmulo de metabólitos-chave para o ajuste osmótico e iônico, mitigando assim os efeitos deletérios da salinidade.

3. OBJETIVOS

3.1 Objetivo geral

Testar se o condicionamento fisiológico (*priming*) das sementes de arroz com tunicamicina induz tolerância à salinidade, durante a fase inicial de crescimento, analisando os mecanismos bioquímicos, fisiológicos e moleculares envolvidos, bem como as alterações no metaboloma.

3.2 Objetivos específicos

- Determinar a concentração de NaCl que permite a germinação das sementes de arroz condicionadas com TM com base no percentual e parâmetros de germinação e biomassa;
- Determinar a concentração de TM que promove melhores índices e parâmetros de germinação e de biomassa em sementes de arroz, submetidas ao estresse salino em concentração de NaCl determinada no item anterior;
- Baseados na concentração de sal e de TM, estabelecer os tratamentos para as análises morfofisiológicas, bioquímicas e metabólicas;
- Mensurar os parâmetros de crescimento e biomassa na parte aérea e raízes de plântulas de arroz pré-tratadas com TM, sob estresse salino;
- Determinar o grau de vazamento de eletrólitos e o potencial osmótico de plântulas de arroz condicionadas ou não com TM na presença de NaCl;
- Quantificar os pigmentos fotossintéticos (clorofilas e carotenoides) da parte aérea de plântulas de arroz condicionadas ou não à TM, sob salinidade;
- Determinar os teores dos íons Na^+, K^+ e Cl^- na parte aérea e raízes de plântulas de arroz condicionadas ou não com TM, na presença de NaCl;

- Determinar e visualizar o acúmulo de íons Na^+ na parte aérea e raízes de plântulas de arroz condicionadas ou não com TM, na presença de NaCl, por meio de fluorescência do íon sódio usando microscopia confocal;
- Mensurar os teores de EROs (H_2O_2 e $\bullet O_2^-$) na parte aérea e raízes de plântulas de arroz pré-tratadas ou não com TM sob estresse salino;
- Avaliar os efeitos da TM na peroxidação de lipídios de membranas na parte aérea e raízes de plântulas de arroz condicionadas ou não com TM. na presença de NaCl;
- Determinar as atividades enzimáticas da dismutase do superóxido (SOD), catalase (CAT), peroxidase do guaiacol (GPOD) e peroxidase do ascorbato (APX) de parte aérea e raízes de plântulas de arroz condicionadas ou não com TM, sob estresse salino;
- Verificar a expressão de genes responsivos ao estresse salino, como *SOS1* e *NHX1*, e da via UPR, como *IRE1* e *bZIP50*, em na parte aérea e raízes de plântulas de arroz condicionadas ou não com TM, na presença de NaCl;
- Investigar como a TM modula o metaboloma da parte aérea e raízes de plântulas de arroz, sob salinidade, e induz metabolitos-chave para a defesa contra o estresse abiótico.

4. CAPÍTULO 1: CAPÍTFUNDAMENTAÇÃO TEÓRICA

4.1 O estresse salino

4.1.1 Salinidade no mundo, no Brasil e nos solos

A salinidade é um dos fatores abióticos que mais limitam a produtividade, qualidade e quantidade das culturas, afetando 7% da área terrestre e 33% das áreas irrigadas no mundo (CHELE et al., 2021; SHOKAT; GROSSKINSKY, 2019). Comumente, o fenômeno de salinização ocorre em zonas áridas ou semiáridas, nas quais a irrigação e/ou a pluviosidade são insuficientes para lixiviar os sais, a drenagem é deficiente e/ou lençóis freáticos rasos existem e onde subsolos salinos se formaram naturalmente de depósitos marinhos (CORWIN; SCUDIERO, 2019). Embora possa ocorrer de forma natural, as principais causas do aumento da salinização do solo são de origem antropogênica, por conta de más práticas de irrigação, uso impróprio de fertilizantes e poluição industrial (AFZAL et al., 2020). Os solos afetados pela salinidade cobrem uma área de aproximadamente 1.060,1 milhões de hectares (Mha) no mundo, e são encontrados principalmente na Ásia Setentrional e Central (211,7 Mha), África (200,6 Mha) e América do Sul, com 129,3 Mha de áreas afetadas (ESWAR; KARUPPUSAMY; CHELLAMUTHU, 2021).

No Brasil, os solos afetados pela salinidade ocupam cerca de 16 Mha, sendo 70% desta área localizada na região semiárida (GHEYI et al., 2022). Problemas de salinização do solo em áreas irrigadas na região Nordeste ocorrem em planícies e onde pelo menos parte da área possui problemas de drenagem naturais (DE ALBUQUERQUE et al., 2018). Por exemplo, para a instalação do Perímetro Irrigado de Morada Nova (CE), no fim dos anos 1960, vastas áreas ocupadas por árvores de carnaúba foram desflorestadas, uma espécie que indica a existência de solos com baixa drenagem natural, e, possivelmente, com problemas naturais de sodicidade. Apesar disso, não se exclui a responsabilidade do homem à salinização de extensas áreas agricultáveis (DE ALBUQUERQUE et al., 2018; SILVA et al., 2019).

Um solo é considerado salino quando a condutividade elétrica do extrato de saturação na zona radicular excede 4 dS/m, o que corresponde a aproximadamente 40 mM de NaCl a 25 ºC e um percentual de sódio trocável de 15% e pH inferior a 8,5 (ISMAYILOV et al., 2021). A acumulação de sais em solos agrícolas é comumente feito através de fertilização e irrigação (SHRIVASTAVA; KUMAR, 2015). Embora os sais presentes nos solos salinos contenham, em geral, os cátions Ca^{2+} e Mg^{2+}, e os ânions SO_4^{2-} e HCO_3^- , os íons Na^+ e Cl^- tendem a ser mais abundantes e também os mais danosos às culturas, causando distúrbios

bioquímicos e fisiológicos a elas (DJAJADI; SYAPUTRA; HIDAYATI, 2022).

4.1.2 Características do estresse salino e seus efeitos nas plantas

Sabe-se que o acúmulo de sais no solo altera a fisiologia e metabolismo das plantas, afetando negativamente a germinação, o crescimento da plântula, a floração e frutificação, o que leva à redução da produtividade (MBARKI et al., 2020).

O estresse salino pode ser divido em duas fases: a osmótica, também chamada de resposta inicial, e a iônica, sendo esta de resposta tardia (MUCHATE et al., 2016; MUNNS; TESTER, 2008). A fase inicial do estresse salino se deve ao sal do lado de fora da zona radicular, baixando seu potencial osmótico e diminuindo o gradiente de potencial hídrico entre a planta e o meio de crescimento. Em consequência disso, ocorre redução da absorção de água, do crescimento da raiz, da elongação celular e do desenvolvimento de folhas, levando também à redução do número de novas folhas, ao fechamento estomático e à danos nas células de folhas transpiratórias (Figura 1), dentre outros (MUNNS; TESTER, 2008; NADEEM et al., 2019). Por outro lado, a fase tardia da salinidade se deve aos efeitos tóxicos do sal (íons Na^+ e Cl^-) dentro da planta, levando à captura destes em folhas mais velhas, o que acarreta a senescência prematura de folhas, inibição da fotossíntese e da atividade enzimática de forma geral (MUCHATE et al., 2016; ROY; NEGRÃO; TESTER, 2014).

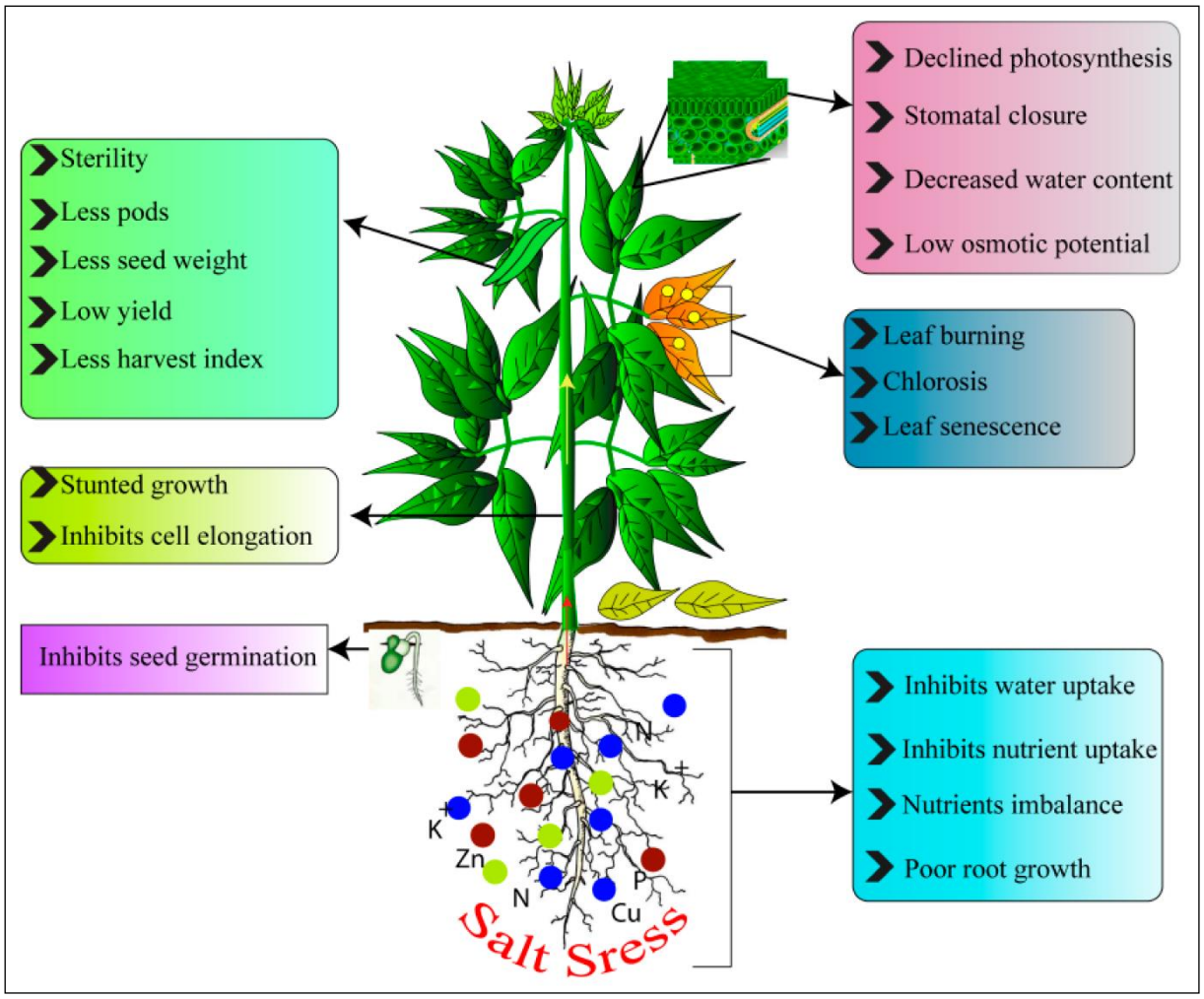

Figura 1 - Efeitos da salinidade nas plantas. Nadeem et al. (2019)

O primeiro efeito fisiológico ocorre devido à absorção dos íons Na^+ e Cl^-, o que reduz o potencial hídrico (Ψw) entre a raiz e a solução do solo, o que determina a disponibilidade de água (ABBASI et al., 2016). Em seguida, altas concentrações de Na^+ induzem toxicidade iônica que afeta a absorção de nutrientes (TAVAKKOLI et al., 2012). Além disso, a toxicidade com NaCl está diretamente associada à condutividade elétrica (CE), que é um indicador de tolerância da planta ao estresse salino (ULLAH; BANO; KHAN, 2021).

A fotossíntese é um processo metabólico que é severamente afetado pelo estresse salino. Os íons Na^+ e Cl^-, quando absorvidos em grandes quantidades nas folhas, levam à perda de água das células-guarda nos estômatos, e, consequentemente, à mudanças na morfologia, na atividade ou densidade numérica dessas estruturas, o que pode acarretar a redução ou prevenção da entrada de CO_2 e limitar a fotossíntese líquida (BEN AMOR et al., 2020). Além disso, a salinidade também pode danificar a integridade estrutural dos cloroplastos, causando danos às membranas dos tilacoides, prejudicando a etapa

fotoquímica da fotossíntese e todo o processo de assimilação de carbono (BOSE et al., 2017). Estudos mostram que, na presença de NaCl, os sistemas de membranas cloroplastidiais são danificados, os formatos dos cloroplastos são alterados passando da forma ovoide para a esférica, as lamelas internas se tornaram levemente inchadas e o número de grãos de amido, as gotículas lipídicas e os glóbulos osmiofílicos aumentam (GOUSSI et al., 2018; LU et al., 2023).

A salinidade também afeta outras organelas e estruturas celulares importantes, como a parede celular e as mitocôndrias. Um acúmulo de íons Na^+ na parede celular pode impedir a associação dos íons Ca^{2+} com as pectinas, perturbando assim sua ultraestrutura primária; em adição a isto, este estresse também induz à inibição da síntese de celulose, principalmente pela despolimerização dos microtúbulos e pela internalização de complexos de celulose-sintase, conforme ilustrado na figura 2 (COLIN et al., 2023). Já nas mitocôndrias, a salinidade pode causar não só redução na síntese de ATP, mas também alterações morfológicas, e assim como nos cloroplastos, pode levar à produção de EROs que, não só afetam a cadeia transportadora de elétrons mitocondrial, como também às dos cloroplastos (TANG; ZHU, 2023).

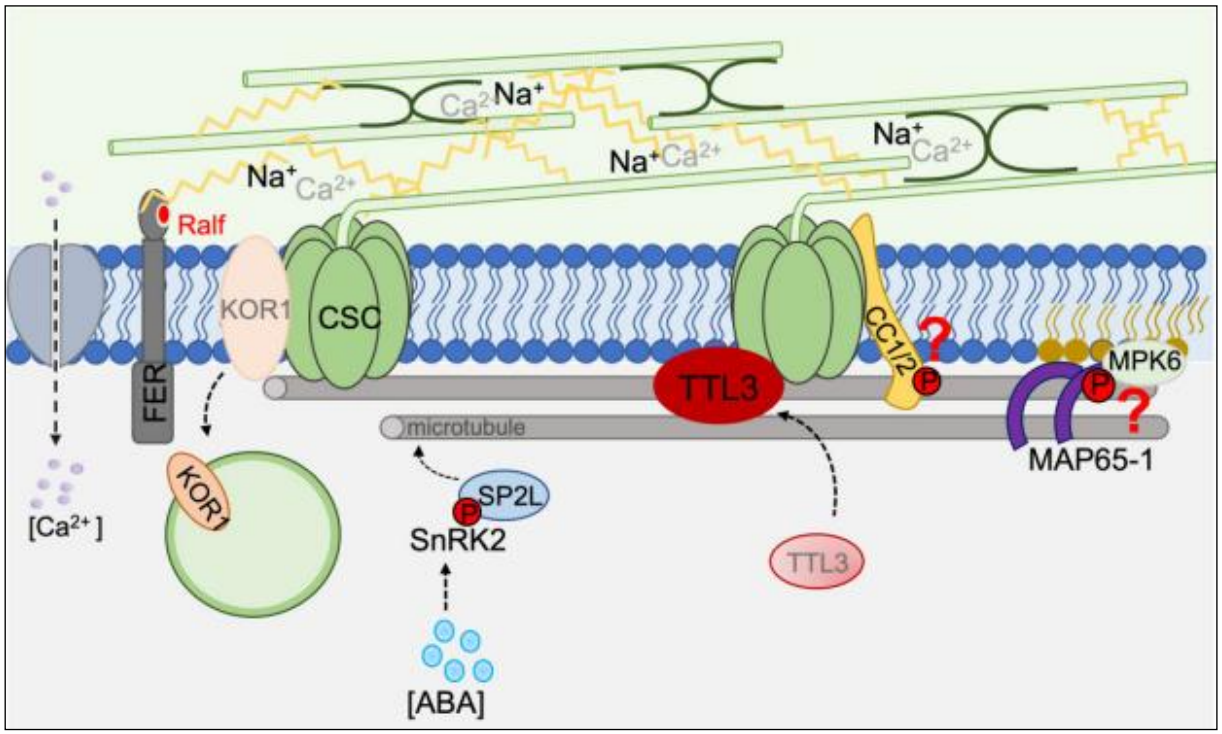

Figura 2 - Efeito da salinidade na estrutura da parede celular. *RAPID ALKALINIZATION FACTOR* (RALF), *FERONIA* (FER), *KORRIGAN* (KOR*), cellulose synthase* (CESA) *complex (CSC), tetratricopeptide thioredoxin-like* (TTL), *mitogen-activated protein kinase* (MPK), *microtubule-associated protein* (MAP), *SPIRAL2 (SPR2)-like (SP2L), sucrose non-fermenting-1-related* (SnRK), *abscisic acid* (ABA). Fonte: Colin et al. (2022).

4.1.3 Espécies reativas de oxigênio (EROS), salinidade e estresse oxidativo

As EROs são espécies químicas que possuem pelo menos um átomo de oxigênio proveniente de reações metabólicas secundárias (oxidações incompletas), e que ocorrem principalmente nas mitocôndrias, cloroplastos, peroxissomos, parede celular e membrana plasmática, como ilustrado na figura 3 (HASANUZZAMAN et al., 2021).

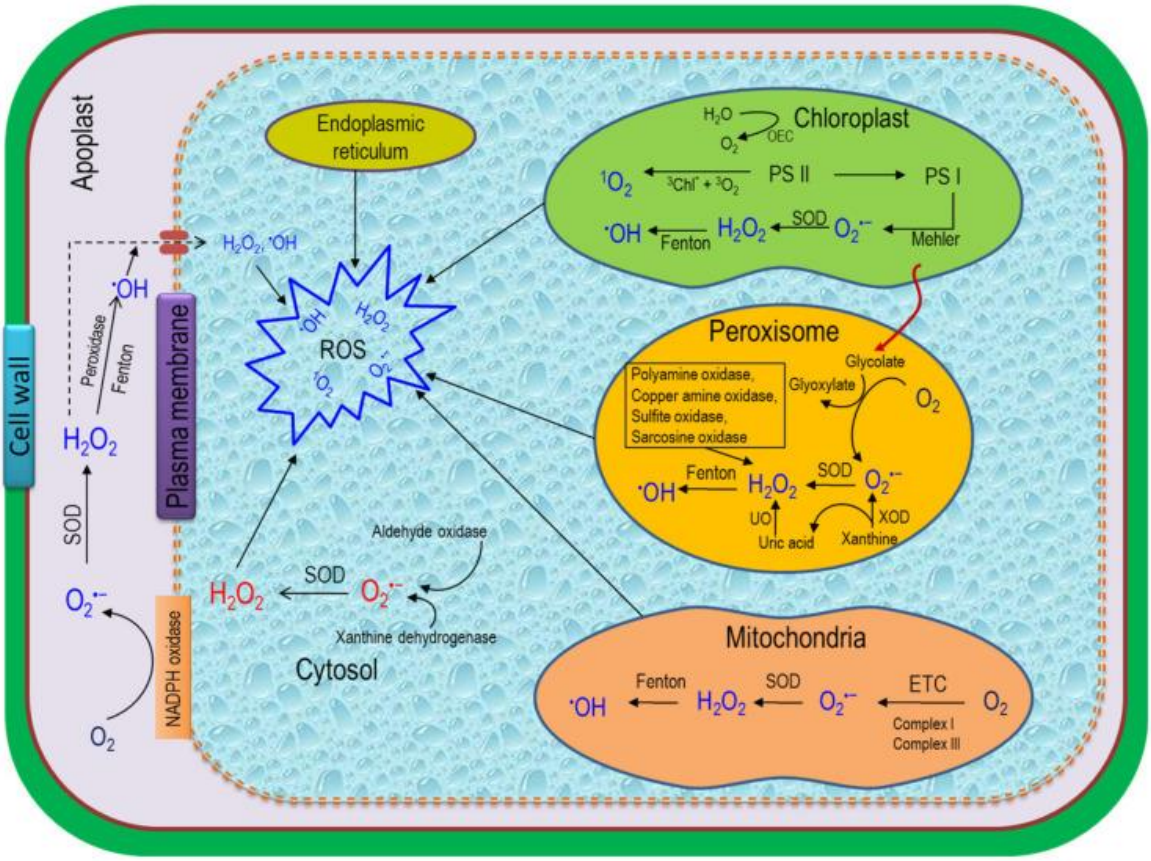

Figura 3 - Sítios de formação de EROs na célula vegetal. Hasanuzzaman et al. (2021)

Dentre as principais EROs, estão os radicais hidroxil ($^{\bullet}OH$), ânions/radicais superóxidos ($\bullet O_2^-$), o oxigênio singleto ($^1O_2$) e o peróxido de hidrogênio (H_2O_2), os quais se acumulam tanto em organelas quanto no citosol (HUANG et al., 2019; MANSOOR et al., 2022). O oxigênio singleto geralmente é produzido no fotossistema II (PSII) dos cloroplastos; o ânion superóxido é produzido nas cadeias transportadoras de elétrons mitocondriais e cloroplastidiais, assim como nas membranas plasmáticas, através da reação da NADPH-oxidase, sendo o precursor da maioria das EROs (YAMAUCHI et al., 2017); o H_2O_2 normalmente é produzido através das reações da SOD, utilizando o $\bullet O_2^-$ como substrato, e é a EROs que possui a meia-vida mais longa (10^{-3} s), e por isso funcionando como molécula sinalizadora, podendo ser também transportada para dentro das células pelas aquaporinas

(HUANG et al., 2019; MITTLER, 2017); e por fim o radical hidroxil, que pode ser formado a partir da quebra da ligação dupla O=O na molécula de H_2O_2 (AL-MAMARY; MOUSSA, 2021). Este radical é muito reativo e age bem próximo de seu sítio de produção, sendo ~~assim~~ a mais ativa das EROs, podendo reagir com quaisquer biomoléculas (AL-MAMARY; MOUSSA, 2021; HUANG et al., 2019).

Os estresses abióticos, dentre eles o salino, tendem a aumentar significativamente os níveis das EROs, as quais em altas concentrações acentuam seu efeito deletério. O acúmulo de $^1O_2$ pode colocar em risco o centro de reação do PSI ao danificar proteínas de membranas, assim como causar perda de atividade do PSII nos cloroplastos (SINGH, 2022). O excesso de $\bullet O_2^-$ pode levar à anomalias nas proteínas Fe-S, importantes centros de reação dos citocromos b_6f nos cloroplastos, e nos complexos I (NADH-desidrogenase), II (succinato desidrogenase) e III (citocromo *bc*1) nas membranas internas mitocondriais, prejudicando assim o transporte de elétrons nestas organelas (DUMANOVIĆ et al., 2021). Embora seja a menos reativa das EROs, o H_2O_2 pode promover a oxidação de proteínas (especialmente nos resíduos de cisteína e metionina), promover a oxidação do DNA (especialmente nos nucleotídeos de guanina) e, principalmente, promover a peroxidação de lipídios de membrana, produzindo malondialdeído (MDA) e consequentemente lisando as membranas como indicado na figura 4 (HASANUZZAMAN et al., 2020).

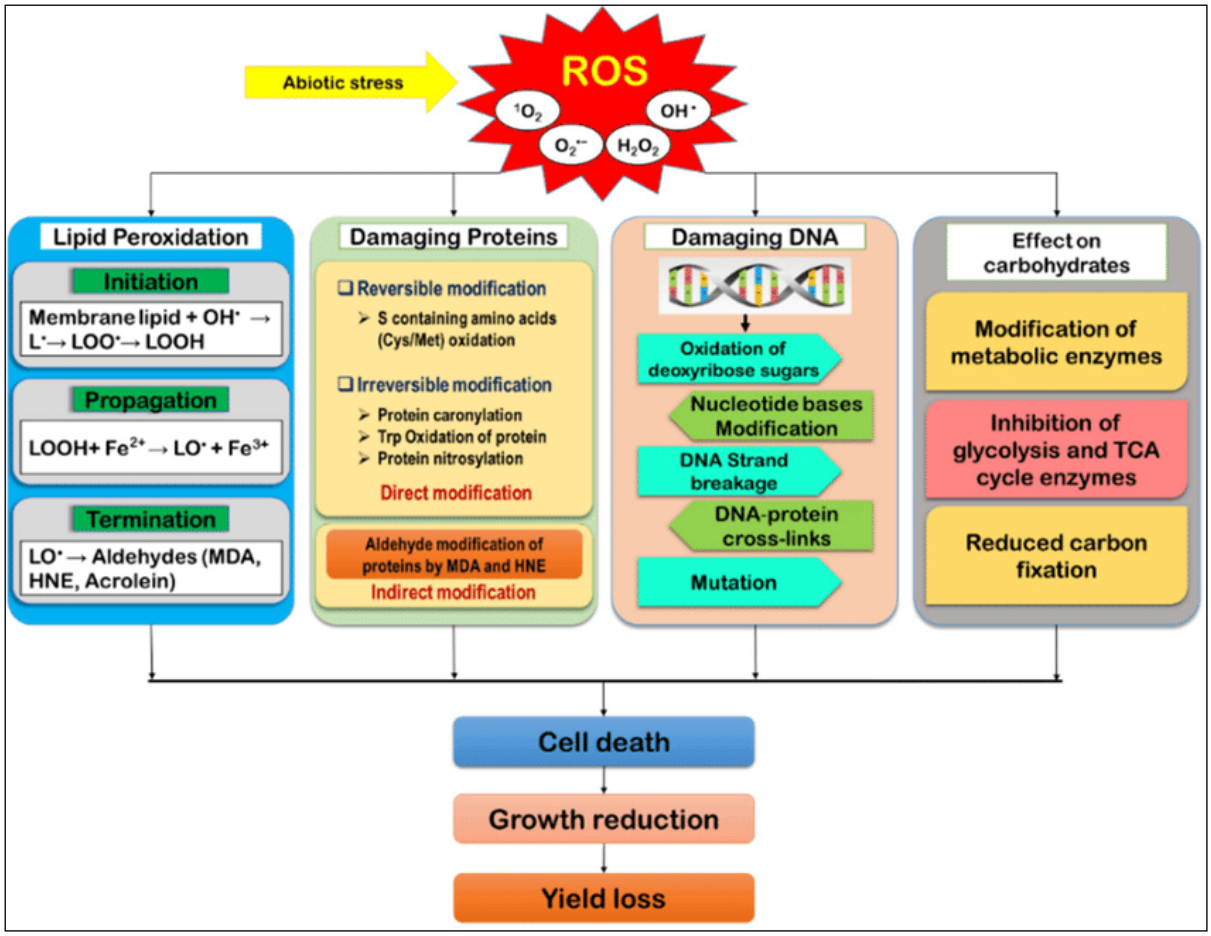

Figura 4 - Danos das EROs às células. Fonte: Hasanuzzaman et al. (2020)

A fim de combater o excesso de EROs e seus danos cumulativos, as células desenvolveram um sistema antioxidante enzimático e não-enzimático para neutralizar seus efeitos prejudiciais estes compostos (MOHAMMADI et al., 2021). Antioxidantes não enzimáticos são moléculas de baixo peso molecular de natureza não-proteica, como o ascorbato (AsA), a glutationa reduzida (GSH), os compostos fenólicos, α-tocoferol (vitamina E) e os carotenoides (LAXA et al., 2019), sendo estes dois últimos capazes de neutralizar o oxigênio singleto, conforme pode ser visto na figura 5 (ADILETTA; DI MATTEO; PETRICCIONE, 2021). Já os antioxidantes AsA e GSH atuam em conjunto com o sistema enzimático de defesa contra o estresse oxidativo, o qual é composto pelas enzimas SOD, CAT, APX, GPOD e glutationa-peroxidase (GPX) (KUMARI et al., 2022).

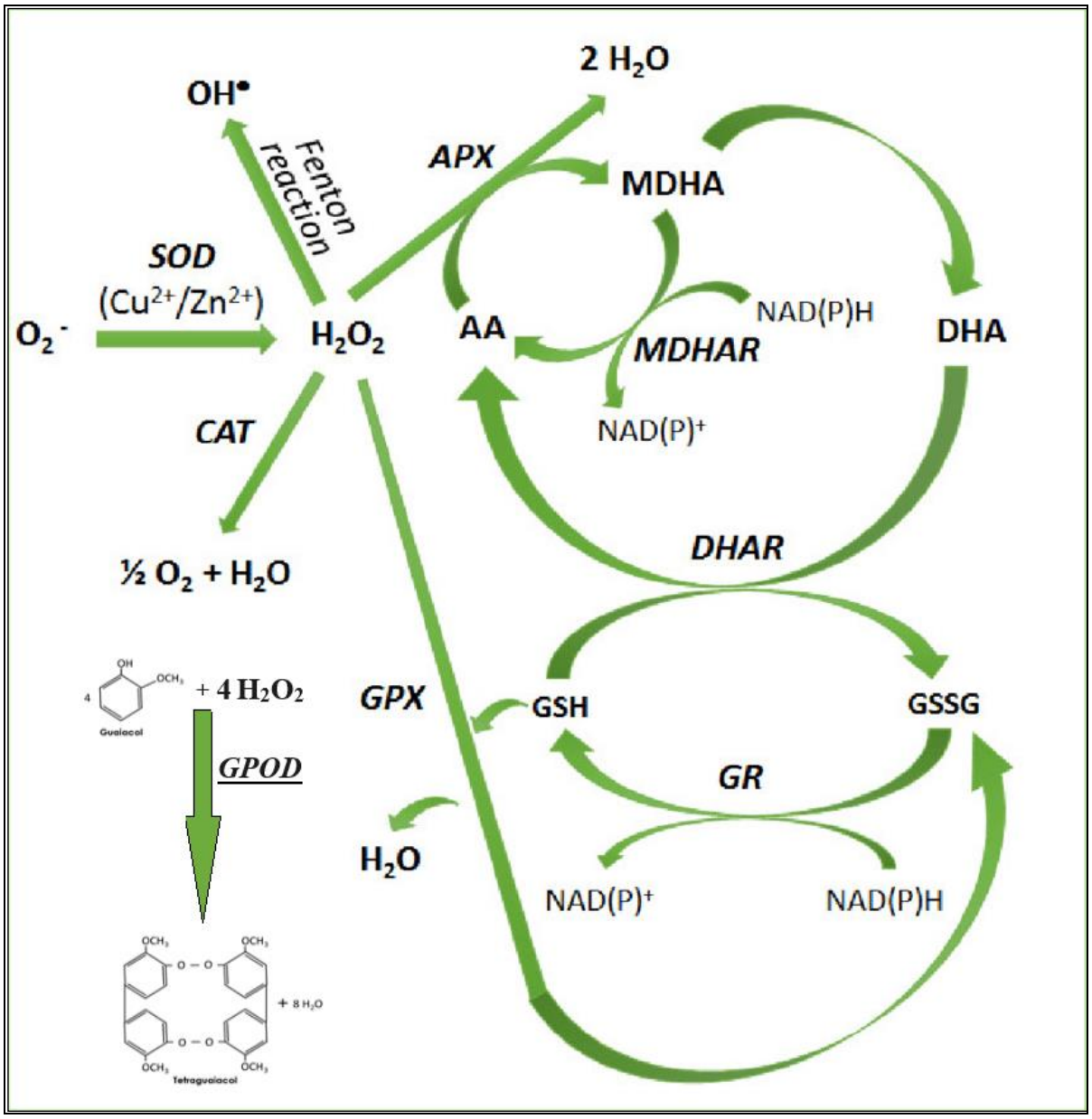

Figura 5 - Enzimas antioxidantes e suas respectivas reações catalisadas. Fonte: (ADILETTA; DI MATTEO; PETRICCIONE, 2021)

A enzima SOD catalisa a reação de dismutação (redução e oxidação ao mesmo tempo de um composto, gerando dois produtos diferentes) do ânion/radical superóxido em O_2 e H_2O_2 (figura 5), com o uso de íons H^+ e representa a primeira linha de defesa antioxidante (WANG et al., 2018b). Essa proteína possui isoformas na parede celular, citosol (isoforma Cu/Zn), cloroplastos (isoforma Cu/Zn e Fe), peroxissomos (isoforma Cu/Zn e Mn) e mitocôndrias (isoforma Mn) (MAHANTY et al., 2012; MISHRA et al., 2023). A enzima CAT quebra a molécula de H_2O_2 em água e gás oxigênio, e possui isoformas nos plastídeos, mitocôndrias, peroxissomos, apoplasto e retículo endoplasmático (KATO; NELSON;

LAUERSEN, 2021; PALMA et al., 2020). A APX catalisa a redução de H_2O_2 a água e oxigênio molecular às custas da oxidação de ascorbato a monodeidroascorbato (MDHA, figura 5), possuindo isoformas no citosol, peroxissomos, cloroplastos (com isoformas tilacoidais e estromais) e mitocôndrias (LI, 2023). A GPOD catalisa, de forma similar à APX, a quebra de H_2O_2 a água e gás oxigênio, mas causando a oxidação de guaiacol a tetraguaiacol (Figura 5), possuindo isoformas no vacúolo, retículo endoplasmático (RE), complexo de Golgi e parede celular, participando inclusive do processo de lignificação deste compartimento (YEMELYANOV et al., 2022). Por fim, a GPX catalisa a conversão de H_2O_2 a H_2O e O_2 pela oxidação de duas glutationas reduzidas (2 GSH => GSSG figura 5), podendo utilizar também tiorredoxina como agente redutor (WU et al., 2021), sendo que essa enzima possui isoformas no citoplasma, cloroplastos, RE e núcleo (WU et al., 2021).

Desta forma, a viabilidade da célula, e possivelmente de um tecido, é dependente do balanço entre a quantidade de EROs e a eficiência do sistema antioxidante (HASANUZZAMAN et al., 2020). Além do estresse oxidativo, a salinidade pode promover outros estresses secundários, como o estresse do RE (ÇAKIR AYDEMIR et al., 2020).

4.2 O estresse do RE

O RE é uma organela que ocupa uma porção significativa do citoplasma com sua membrana constituindo-se em torno de 50% das membranas celulares totais (STEFANO; BRANDIZZI, 2018), e possui papel crucial na síntese e dobramento de proteínas, modificações pós-traducionais, síntese de lipídios e armazenamento e homeostase de íons Ca^{2+} (SCHWARZ; BLOWER, 2016). Além disso, essa organela fornece um ambiente oxidativo para facilitar a formação de ligações dissulfeto e está repleta de chaperonas moleculares, ~~sendo~~ estando também envolvida na regulação de respostas a estresses em células animais e vegetais (BENHAM, 2019). De fato, a ação de patógenos, estresse térmico e de salinidade induzem um acúmulo de proteínas mal dobradas ou não-dobradas, o que ~~induz~~ leva a um desequilíbrio da homeostase do RE, caracterizando o estresse do RE (CHEN; YU; XIE, 2020), que, se não tratado de modo eficiente e em tempo, pode acarretar morte celular programada (SIMONI et al., 2022). Quando surge o estresse do RE, sensores localizados na membrana do RE ativam vias sinalizadoras de organela-núcleo *downstream* para desencadear uma resposta citoprotetora denominada como via UPR, do inglês *unfolded protein response* (MANGHWAR; LI, 2022).

A via UPR, em plantas, possui duas ramificações (Figura 6). Uma é a principal via transdutora de sinais da UPR, que é composta pela proteína Zíper de Leucina Básica 60

(bZIP60) e uma proteína fator de *splicing* de RNA, a quinase e ribonuclease dependente de inositol 1 (IRE1), do inglês *Inositol Requiring Enzyme 1* (LIU et al., 2022a), que possui um domínio sensor N-terminal voltado para o lúmen do RE, uma única hélice transmembrana inserida na membrana da organela e os domínios quinase e RNase na extremidade C-terminal, no citosol (MISHIBA et al., 2019). A outra ramificação envolve fatores de transcrição associados a membranas, como as proteínas bZIP17 e bZIP28 (BAO; BASSHAM; HOWELL, 2019).

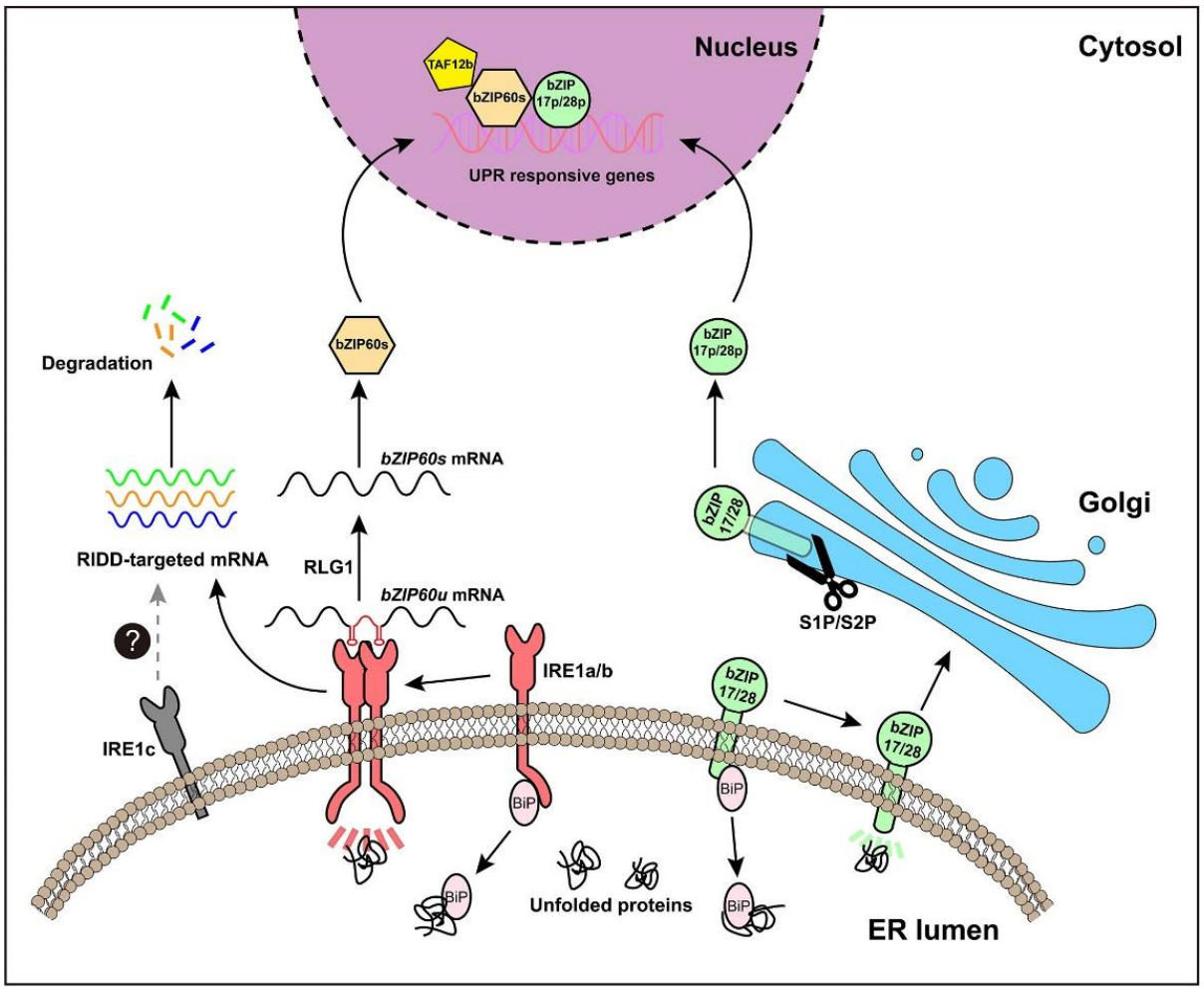

Figura 6 - Esquematização dos componentes da via de resposta às proteínas mal dobradas (UPR) do reticulo endoplasmático e a comunicação com o núcleo em plantas. Zíper de Leucina Básica (bZIP), quinase e ribonuclease dependente de inositol 1 (IRE1), *Binding protein* (BiP), *regulated IRE1-dependent decay* (RIDD), *site-1 protease* (S1P), *site-2 protease* (S1P), *tRNA ligase* (RLG). Fonte: (LIU et al., 2022a)

Quando a célula se encontra sob estresse, IRE1 detecta proteínas mal dobradas no lúmen do RE, levando a uma dimerização, auto fosforilação (em função do domínio quinase) e ativação da função RNásica, o que catalisa o *splicing* alternativo citoplasmático (MISHIBA et al., 2019). Dentre os transcritos (RNAm) alvos de *splicing* citoplasmático (também chamado de não-convencional) pode-se citar fatores de transcrição específicos da via UPR,

como o *bZIP60* (MISHIBA et al., 2019; NAGASHIMA et al., 2011). No núcleo, a proteína bZIP60 superregula a expressão de genes que codificam várias chaperonas nativas do RE, como a proteína-dissulfeto-isomerase (PDI), calnexina (CNX), calreticulina (CRT) e a proteína de ligação (BiP), uma imunoglobulina similar a HSP-70 (GAYRAL et al., 2019). Além do *splicing* do *bZIP60*, IRE1 ativada também degrada RNAm codificadores do controle de qualidade das vias secretórias, processo denominado como "decaimento regulado de transcritos dependente de IRE1", ou RIDD (HAYASHI et al., 2016; ZHU et al., 2019). A atividade RIDD de IRE1 está conectada à ativação da autofagia em resposta ao estresse no RE (ZENG et al., 2019).

No segundo ramo da UPR, as proteínas fatores de transcrição bZIP17 e bZIP28 são ativadas com o auxílio de uma maquinaria pós-traducional encontrada no RE e no complexo de Golgi, denominada "Proteólise de Intra domínio Regulada", ou RIP (KIM; YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2018; LI; HOWELL, 2021). Este aparato consiste em duas proteases de sítios do complexo de Golgi: protease de sítio 1 (S1P) e protease do sítio 2 (S2P). Ambas participam da remoção de domínios transmembranas de bZIP17 e bZIP28, liberando assim seus domínios de ativação da transcrição da membrana do RE (KIM; YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2018). Em condições normais, bZIP28 e BiP estão relacionadas à proteína atanogênica associada à Bcl-2, isoforma 7 (BAG7) na membrana do RE. Sob estresses químicos ou térmicos o complexo das proteases S1P e S2P removem os domínios transmembrana dos fatores de transcrição bZIP17 e bZIP28, o que permite que eles entrem no núcleo e ativem a expressão de BiPs e de outras chaperonas similar a bZIP60 de modo a aumentar o processamento proteico ou ativar a morte celular programada (NAWKAR et al., 2018). Tais componentes de membrana têm sido amplamente descritos em *Arabidopis thaliana* e mais recentemente em outras culturas como arroz, soja e milho, de fato em arroz foram identificados apenas uma isoforma de IRE1, bZIP60 e bZIP17 (HOWELL, 2021).

Embora comumente o estresse do RE seja gerado por fatores abióticos, substâncias estressoras específicas, como a tunicamicina (TM), o ditiotreitol (DTT) e a azidotimidina (AZT) podem induzir esse tipo de estresse na célula (PARK; PARK, 2019b). TM é um antibiótico produzido por bactérias como *Streptomyces clavuligerus* e *Streptomyces lysosuperficus*, sendo apresentado comercialmente como um pó cristalino branco, solúvel em água alcalina, piridina, metanol quente e DMSO, é levemente solúvel em etanol e n-butanol e insolúvel em acetona, acetato de etila, clorofórmio, benzeno e água acídica (DAWOOD; ALTOBJE, 2020). TM é um inibidor competitivo análogo a um nucleosídeo que disputa com

o substrato natural, o UDP-N-acetilglicosamina (UDP-GlcNAc), o sítio ativo da enzima N-acetilglicosamina-1-P-transferase (GPT), que catalisa o primeiro e crítico passo da reação de síntese de N-glicosilação de proteínas no RE, motivo pelo qual é utilizada amplamente como agente estressor específico do RE (ASTANI et al., 2022).

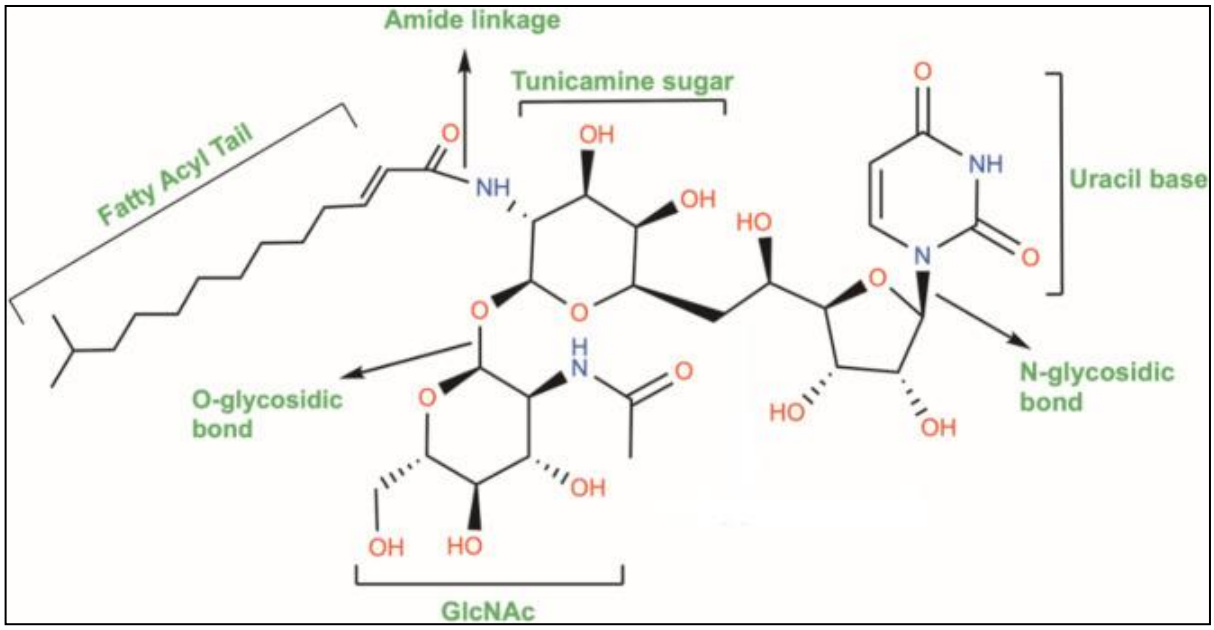

Figura 7 - Estrutura da tunicamicina. Astani et al. (2022).

Experimentos com TM demonstram que os efeitos do estresse são agravados com o tempo de exposição, promovendo diminuição da expressão de genes relacionados ao RE como IRE1, BiP e PDI, redução de vias metabólicas relacionadas ao metabolismo primário e consequentemente o crescimento da plântula (LIMA et al., 2022). No entanto, concentrações menores de TM (0,5 ug.L^{-1}) possuem um efeito de manutenção da biomassa, comprimento, e xpressão desses genes relacionados ao RE, enquanto que concentrações maiores impactam negativamente o metabolismo (CAVALCANTE et al., 2023). Torna-se evidente a dupla função do RE em ativar mecanismos de sobrevivência ou morte celular em respostas ao estresse (CARVALHO et al., 2014). Desta forma, embora possa causar diretamente o estresse do RE, quando em altas concentrações, a TM, em pequenas concentrações, poderia ativar reações metabólicas, preparando a planta para estresses mais severos, processo esse conhecido como condicionamento fisiológico (KHAN; YUSUF; FARIDUDDIN, 2018).

4.3. O condicionamento fisiológico em plantas

Em fisiologia vegetal, o termo condicionamento fisiológico, também chamado de

priming, se refere a um pré-condicionamento ou pré-tratamento com compostos químicos, agentes biológicos ou fatores físicos a fim de se adquirir sobrevivência melhorada e uma ação eficiente dos mecanismos de defesa sob estresses subsequentemente encontrados (MÉNDEZ et al., 2016). Em contrapartida, respostas de defesa adquiridas durante longos períodos de duração de um estresse, ao qual o metabolismo das plantas se ajusta, são relacionados ao fenômeno de aclimatação (WISZNIEWSKA et al., 2019).

O condicionamento fisiológico de sementes é um processo de três estágios que se inicia com a embebição da semente no agente condicionante, por um determinado tempo, que é padronizado através do método de tentativa e erro (BISWAS et al., 2023). A segunda fase, chamada "fase de ativação", está relacionada com a indução de uma cascata de eventos metabólicos a nível celular, tais como síntese proteica, ativação do sistema antioxidante, formação de novas mitocôndrias e reparo de DNA. Na já na terceira etapa, chamada de reidratação, o *priming* ativa a divisão celular, síntese de ácidos nucleicos e ATP para aumentar a energia celular (DEVIKA et al., 2021). Embora tenha havido relatos de plântulas cultivadas com sementes condicionadas com alterações no conteúdo de água (déficit hídrico), uma regulação aprimorada do ciclo celular, um gerenciamento do estresse oxidativo e mobilização de reserva energética, a eficiência do condicionamento fisiológico de sementes depende fortemente da espécie vegetal e do método de *priming* (JOHNSON; PUTHUR, 2021).

Mecanismos por trás da memória do estresse em plantas podem ser descritos a níveis epigenéticos, transcricionais, traducionais ou metabólicos (GANIE; MCMULKIN; DEVOTO, 2024). Alterações fisiológicas e metabólicas, que por sua vez são governadas pelas mudanças epigenéticas, transcriptômicas e proteômicas, durante exposição anterior ao estresse, podem atuar como marcadores de estresse que podem afetar as respostas da planta a episódios de estresses posteriores (GANIE; MCMULKIN; DEVOTO, 2024). Dentre as respostas fisiológicas e bioquímicas, pode-se citar o conteúdo de clorofila, a relação clorofila *a*/clorofila *b*, osmoprotetores e sinalização com fitormônios, os quais foram ligados a estresses como o déficit hídrico e o salino em diferentes espécies vegetais, como arroz, arabidopsis, trigo, soja e feijão-de-corda, respectivamente (BRENYA et al., 2023; FLETA-SORIANO; MUNNÉ-BOSCH, 2016; LIU; ABLE; ABLE, 2022; SHARMA et al., 2022; TRASOLETTI et al., 2022).

Diversos trabalhos mostram a eficiência do condicionamento fisiológico em culturas de interesse. Estudos envolvendo o condicionamento fisiológico de sementes de arroz com ácido salicílico mostram que, plântulas sob estresse salino e condicionadas com fitormônio, não só aumentam a expressão de genes de proteínas antiportes de sódio *OsHKT1;1, OsHKT1;5, OsHKT2;1* e *OsSOS1*, mas também aumentam as atividades das enzimas

antioxidantes SOD, GPOD e CAT, alterações que contribuem tanto para a redução da absorção de Na^+ pelas sementes, como também para redução do conteúdo das EROs H_2O_2 e $\bullet O_2^-$, e diminuição da peroxidação lipídica (LIU et al., 2022b). Akter et al. (2023), ao pré-tratarem sementes de arroz com uma linhagem de fungos *Beauveria bassiana* BeauA1 e depois submeterem-nas a uma solução de 120 mM de NaCl, verificaram que o *priming* promoveu aumento do número de pigmentos fotossintéticos, área foliar, teor relativo de água e também aumentou o conteúdo de prolina, açúcares totais e a razão K^+/Na^+ nas folhas, além de incrementar também as atividades das enzimas antioxidantes APX, CAT, GPOD e glutationa-S-transferase (GST), em comparação àquelas não co-cultivadas com o fungo. O *priming* com KNO_3 e SiO_2 em plântulas de arroz submetidas ao déficit hídrico leve (75% da capacidade de campo, CC), moderado (50% CC) e severo (25% CC), verificaram que o condicionamento fisiológico com o sal de potássio e o óxido de silício aumentaram, em comparação a seus respectivos controles e em todas as condições estressantes (leve, moderado e severo), a percentagem de germinação, o vigor da semente, a massa seca de partes aéreas e raízes, além de aumento no teor de pigmentos fotossintéticos, de carboidratos totais, açúcares solúveis e de proteínas solúveis totais. Além disso, foi observado, também, aumentos nas atividades das enzimas antioxidantes SOD, CAT e APX, provando que o condicionamento funciona para vários tipos de estresses ambientais (ALI et al., 2021). Dessa forma, o condicionamento fisiológico pode também levar a memória de estresse em culturas de interesse para a humanidade, como o arroz (*Oryza sativa*) de forma somática ou intergeracional???, podendo ser regulada a distintos níveis hierárquicos como epigenético, transcriptômico, proteômico ou metabólico (Figura 8), gerando assim culturas mais tolerantes aos estressores abióticos ambientais (GANIE; MCMULKIN; DEVOTO, 2024), especialmente quando combinadas com outras abordagens multivariadas, como as -ômicas.

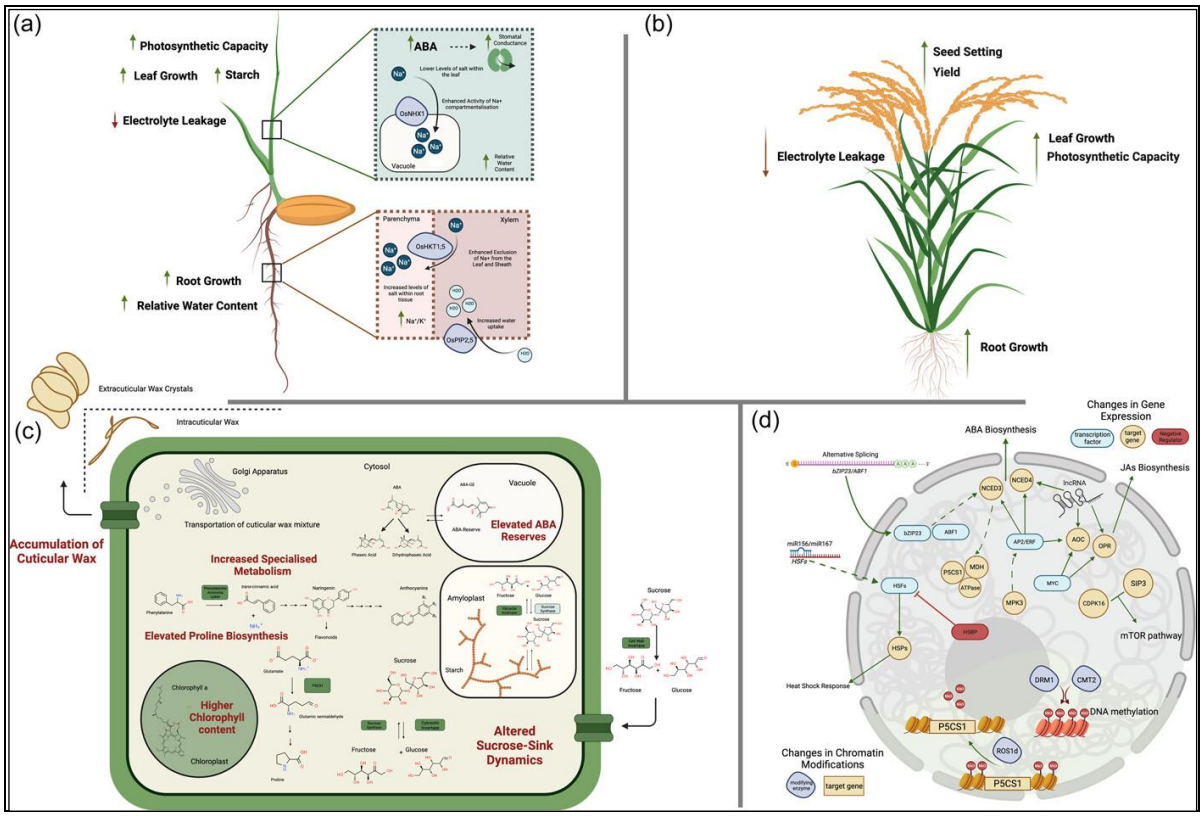

Figura 8 - Alterações no *status* fisiológico, metabólico, transcriptômico e epigenômico de plantas de arroz condicionadas com uma variedade de estresses abióticos. Fonte: Ganie; McMulkin; Devoto, 2024

4.4. As -ômicas na elucidação de respostas a estresses em plantas

Desde seu surgimento, as plantas têm desenvolvido estratégias moleculares e fisiológicas intrincadas para lidar com os estresses abióticos, como salinidade, seca e metais pesados, e bióticos, como infecções virais ou fúngicas (YOLCU et al., 2016). Sabe-se que ambos os tipos de estresses são responsáveis por perdas significativas na agricultura a nível mundial. Assim, entender as estratégias adaptativas adotadas pelas plantas para tolerar estes estresses é fundamental para aprimorar a produtividade das culturas nestas condições (IBRAHEEM; ADIGUN; OLATUNJI, 2018). Recentemente, as abordagens e tecnologias "ômicas", que serão discutidas a seguir, colaboraram significantemente no esclarecimento destes mecanismos de aclimatação a estresses (ZHANG; NAN; YU, 2016).

O termo "ômica" se refere ao estudo de conjuntos de genes ou moléculas biológicas de um órgão, tecido ou célula, podendo ser dividido em: genômica – conjunto total de genes, transcritômica – conjunto de transcritos ou RNAm totais, proteômica –conjunto de proteínas totais, metabolômica – conjunto de metabólitos totais, dentre outras (YANG et al., 2021). Com as ômicas, pode-se compreender as complexas interações entre genes, proteínas e metabólitos que determinam um fenótipo de uma espécie de interesse, como por exemplo,

resultante dos efeitos de um determinado estresse, valendo-se de ensaios bioquímicos, fisiológicos, moleculares, de bioinformática e de outros ramos da Biologia, o que pode levar a aprimoramentos das culturas de interesse (IBRAHEEM; ADIGUN; OLATUNJI, 2018; VAN EMON, 2016).

Wang et al. (2018a), ao compararem o transcritoma de dois genótipos de arroz, um tolerante e outro sensível à salinidade, e em três tempos distintos (0, 48 e 72 h), conseguiram não só identificar 1375 novos genes como obtiveram um total de 5273 genes diferencialmente expressos, sendo 286 deles exclusivos do genótipo tolerante. Dentre as funções moleculares destes genes, as categorias de ligação (a compostos como os grupos heme e ATP) e de atividade catalítica (como as peroxidase e transferase) foram as mais significativas nestes transcritos.

Uma vez que o estresse salino induz estresses secundários, como o oxidativo e o do RE, estudos de abordagens -ômicas que façam a integração desses estressores fazem-se necessárias (ÇAKIR AYDEMIR et al., 2020). Rajkumari et al. (2023) relatam que metabólitos secundários podem funcionar como moléculas sinalizadoras a fim de ativar mecanismos *downstream* de tolerância à salinidade, ao passo que metabólitos primários, como açúcares, ácidos orgânicos e aminoácidos atuam diretamente como agentes protetores para manter o equilíbrio osmótico e eliminar as EROs, durante o estresse salino. De forma similar, Xie et al. (2020) ao estudarem os metabolomas de linhagens tolerantes e sensíveis de arroz à salinidade, constataram que, além de um maior conteúdo de aminoácidos nas linhagens tolerantes, havia um maior conteúdo de alantoína, um metabólito intermediário do catabolismo de purinas e que funciona como um carreador de nitrogênio, o que pode ter contribuído para um melhor crescimento de plantas destas linhagens. Além disso, houve um aumento nos conteúdos de açúcares, como o sorbitol, a melezitose e o ácido pipecólico em todas as linhagens, indicando que estes compostos contribuem para as respostas do arroz à salinidade (XIE et al., 2020).

De forma geral, os estudos ômicos podem oferecer informações capazes de esclarecer os efeitos de estresses como o salino em culturas de importância mundial, como o arroz ((RAJKUMARI et al., 2023).

4.5. O arroz (*Oryza sativa*)

O arroz (*Oryza sativa* L.) é uma espécie anual pertencente à família *Poaceae* (gramíneas) de metabolismo fotossintético do tipo C3 (RANGAN; FURTADO; HENRY, 2016). É o terceiro cereal mais produzido no mundo, atrás apenas do milho e trigo, e no ano

de 2022 obteve uma produção mundial de aproximadamente 800 milhões de toneladas (FAOSTAT, 2022). A Ásia liderou a produção de arroz, com os três principais produtores do grão na região: China, com aproximadamente 216 milhões de toneladas (27% da produção global), Índia com 200 milhões de toneladas (25% da produção global) e Bangladesh com 56 milhões, equivalendo a 7% da produção total (FAOSTAT, 2022), ao passo que o Brasil, neste período, gerou 10,78 milhões de toneladas, o que equivale a 1,34% da produção total (STATISTA, 2024).

O Brasil é o maior produtor da cultura fora do ambiente asiático, e no país, o estado do Rio Grande do Sul (RS) sustenta a maior produção do cereal (COLTRO et al., 2017). No período 2022/2023, o país obteve uma produção de 10,033 milhões de toneladas, em uma área cultivada de arroz de 1,48 milhões de hectares, e o RS contribuiu com 862,6 mil hectares, representando 55,8% da produção nacional (TOLEDO, 2024).

No Nordeste, a produção de arroz atingiu, na safra 2022/2023, o montante de 353,6 mil toneladas. Na região, o estado do Maranhão é o principal destaque, produzindo 187,8 mil toneladas de arroz, representando 53% da produção da região e contribuindo com 1,81% da produção nacional (COMPANHIA NACIONAL DE ABASTECIMENTO, 2022). Na produção da cultura, o Ceará (CE) vem em quarto lugar no Nordeste, ao produzir 5 mil toneladas no ano (safra 2023), o que constitui 0,04% da produção nacional (COMPANHIA NACIONAL DE ABASTECIMENTO, 2022).

O arroz constitui um alimento básico para mais da metade da população mundial e provê mais de 20% das calorias consumidas mundialmente, especialmente na Ásia e América Latina (FUKAGAWA; ZISKA, 2019). O cereal é constituído majoritariamente de carboidratos complexos, representa uma importante fonte de proteínas (ricas em lisina e 7 outros aminoácidos essenciais) e também de vitaminas do complexo B (B1, B2 e B3) e tocoferol ou vitamina E (CHAUDHARI et al., 2018). Além de constituir a base alimentar da população, o arroz também é uma fonte de renda para muitos brasileiros, especialmente os de agricultura familiar, que compõem 34% da produção nacional do arroz (SARAIVA et al., 2013).

Assim, uma melhor aclimatação ao estresse salino visa garantir o cultivo do arroz não só em ambientes áridos, como os do Nordeste brasileiro, desenvolvendo estratégias de manejo mais eficientes de forma a minimizar os impactos ambientais e econômicos, além de garantir a segurança alimentar da população pela produção em áreas com risco de salinização ou o uso de águas de menor qualidade. Uma vez que esse alimento é base de 1/3 da população mundial e em vista da crescente salinização dos solos, estratégias que aliviem os efeitos

deletérios do NaCl em plantas se tornam cada vez mais necessárias. Para este trabalho, foi escolhida a cultivar de arroz irrigado SCSBRS 113, também chamada de "Tio Taka", que possui uma elevada resistência ao acamamento, tem ciclo inferior a 150 dias, apresenta teor de amilose e temperatura de gelatinização elevados que reflete em uma ótima alternativa para cultivo pré-germinado da cultura (YANG; ZHAO; LIU, 2020).

5. ESTRATÉGIA EXPERIMENTAL

Para testar a hipótese levantada, este estudo foi desenvolvido em duas etapas consecutivas, nas quais sementes de arroz foram submetidas ou não ao condicionamento fisiológico com TM, na presença ou não de NaCl. A primeira etapa teve o objetivo de determinar a concentração de NaCl que inibisse a germinação, mas que permitisse a viabilidade das plântulas, e determinar, logo em seguida, a concentração de TM que melhor reduzisse os efeitos deletérios do estresse severo por NaCl. Para isso, foram realizados três experimentos de *screening*: os primeiro e segundo experimentos utilizaram duas concentrações de TM (0 e 0,25 µg. mL^{-1}) e para cada uma dessas concentrações foi feito um *screening* com cinco concentrações crescentes de NaCl: 0, 100, 150, 200 e 250 mM. Em seguida, após determinar a concentração de NaCl (150 mM), fez-se um terceiro *screening* utilizando plantas submetidas a *priming* com concentrações crescentes de TM (0; 0,125; 0,250; 0,375; e 0,500 µg. mL^{-1}) por 10 dias em soluções de NaCl a 150 mM. Neste ínterim e para cada experimento de *screening*, foram verificados os seguintes parâmetros: percentual de germinação, plântulas anormais, índice de velocidade de germinação (IVG), tempo médio de germinação (TMG), índice de velocidade de emissão de parte aérea (IVEPA), tempo médio de emissão de parte aérea (TMEPA), massa seca da parte aérea (MSPA), massa seca da raiz (MSR), comprimento da parte aérea (CPA) e comprimento da raiz (CR). Com base nestes dados, foram estabelecidas as concentrações ideais de NaCl (0 e 150 mM) e de TM (0 e 0,25 µg, mL^{-1}) para utilização na segunda etapa. A segunda etapa deste projeto foi realizada com a utilização de quatro tratamentos, resultantes do fatorial 2 x 2 (duas concentrações de NaCl e duas de TM) e teve por finalidade a mensuração de MSPA, MSR, CPA e CR, bem como o grau de vazamento de eletrólitos em parte aérea (PA) e raízes, o potencial osmótico (Ψs), e os teores de pigmentos fotossintetizantes (clorofila *a*, clorofila *b*, clorofila *total* e carotenoides). Além disso, foram determinados os teores dos íons inorgânicos (Na^+, K^+ e Cl^-), sendo o acúmulo de Na^+ em tecidos de PA e raízes também avalidado por microscopia confocal, os teores de EROS (H_2O_2 e $\bullet O_2^-$), o grau de peroxidação lipídica, a atividade das enzimas antioxidantes SOD, CAT, APX e GPOD, os perfis de metabólitos e expressão dos genes *SOS1*,

NHX1, IRE1 e *bZIP50* em PA e raízes de plântulas de arroz aos 10 DAT condicionadas ou não com TM e submetidas ou não ao estresse salino. Todos os resultados da etapa 1 e 2 estão descritos no item 6 deste trabalho.

6. CAPÍTULO 2: MANUSCRITO SUBMETIDO À REVISTA PLANT SCIENCE

Title:
Endoplasmic reticulum activation via tunicamycin seed priming enhances salt acclimation in rice seedlings

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Abstract

Seed priming is a promising alternative to increasing salt acclimation, but the role of endoplasmic reticulum (ER) remains unclear. This study investigated if ER activation by tunicamycin (TM) as a seed priming agent promotes salt acclimation in rice (*Oryza sativa* L.) seedlings. The results showed that salinity (150 mM NaCl) decreased the seedling growth. However, priming seeds with TM and salt treatment increased dry mass, length, photosynthetic pigments and K^+ contents, osmotic potential, and antioxidant enzyme activities. Conversely, it decreased intracellular Na^+ accumulation, electrolyte leakage, lipid peroxidation, and ROS levels. Additionally, TM priming enhanced the expression of ER response gene markers *OsIRE1*, *OsbZIP50,* and *OsbZIP60* in seedlings under salinity. Metabolomic profiling revealed that TM priming and salinity positively modulated salt-defensive metabolites in shoots, including the osmoprotectants β-alanine and maltose. In roots, it led to a higher accumulation of phosphoric acid, the amino acids O-acetylserine and N-acetylserine (involved in Fe and S metabolism), and the sugars maltose, raffinose, and sorbitol, which also function as osmoprotectants and energy sources. In conclusion, TM seed priming followed by salt stress induced the ER response activated ER unfolded protein response (UPR). This may enhance antioxidant enzyme activity, reducing ROS levels and intracellular Na^+ content, thereby mitigating salt stress through the positive modulation of defense-related and energy-related metabolites. These findings suggest an efficient strategy to improve salt acclimation during the early growth stages of rice seedlings.

Keywords: seed priming; tunicamycin, antioxidants, *Oryza sativa* L, metabolic profiles.

6.1 Introduction

Adverse conditions such as pathogen attacks, heat, salinity, drought, or exposure to specific chemicals, including the natural antibiotic tunicamycin (TM), can lead to the accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) lumen, a state known as ER stress (JE; LEE; YAMAOKA, 2023; PARK; PARK, 2019; LIU; TIAN; CHEN, 2025). It triggers a cytoprotective, nucleus-integrated pathway known as the unfolded protein response (UPR). The UPR involves transcription factors, protease, and RNAse activities to re-establish ER homeostasis, maintaining the quality control of exported proteins (MANGHWAR; LI, 2022).

In plants, UPR involves two main branches (AYAZ; HU; LI, 2024). The first one includes a Basic Leucine Zipper 60 (bZIP60) protein and an RNA splicing factor protein, the inositol-requiring enzyme 1 (IRE1), which has an N-terminal sensor domain facing the ER lumen and a C-terminal end facing the cytosol (LIU et al., 2022; MISHIBA et al., 2019). The other one comprises membrane-associated transcription factors, such as the bZIP17 and bZIP28 proteins (BAO; HASSAM; HOWELL, 2019). Under stress conditions, IRE1 detects misfolded proteins in the ER lumen, leading to its dimerization and activation of RNase function. It catalyzes the alternative cytoplasmic splicing of the bZIP60 transcription factor (MISHIBA et al., 2019). In the nucleus, the bZIP60 protein upregulates the expression of genes encoding various native ER chaperones, such as binding protein (*BiP*), which helps to decrease ER stress (NAGASHIMA et al., 2011). Additionally, bZIP17 and bZIP28 migrate to the Golgi complex, where their transmembrane domains are cleaved, enabling their transcription factor domains to enter the nucleus and activate the chaperone's expression. Like bZIP60, these transcription factors enhance protein processing or, in severe cases, activate programmed cell death (AYAZ; HU; LI, 2024; NAWKAR et al., 2018). Such membrane components have been described in *Arabidopsis thaliana* and recently demonstrated to be conserved among crops such as rice, soybean, and maize. In rice, only one ortholog of each has been recognized (HOWELL, 2021; HAYASHI; WAKASA; TAIKAWA, 2013): *Os*IRE1 is the *At*IRE1a/b isoform (HAYASHI et al., 2012), *Atb*ZIP60 is *Osb*ZIP50 also known as *Osb*ZIP74 (LU et al., 2012), the isoform of *Atb*ZIP17 is *Os*bZIP17 also known as *Osb*ZIP39, and for *Atb*ZIP28 is *Osb*ZIP60 also known as *Osb*ZIP16 (HAYASHI et al., 2012; WANG et al., 2022).

Several studies have examined the impact of ER stress on plant metabolism. TM aggravates stress effects with prolonged exposure, leading to decreased expression of IRE1 and BiP chaperones, suppression of metabolic pathways related to primary metabolism, and impaired seedling growth (LIMA et al., 2022). However, moderate ER stress has been associated with plant biomass maintenance, positive modulation of primary metabolites (such as glycolysis and TCA cycle intermediates), and an increase in ER-related gene expression (e.g. *BiP* and *IRE1*), while excessive stress induces deleterious effects (CAVALCANTE et al., 2023). Additionally, low to moderate levels of ER stress may handle the UPR pathway to mitigate the harmful effects of environmental stress on cells, enhancing the tolerance response and increasing plant performance under saline stress

(BEAUGELIN et al., 2020; OHTA; TAIKAWA, 2020). Queiroz et al. (2020) proved that ER stress reduced sodium accumulation in *Sorghum bicolor*, increasing the expression of *SOS* genes in roots. Beyond ER stress, salinity can also lead to an overproduction of reactive oxygen species (ROS), which cause oxidative stress and disrupt the chloroplast electron transport chain (SINGH, 2022). This oxidative imbalance results in protein oxidation, DNA damage, and, most notably, lipid peroxidation in membranes (HASANUZZAMAN et al., 2020). To counteract these effects, cells have developed an antioxidant system, which is comprised of non-enzymatic compounds, such as phenolic compounds, ascorbic acid, and carotenoids (LAXA et al., 2019), and antioxidant enzymes (KUMARI et al., 2022). Considering that most of these molecules (proteins) are synthesized in the ER, it is worth pointing out that this organelle is crucial for modulating plant stress responses (KIM; MOCHIDA; SHINOZAKI, 2022). Since molecular responses to ER stress are not well comprehended in plants as in animals, studies that clarify these mechanisms are necessary (HOWELL, 2017).

Salinity is one of the most challenging environmental factors that affect crop yield, especially in arid and semiarid regions (MUKHOPADHYAY et al., 2021). It is widely known that salt stress can impair crucial cellular processes, such as photosynthesis (BEN-AMOR et al., 2020), cell wall integrity (COLIN et al., 2023), and mitochondrial electron transport chain (TANG; ZHU, 2023), ultimately inhibiting plant growth and development (ZHAO et al., 2021). In response to salt stress, plants have evolved physiological and biochemical mechanisms, such as the activation of mechanisms to control ionic homeostasis, which include the reduction of Na^+ content in the cytosol, via compartmentation in the vacuole, carried out by antiport proteins called NHX, Na^+/H^+ exchange (BASSIL et al., 2019), or the efflux of this ion to the apoplast, performed by the SOS (Salt Overly sensitive) antiporters (ALI et al., 2023). Besides its harmful effects on the cell, it is known that salinity induces several other secondary stresses, such as oxidative stress (KHAN et al., 2022). Equally important, its is known that salinity induces several secondary stresses, such as oxidative stress (AZEEM et al., 2023), and also ER stress (AYDEMIR et al., 2020; LI et al., 2021), which is why the crosstalk among these stresses make an important issue.

Several approaches have been applied to produce more tolerant crops to environmental stresses, such as priming, which is a short-period pretreating of seeds or leaves with biological, chemical, or physical agents to withstand severe stresses subsequently found (MÉNDEZ et al., 2016). Seed priming can induce physiological alterations, such as increased chlorophyll content, osmoprotectant levels, ROS, and defense metabolites that might lead to tolerance to abiotic stresses such as salinity (BRENYA et al., 2023). Therefore, priming can lead to more tolerant species through generation of stress memory in important crops, such as rice (*Oryza sativa* L.) (GANIE; MCCULKIN; DEVOTO, 2024). In this way, priming upregulates UPR and ER quality control genes, and their expression is maintained after recovery, enabling salt-primed plants to exhibit improved growth upon subsequent salt exposure (TIAN et al., 2019).

Thus, we wondered if TM, an ER stress inductor could act as a priming to induce salt

acclimation in rice. Given this question, this work aimed to test the hypothesis that TM seed priming activates ER responses that help seedling establishment under salt stress. Understanding the responses of rice plants to abiotic stresses is crucial for breeding and genetic engineering programs, ultimately contributing to increased crop quality and yield, ensuring global food security, and promoting environmental sustainability.

6.2. Material and methods

6.2.1 Plant material and screenings

Rice seeds (*Oryza sativa* L.) of SCSBRS Tio Taka cultivar were provided by *Agrogiusti Indústria e Com. de Sementes Ltda.* (Turvo, SC, Brazil - 28° 56' 22.06222'' S, 49° 44' 9.64866" W). The experiment was carried out in a BOD (Biochemical Oxygen Demand) incubator with a mean temperature of 25 ºC in a photoperiod of 12/12 h.

First, screening experiments were carried out to determine the optimal NaCl and Tunicamycin (TM) concentrations for further assays. The first and second ones were NaCl screenings, performed at the same time, with either 0 or 0.5 µg. mL^{-1} TM, being the latter chosen based on previous studies [17]. For this part, seeds were previously surface sanitized with a 0.5 % (v/v) sodium hypochlorite solution, washed three times with tap water, and rinsed with distilled water. Then, they were pre-treated at a fixed 0.5 µg. mL^{-1} TM for 24 hours (T050), or not, only with distilled water for 24h (T0 group). They were dried at room temperature for 24 hours and sown in a moistened germitest paper. The first screening test used unprimed (T0) rice seeds grown in crescent concentrations of NaCl (0, 100, 150, 200, and 250 mM NaCl), whereas the second one used 0.50 TM-primed seeds grown in the same five NaCl concentrations (0-250 mM). In this way, the seed germination was followed by 10 days [16]. Based on the results, the NaCl concentration was fixed to 150 mM. Then, a third screening was performed using five distinct TM concentrations (0, 0.125, 0.250, 0.375, and 0.500 µg.mL^{-1}) submitted to 150 mM NaCl. Just like the two previous screening tests, seed germination tests were conducted in 10 days.

The following parameters were used to assess seed germination screenings: germination percentage, nº of abnormal seedlings, germination speed index (GSI), according to Maguire (1962):

GSI = $\sum_{i=1}^{n}(\frac{Gi}{Ni})$, where:

Gi = number of germinated seeds counted per day

Ni = number of days after sowing

n = nth time (day) of counting

Mean germination time (MGT), according to Labouriau (1983):

MGT = $\sum_{i=1}^{n} \mathrm{Gi} * \mathrm{Ni} \,/\, \Sigma\, \mathrm{Gn}$, where:

Gi = number of seeds germinated per day of counting;

Ni = time between the first day of germination and the nth counting;

n = "n^{th}" time (day) of counting,

Shoot emergence speed index (SESI) was adapted from Maguire (1962), while mean shoot emergence time (MSET) was adapted from Labouriau (1983). Additionally, after 10 days of sowing,

we measured shoot length (SL), root length (RL), shoot dry mass (SDM), and root dry mass (RDM).

6.2.2 Seed pre-treatment and salt stress treatment establishment

According to the screening results, the next steps were performed using 0.25 $\mu g.mL^{-1}$ TM as a seed pre-treatment and 150 mM NaCl as salt stress treatment during seed germination. Rice seeds were pre-treated with 0.25 $\mu g.mL^{-1}$ TM (TM 0.25) for 24 h, being after that left to dry at room temperature), and included a control group without priming. Then, seeds were sown in germination paper moistened to either 0 (distilled water) or 150 mM NaCl solution in a 2 x 2 factorial scheme. It was rolled and placed in the BOD incubator for 10 days in the same condition previously described. The germination papers were rewatered every 3 days to keep moistening. Seedlings were evaluated after 10 days of sowing.

6.2.3 Seedling growth parameters

After this time, rice seedlings were collected and divided into shoots and roots for dry mass and length measure. For length determination it was used the ImageJ software (RASBAND, 2016), whereas for dry masses, shoots and roots were placed in paper bags and transferred to a hot air oven, at 65 ºC, for 3 days, until the uniform weight. After that, it was used an electronic scale for dry mass measurement.

6.2.4 Measurement of osmotic potential

The osmotic potential (Ψ_s) was measured in the xylem sap according to Callister, Arndt & Adams (2006). Approximately 350 mg of fresh shoot material was harvested and compressed with a disposable syringe. Then, the obtained extract was centrifuged at 6.000 g for 5 min, and the supernatant used to determine the Ψ_s using the osmometer (VAPRO 5520, Wescor, Utah, USA), which provides molality values of the extracted saps, and the values were calculated using the Van't Hoff equation, and the values were expressed in MPa (Mega Pascal).

$\pi = (- R) . (T) . (C.i)$, where:

π = osmotic pressure (MPa);

R = the universal gas constant (0.0821 L * atm* $mole^{-1}$ * K^{-1})

C = the molal concentration of the solution (mmol. kg^{-1});

i = the Van't Hoff Fator (usually for ions is 1).

6.2.5 Electrolyte leakage

Electrolyte leakage (EL) was determined in rice shoots and roots according to Dionisio-Sese & Tobita (1998). Samples were incubated in deionized water, and the electrical conductivity 1 (EC1) was measured after 4 h using an electrical conductivity meter (Mp513, Sanxin®). The samples

were then incubated in a water bath at 95 ºC for 30 min to completely disrupt the plasma membrane, after which electrical conductivity was measured again (EC2). The percentage of EL was determined using the formula:

EL (%) = 100 × (EC1 / EC2).

6.2.6 Measurement of photosynthetic pigments

For the determination of photosynthetic pigment contents, approximately 25 mg of fresh tissues were incubated in 10 ml of dimethyl sulfoxide (DMSO) saturated with calcium carbonate ($CaCO_3$) to obtain the plant extract, according to Wellburn (1994). After 24 h in the dark, samples were incubated at 65 °C for 45 min. The absorbance readings were obtained at 665, 649, and 480 nm in an Ultrospec 6300 pro spectrophotometer (Amersham Biosciences, Slough, United Kingdom). The pigment contents were expressed as mg. g^{-1} of dry mass using the following equations:

Chlorophyll *a* = [12.47 x (A_{665})] – [3.62 x (A_{649})];

Chlorophyll *b* = [25.06 x (A_{649})] – [6.50 x (A_{665})];

Total chlorophyll (a + b) = [7.15 x (A_{665})] – [18.71 x (A649)];

Carotenoids = {[1000 x (A_{480})] – [1.29 x (chlorophyll *a*)] – [53.78 (chlorophyll *b*)]} / 220.

6.2.7 Inorganic ion contents

Inorganic ions were extracted by homogenizing 20 mg of dried and powdered rice shoots and roots in 2.0 mL of deionized water for 1.0 h at 75 °C, vortexing each sample every fifteen minutes. The homogenate was centrifuged at 3000 x g for 10 min at room temperature. The Na^+ and K^+ and contents were determined by flame photometry, according to Malavolta, Vitti & Oliveira (1989). The ion Cl^- content was determined using a spectrophotometric method with NaCl as a standard and reaction with mercury (II) thiocyanate and iron (III) nitrate, with readings at 460 nm according to Iwasaki, Utsumi & Ozawa (1952). The contents were expressed as mg. g^{-1} of dry mass.

6.2.8 Detection of Na^+ by confocal microscopy

For the confocal microscopy analyses, root and shoot sections were placed on 0.01 M phosphate-buffered saline (PBS) at pH 7.4 (Sigma-Aldrich) and later incubated with a cell membrane permeable cytosolic Na^+ indicator, 5 µM Asante NaTRIUM Green-2 AM, for 1 h in the dark (GADELHA et al., 2021; RODER; HILLE, 2014) . Fluorescence emission by Asante NaTRIUM Green-2 AM was collected from 535 to 555 nm.

Sections were washed three times each 5 min with PBS and incubated with Calcofluor White (0.2 µg/mL) (Fluorescent Brightener 28, Sigma, UK) for 5 min in the dark. Fluorescence emission by calcofluor white is blue and indicates cellulose in the cell walls. They were washed three times each 5 min with PBS at pH 7.4, and sealed with coverslip sealant (CoverGrip™ Biotium - Fisher

Scientific). Samples were analyzed on a confocal scanning laser microscope (LSM710, Carl Zeiss, Jena) using appropriate lasers. Images were captured with Zen software (Zeiss). For measuring the mean intensity of emission of NaTRIUM Green-2AM, three image captures for each treatment, an unstained (not treated with any dye) image was kept in potassium phosphate-buffered saline only to capture natural fluorescence. The fluorescence intensity was measured using Zen software.

6.2.9 Lipid peroxidation

Lipid peroxidation was measured by evaluating the malondialdehyde (MDA) absorbance, according to Heath & Packer (1968). Fresh rice shoots and roots (0.2 g) were macerated in 5% trichloroacetic acid, at 4 °C. After centrifugation at 10,000 x g for 15 min, an aliquot of the supernatant was mixed with a solution of 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) trichloroacetic acid, then it was heated at 95°C in water bath for 30 min. MDA content was calculated through the difference between the absorbance at 532 and 600 nm, using a molar extinction coefficient of 155 mM^{-1}. cm^{-1} expressed in µmol MDA . g^{-1} of fresh mass.

6.2.10 Reactive oxygen species contents ($\bullet O_2^-$ and H_2O_2)

Crude extracts were prepared by homogenizing 0.2 g of fresh mass from shoots and roots in 50 mM potassium phosphate buffer (pH 7.8) and in 0.1% (w/v) trichloroacetic acid, with 5 mM KCN, respectively, for superoxide radical ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2) extraction (CHEESEMAN, 2006; XU et al., 2010). Spectrophotometric readings at 530 nm were performed in supernatant in order to measure superoxide ($\bullet O_2^-$) content using $NaNO_2$ solution as standard curve (ELSTNER; HEUPEL, 1976). For determining H_2O_2 concentration it was used a standard curve prepared with H_2O_2 solutions, potassium phosphate pH 7.0 and potassium iodide (SERGIEV; ALEXIEVA; KARANOV, 1997).

6.2.11 Antioxidant enzymes

For antioxidant enzyme activities, 0.2 g of rice shoots and roots were homogenized in 100 mM phosphate buffer (pH 7.0) with 0.1 mM ethylenediaminetetraacetic acid (EDTA) at 4°C. After centrifugation at 12000 x g, at 4°C, the supernatant was collected and used to measure the enzyme activity.

Enzyme activities were assayed in a 96-well microplate reader (Synergy HTX, BioTek®), performing a kinetic activity during 30 min at 30 °C, except for superoxide dismutase (SOD; EC 1.15.1.1) which was performed in a glass cuvette and spectrophotometer.

Ascorbate peroxidase (APX; EC 1.11.1.11) absorbance decay was measured at 290 nm and the activity was calculated using the molar extinction coefficient for ascorbate (ε = 2.8 $mM^{-1}.cm^{-1}$), according to Nakano & Asada (1981). Catalase (CAT; EC 1.11.1.6) activity was determined by consumption of H_2O_2 monitored at 240 nm using the molar extinction coefficient (ε = 36 $M^{-1}. cm^{-1}$) as described by Beers and Sizer (1952). Guaiacol peroxidase (GPOD; EC 1.11.1.7) activity was measured using the molar extinction coefficient for tetraguaiacol (ε = 26.6 $mM^{-1}. cm^{-1}$) (URBANEK; KUZNIAK-GEBAROWSKA; (POLAND)., 1991). APX, CAT and GPOD activities were expressed as μmol H_2O_2. min^{-1}. mg $protein^{-1}$. SOD activity was measured by absorbance reading of blue formazan at 560 nm, produced by photoreduction of the nitroblue tetrazolium (NBT), after 15 min under light (two 20-W fluorescent tubes) at 25 °C (BEYER; FRIDOVICH, 1987). SOD activity was determined as the amount of enzyme required to cause 50% inhibition of the NBT photoreduction, it was expressed as enzyme unit (EU. mg $protein^{-1}$).

6.2.12 Expression analysis by qPCR

Total RNA was isolated using TRizol (Sigma Aldrich), the quantification and integrity in a NanoDrop 2000 spectrophotometer (Thermo Scientific TM, Waltham, USA), and electrophoresis in 1.5% (m/v) agarose gel electrophoresis system, at 50 mA, 100 V. cDNA libraries were built employing M-MLV reverse transcriptase. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using RNase-free water and oligo(dT)s incubation at 70 ºC for 5 min and cooling at 4 ºC for 5 min. It was mixed with RNase inhibitor, Reverse Transcription Mix, oligo(dTs) and submitted to 25 ºC annealing temperature for 5 min, followed by elongation at 42 ºC for 60 min and enzyme denaturation at 70 ºC for 15 min. Synthesized cDNA was stored under -20 ºC until used. The qPCR amplifications were carried out in a real-time thermal cycler (Esco Swift, Esco), composed of initial denaturation at 95 °C for 2 min and 40 thermal cycles of 15 s at 95 °C, followed by 15 s at a specific annealing temperature for each primer (Supplementary Table S1) and finally at 20 s at 60 °C in a total volume of 10 μL, according to the manufacturer's instructions of GoTaq qPCR Master Mix (Promega) in biological triplicates.

The efficiency of the primers was determined by the serial dilution method of cDNAs and calculated based on the slope, with values obtained between 95 and 100%. Melting curves confirmed the absence of nonspecific products and dimer formation. The relative quantification of transcripts was performed using the mean (LIVAK; SCHMITTGEN, 2001).

6.2.13 Metabolic profiles by gas chromatography coupled to mass spectrometry (GC-MS)

Approximately 50 mg of the samples were ground and mixed in methanol,

chloroform, and ultrapure water (2: 1: 2, v/v) solution for polar metabolite extraction (LISEC et al., 2006). Then, 30 µL of the internal standard ribitol (0.2 mg ml^{-1}) was added to the mixture, and 150 µl aliquot of the upper (polar) water-methanol phase was transferred to a new tube and dried in a vacuum concentrator at room temperature (SpeedVac Concentrator, Eppendorf, Hamburg, Germany). In the derivatization stage, the dried samples were treated with methoxylamine hydrochloride (10 mg/0.5 mL in pyridine) with stirring at 37 °C for 2 h, followed by the addition of N-methyl-N- (trimethylsilyl) -trifuoro acetamide (MSTFA) with stirring at 37 °C for 30 min. Then the samples were conducted for GC-MS analyses.

GC–MS analyses were carried out on a QP–PLUS 2010 Shimadzu GC– MS instrument. One microliter of the sample was injected in split mode (1:10 ratio) with helium as carrier gas at a flow rate of 1.0 mL min"1 in an RTX-5MS capillary column (30 mm # 0.25 mm # 0.25 µm) to separate the metabolites. Chromatographic runs were carried out in five replicates at 80 ºC for 5 min, then increased to 310 ºC by 8 ºC.$min^{-1}$and maintained for 1 min at this temperature. The injection, ions source, and MS interface temperatures were 230, 200, and 250 ºC, respectively. Mass spectrometer was operated at 70 eV (EI) in a scanning range of 80–700 (m/z), initiated after a solvent cut-off time of 4.0 min. Chromatograms and mass spectra were processed using the Xcalibur™ 2.1 software (Thermo Fisher Scientific). Metabolites were identified based on the comparison of their retention times and fragmentation patterns of amino acids, carbohydrates, and organic acids previously obtained (CAVALCANTE et al., 2023; COSTA et al., 2023; LIMA et al., 2022), an internal MS library composed of metabolite standards and as well with those of Golm's metabolome database Arabidopsis fragmentation patterns (http://gmd.mpimp-golm.mpg.de/analysisinput.aspx) . The relative value of each metabolite was determined by dividing their respective peak areas by the internal standard ribitol peak area, then divided by the fresh mass of the sample. Then, metabolites were identified and classified using either the Kyoto Gene and Genome Encyclopedia (KEGG) or the PUBChem databases.

6.2.14. Experimental design and statistical analysis

The experiment comprised a completely randomized design. For the NaCl screening, two sets of experiments were performed, both with five NaCl levels (0, 100, 150, 200, and 250 mM) treatments: the first one was performed without TM (unprimed seeds), and in the second one seeds were primed with 0.500 µg.mL^{-1}, to determine the most appropriate NaCl concentration for 2x2 experiments. The experiment was carried out from 5 TM-primed treatments (TM 0, 0.125, 0,250, 0.375, and 0.500), with 4 repetitions per treatment and 50 rice seeds for each repetition, with NaCl at 150 mM, to

determine the best TM concentration for further analyses. Data were submitted to regression analysis using the Sisvar Software (FERREIRA, 2011). The outcome of this analysis was obtained through linear, quadratic, and cubic polynomial regressions, in a 5% probability level, adjusted for all variables.

After the screening tests, all further analyses were carried out with a factorial scheme (2 × 2), two concentrations of TM (0 and 0.25 µg.mL^{-1}) and two concentrations of NaCl (0 and 150 mM NaCl), generating thus four treatments: T0S0 (TM 0 µg.mL^{-1}, 0 mM NaCl), T025S0 (TM 0.25 µg.mL^{-1}, 0 mM NaCl), T0S150 (TM 0 µg.mL^{-1}, 150 mM NaCl), and T025S150 (TM 0.25 µg.mL^{-1}, 150 mM NaCl). Results were subjected to ANOVA analysis of variance, and the data means were compared by Tukey test ($p \leq 0.05$) using the SISVAR statistical program.

Metabolic data were normalized by log transformation and Range scaling. Mean values of the metabolites were compared using the T-tests to assess the effect within each treatment. Principal Component Analysis (PCA) was performed to identify the differences between the four treatments (T0S0, T025S0, T0S150, and T025S150) within each tissue (shoots and roots), and the most contributing metabolites were highlighted in the loading plots. To verify the effect of each TM treatment compared to their salt counterpart on the metabolic profiles, Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) was performed for shoots and roots of each variety, and variable importance in projections (VIP scores) were performed separately for each treatment, obtaining the principal discriminating metabolites. Hierarchical heatmaps were constructed by Euclidean distance based on a one-way analysis of variance (ANOVA).

6.3 Results

6.3.1 NaCl and TM screenings

Growing concentrations of NaCl from 100 to 250 mM decreased all germination parameters, such as the physiological ones (Figure 1, A-J). However, TM priming displayed better performances than the untreated groups. Aside from abnormal seedlings, exogenous TM priming significantly (with $p < 0.05$) improved, especially at 150 mM NaCl and compared to T0 seedlings, germination percentage, GSI, SESI, RDM, SDM, SL and RL. Moreover, TM reduced mean germination time and mean shoot emergence time (Figure 1D and 1F). Other than that, there were little or no significant changes, although there was a tendency to TM ameliorate the deleterious effects of salinity. (Figures 1A – 1J). Therefore, this experiment showed that 150 mM NaCl could allow the seedlings to establish although it was severely repressed, but also had its parameters benefited by TM-priming, which is why it was chosen for further experiments. These data can be accessed in Supplementary Data (Table S2 and Table S3). The germination rate of non-primed seeds (TM0) displayed a quadratic behavior in function of NaCl concentrations, getting lower as the saline concentration increased (Figure 1A). On the other hand, TM050 increased the germination rate in all NaCl treatments (Figure 1A). Concerning

GSI, both TM0 and TM050 displayed quadratic behaviors, in which T050 at 150 mM showed higher values than its counterpart T0 (Figure 1C). MGT regressions revealed interesting behaviors: TM0 displayed a decreasing quadratic function as the NaCl concentrations grew, whereas TM050 showed an increasing quadratic one (Figure 1D), with the last point (at 250 mM NaCl) being nearly at the same place as that one in TM0 function (Figure 1D). SESI exhibited increasing quadratic behaviors, although it was higher in TM050 and 150 mM (Figure 1E). MSET had the same pattern for both regressions. It showed increased linear behaviors as the NaCl concentrations augmented, with TM050 having a significantly lower value than this parameter at 150 mM NaCl, in contrast to T0 at the same saline point (Figure 1F). Both root and shoot dry masses exhibited similar results: increasing quadratic functions for the TM treatments (Figures 1G and 1H), with TM050 at 100 mM, showing much higher values than those found on TM0 (Figures 1G and 1H). There were similar regressions for shoot and root lengths (Figures 1I and 1J). It displayed quadratic behaviors in function of NaCl concentrations (Figures 1I and 1J), but TM050 displayed higher results, especially at 100 and 150 mM NaCl in RL (Figures 1J).

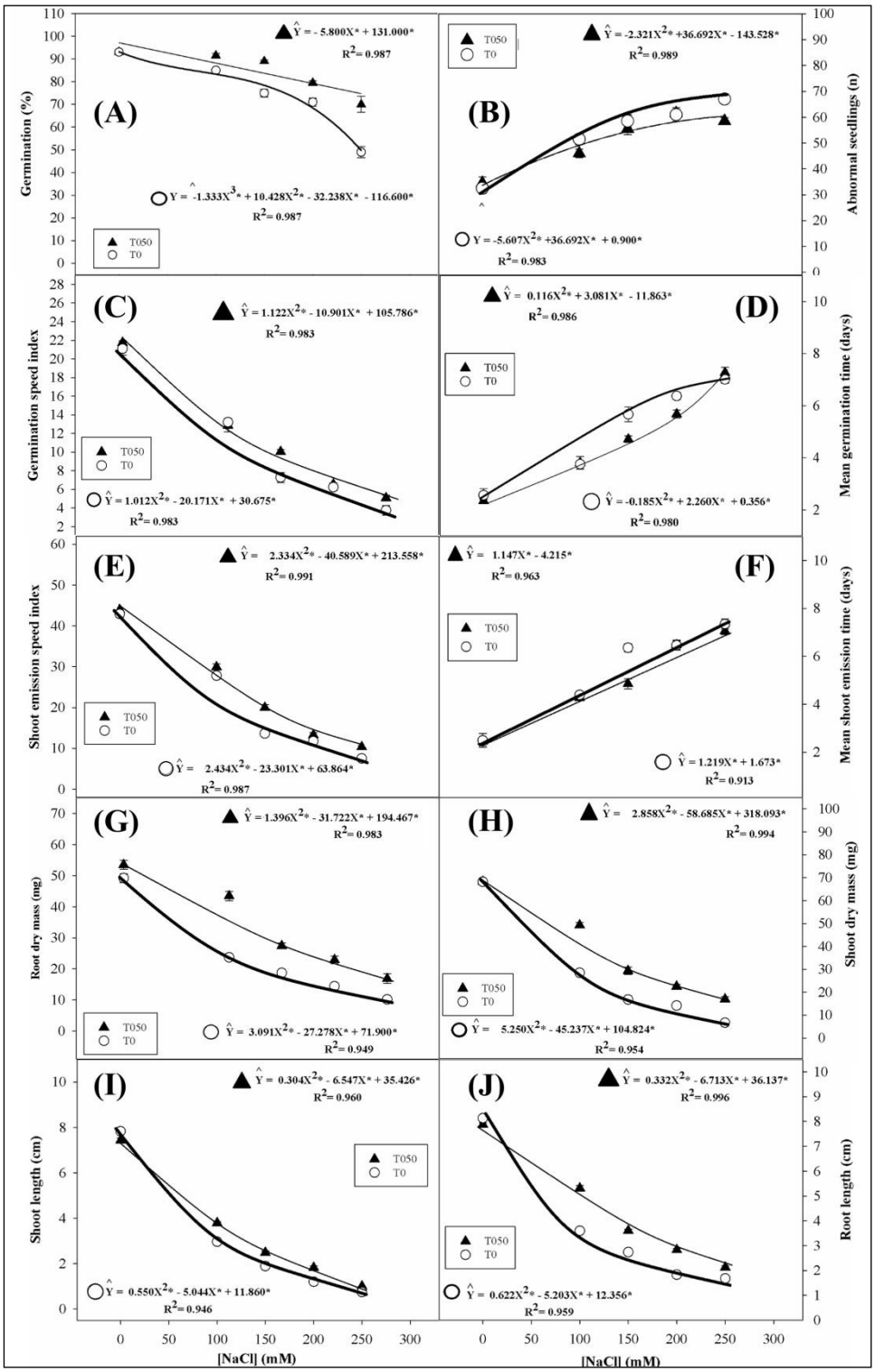

Figure 1 NaCl screening regressions. Percentual of germination (A), number of abnormal seedlings (B), germination speed index (C), mean germination time (D), shoot emission speed index (E), mean shoot emission time (F), root dry mass (G), shoot dry mass (H), shoot length (I) and root length (J) of rice seeds and seedlings, primed with either 0 µg. mL^{-1} (O, thicker lines) or 0.50 µg. mL^{-1} (▲, thin lines) TM submitted to crescent concentrations of NaCl (0 – 250 mM). Circumflex accent (^) over Y means the function was statistically significant. Regression points indicate means of 4 repetitions ± standard error; statistical analyses were represented by * as significant with $p < 0.05$.

For TM screening, we primed seeds with increasing TM concentrations and tested their germination under 150 mM NaCl. Although TM significantly increased all parameters in comparison to 0 TM, regressions showed that only five parameters achieved statistical significance (Table S4): germination percentage (Figure 2A), GSI (Figure 2B), MGT (Figure 2C), SDM (Figure 2D) and RDM (Figure 2E). However, it is relevant to point out that, aside from GSI, 0.25 TM displayed the highest values in all those parameters (Figures 2A – 2E). Regressions showed that TM concentrations induced a decreasing quadratic function of germination percentage, with the value at 0.25 TM being the highest and nearest the vortex of the equation (Figure 2A). GSI regressions also displayed a quadratic function as TM concentrations increased, with the points 0.250 and 0.500 displaying very close values (figure 2B). MGT showed an increasing cubic function of regression, but only with the point at 0.250 TM showing the smallest value (figure 2C). In both shoot and root dry masses (Figures 2D and 2E), regressions also showed decreasing cubic patterns, with 0.250 TM showing the highest values of these parameters (Figures 2D and 2E). Therefore, 0.25 µg. mL^{-1} TM concentration was selected for further analyses as well

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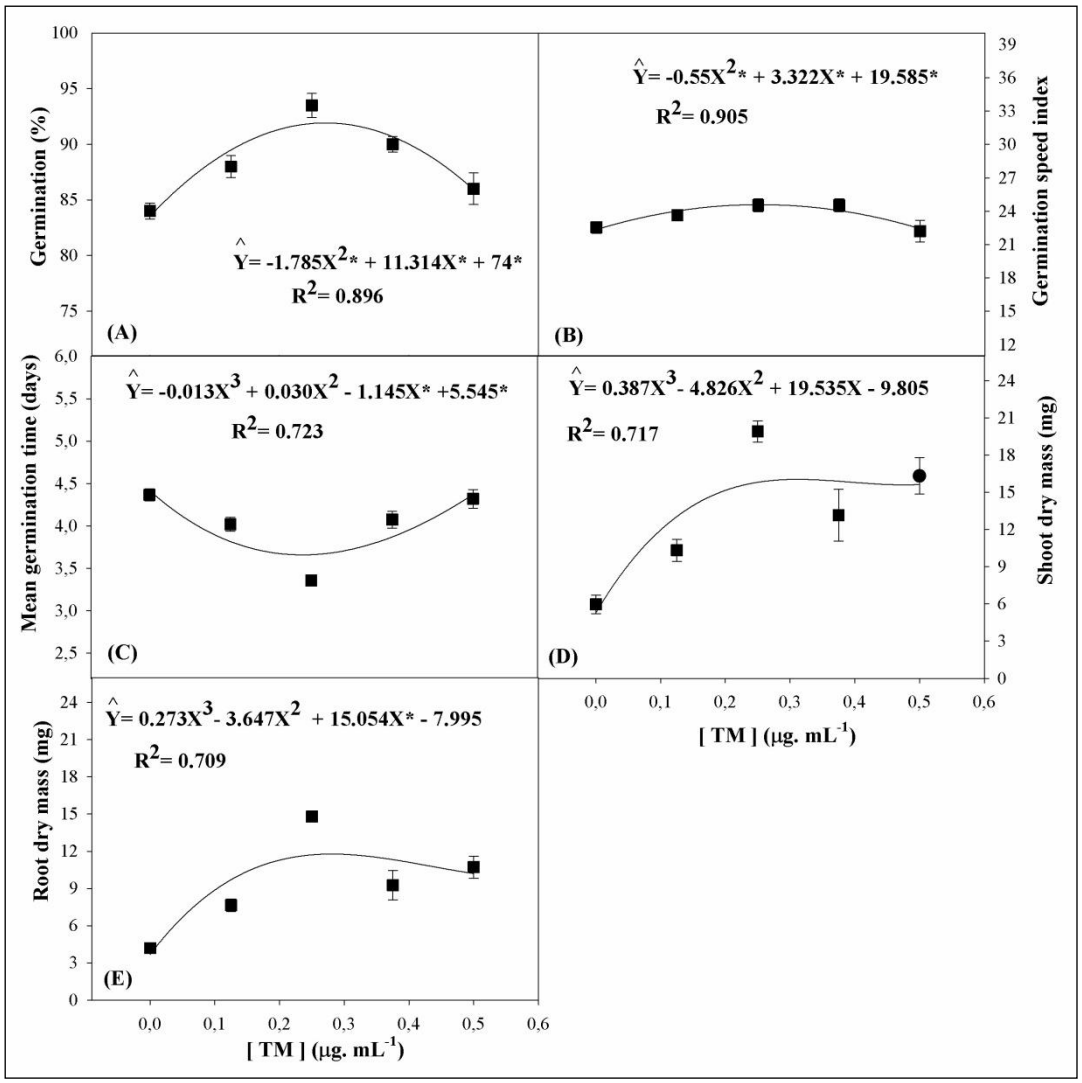

Figure 2 Tunicamycin screening regressions. Percentual of germination (A), germination speed index (B), mean germination time (C), shoot dry mass (D) and root dry mass (E) of rice seeds and seedlings, primed with either 0 µg. mL^{-1} (O, thicker lines) or 0.50 µg. mL^{-1} (▲, thin lines) TM submitted to growing concentrations of NaCl (0 – 250 mM). Regression points indicate means of 4 repetitions ± standard error; statistical analyses were represented by * as significant with $p < 0.05$.

6.3.2 Physiological parameters

In the absence of salinity (0 mM NaCl treatments), there was no significant difference between non-pre-treated and TM pre-treated seedlings (Figure 3). However, salt stress decreased all physiological parameters in comparison to non-pre-treated seedlings. Conversely, TM-pre-treated ones alleviated the deleterious effects of salinity for all parameters. The increases of shoot and root, as well as dry masses of shoot and root lengths of TM pre-treated seedlings under salt treatment, were 45%, 62%, 40%, and 50%, respectively, compared to non-pre-treated seedlings under salinity (Figure 3A-D, and Supplementary Figure S1).

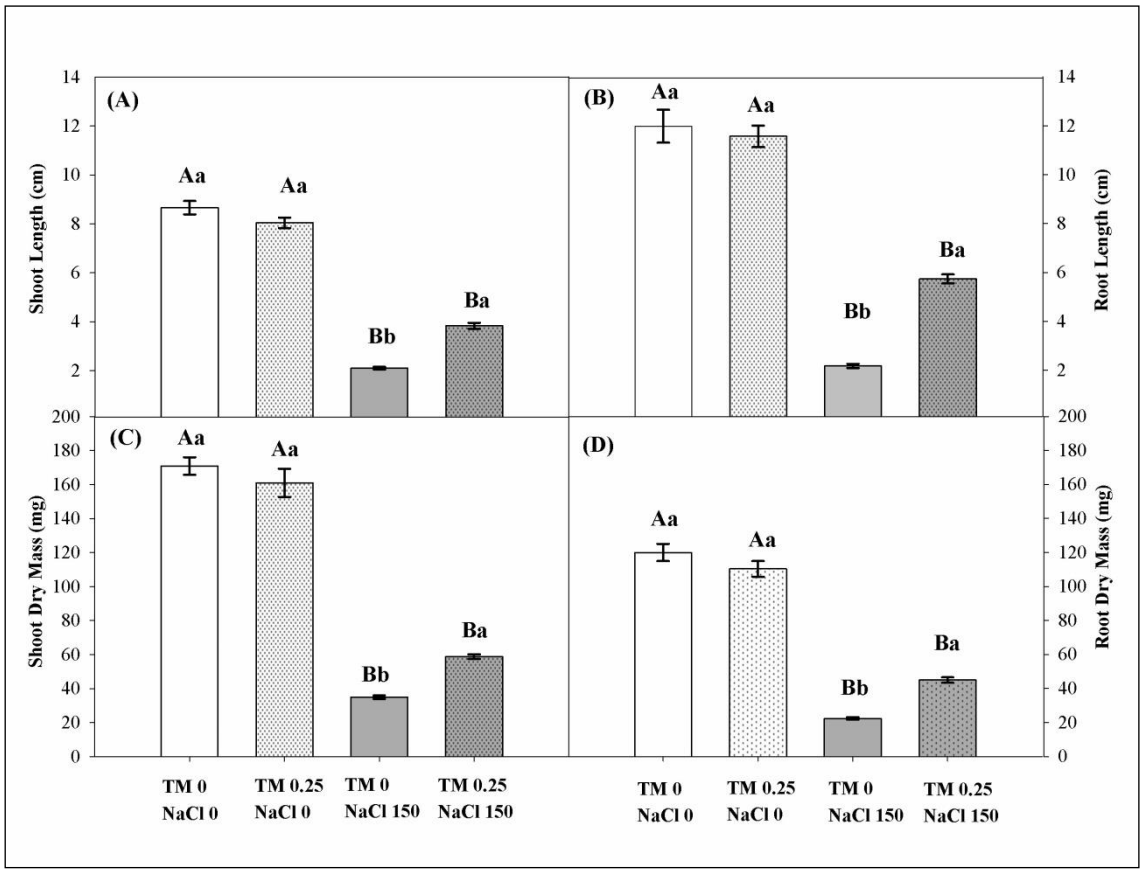

Figure 3 Growth parameters of pre-treated seeds with tunicamycin (0 or 0.25 µg.mL) germinated in NaCl (0 or 150 mM) for 10 days. A) Shoot length; B) Shoot dry mass, C) Root length; D) Root dry mass. Different uppercase letters indicate statistical difference between distinct salt conditions within the same tunicamycin concentration, and lowercase letters indicate statistical difference among TM pre-treatments in the same NaCl concentration using Tukey test ($p<0.05$). Error bars represent standard error (SE) in all groups.

6.3.3 Osmotic potential and electrolyte leakage

Salinity reduced the osmotic potential of shoots, and TM priming increased it by 25% (Figure 4A). Furthermore, NaCl treatment increased electrolyte leakage in the shoots and roots of seedlings. However, TM priming significantly increased these effects in shoots and roots, 32% and 25%, compared to the absence of priming (Figure 4B and 4C).

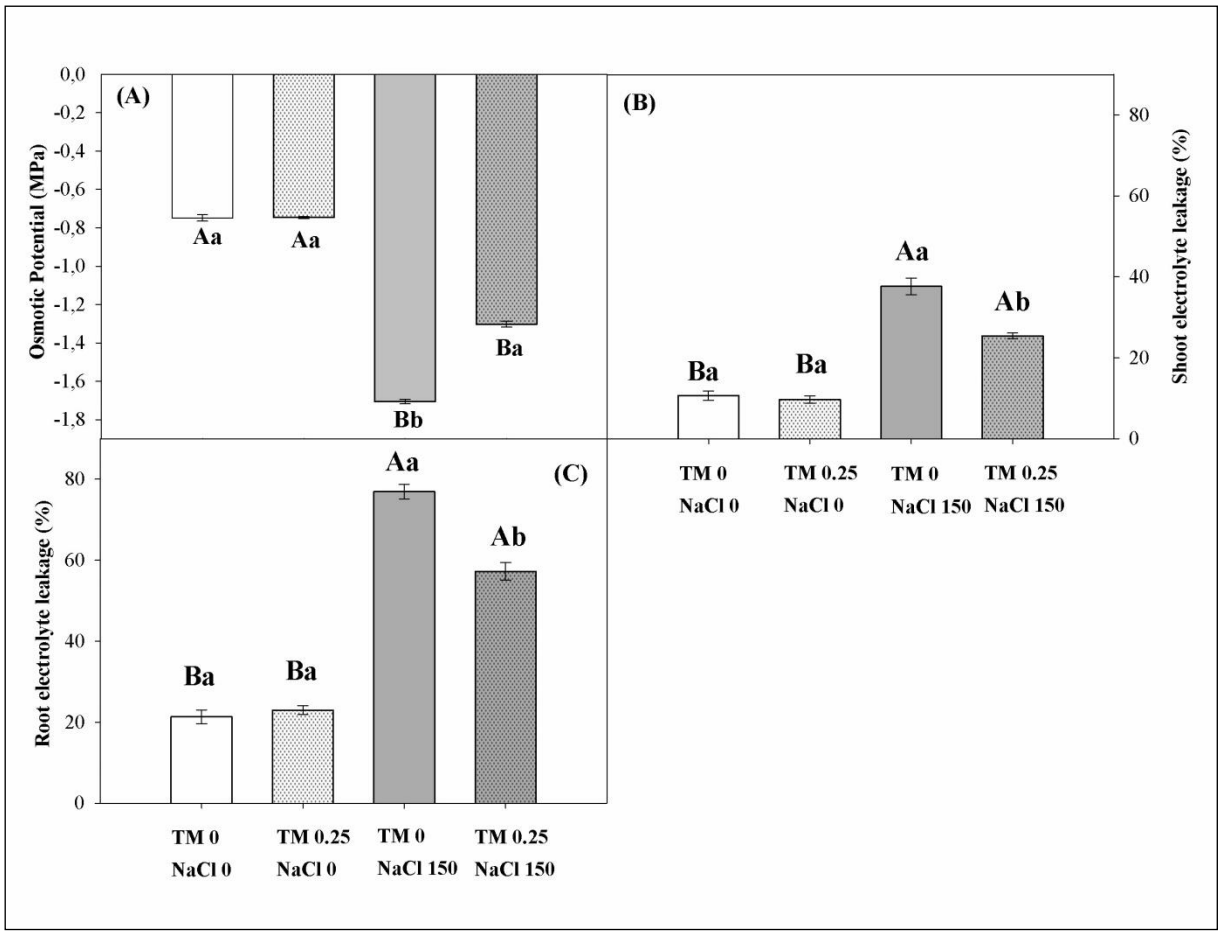

Figure 4 Osmotic potential (A), and electrolyte leakage of shoots (B), and roots (C) of seedlings pre-treated with tunicamycin (0 or 0.25 µg.mL) germinated in NaCl (0 or 150 mM) for 10 days. Different uppercase letters indicate statistical difference between distinct salt conditions within the same tunicamycin concentration, and lowercase letters indicate statistical difference among TM pre-treatments in the same NaCl concentration using Tukey test ($p<0.05$). Error bars represent standard error (SE) in all groups.

6.3.4 Photosynthetic pigments

Salt stress decreased all photosynthetic pigment contents, but the TM pre-treatment ameliorated this effect compared to non-pretreated shoots. There were increases of 61% in chlorophyll *A* content (figure 5A), 30% in chlorophyll *B* content (Figure 5B), 31% in total chlorophyll content (Figure 5C), and 26% in carotenoids contents (Figure 5D) promoted by seed pre-treatment.

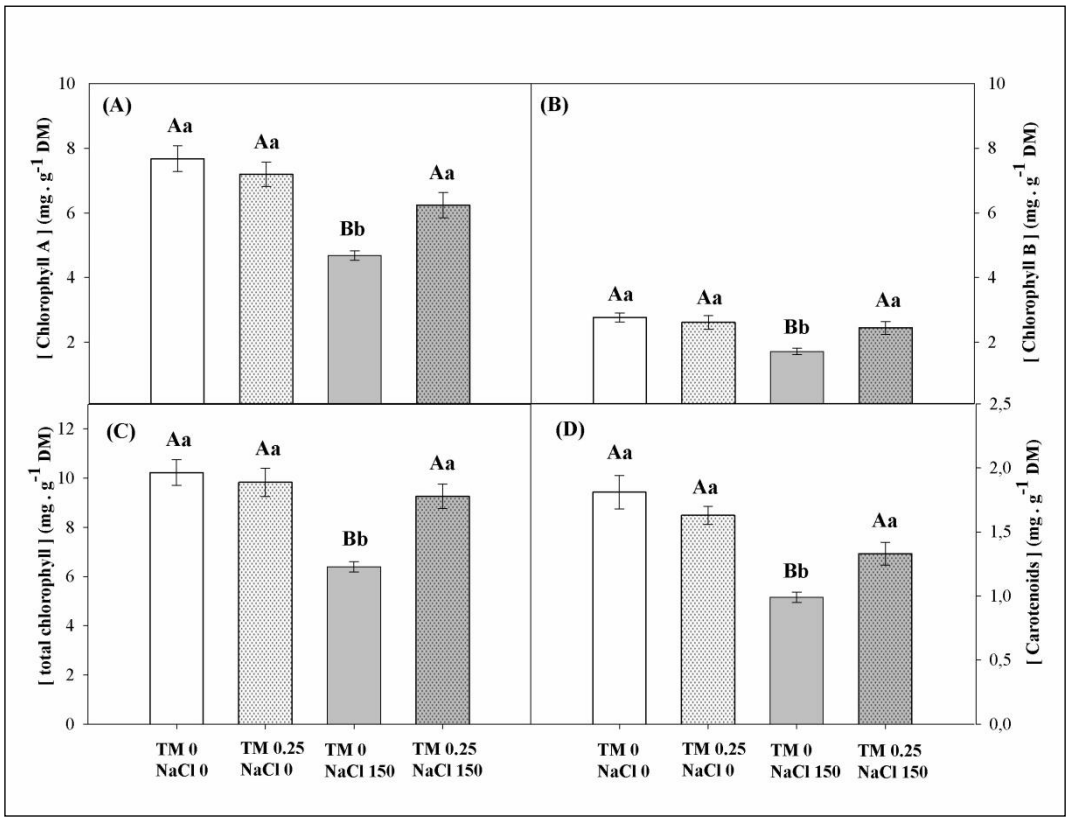

Figure 5 Photosynthetic pigment contents of pre-treated seeds with tunicamycin (0 or 0.25 µg.mL) and germinated in NaCl (0 or 150 mM) for 10 days. A) chlorophyll *A* content, B) chlorophyll *B* content, C) total chlorophyll content, and D) carotenoid content. Different uppercase letters indicate statistical difference between distinct salt conditions within the same tunicamycin concentration, and lowercase letters indicate statistical difference among TM pre-treatments in the same NaCl concentration using Tukey test ($p<0.05$). Error bars represent standard error (SE) in all groups.

6.3.5 Inorganic ion contents (Na^+, K^+, and Cl^-)

The Na^+ content in 150 mM NaCl-treated seedlings increased in shoots and roots. However, TM-primed reduced Na^+ levels by 36% in shoots (Figure 6A) and 22% in roots (Figure 6B). In contrast, K^+ content in shoots was not modified by salinity between TM-primed groups (T025_S0 and T025_S150). Comparing both stressed treatments, TM induced an increase of this ion by 33% when compared to T0_S150 shoots (Figure 6C). In roots, levels of this ion (K^+) decreased with the saline treatment (Figure 6D). Nevertheless, TM-conditioning alleviated this condition, expressing an increase of 29% (Figure 6C) in shoots and 24% (Figure 6D) in roots, when compared to TM 0 ones. Concerning K^+/Na^+ ratio, NaCl-treated seedlings underwent a decrease in this parameter in both shoots and roots. Despite that, TM-primed group revealed an elevation of this ratio, when compared to TM-untreated one, of 50% in shoots (Figure 6E) and 44% in roots, respectively (Figure 6F). Similarly, to Na^+, Cl^- content significantly increased in shoots and roots in the presence of 150 mM NaCl. Nevertheless, TM-pre-treated seedlings under salt stress exhibited a reduction in Cl^- of 26% in shoots (Figure 6G), and 24% in roots (Figure 6H), in comparison to non-pre-treated under salinity.

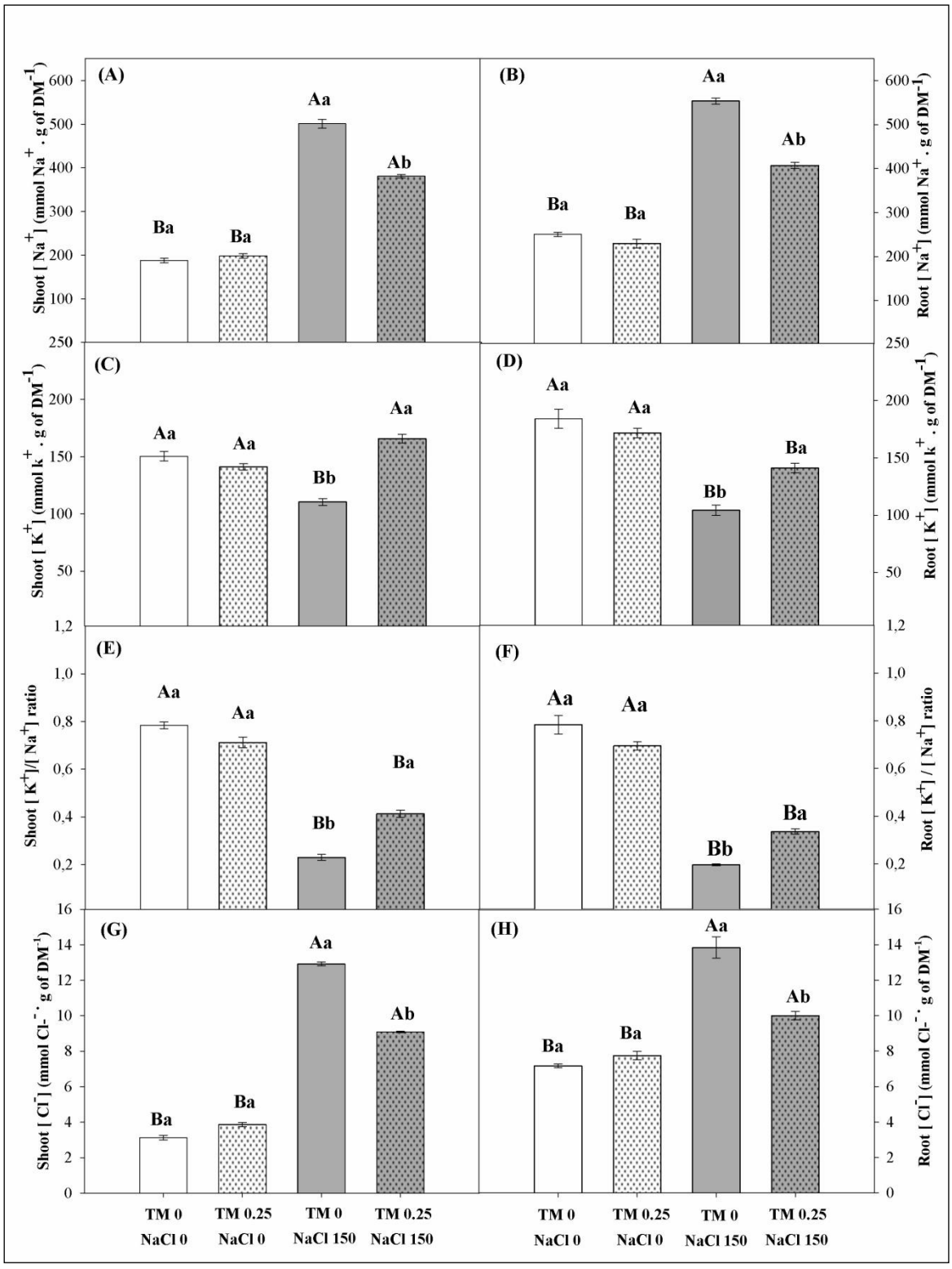

Figure 6 Inorganic ion contents of pre-treated seeds with tunicamycin (0 or 0.25 µg.mL) and germinated in NaCl (0 or 150 mM) for 10 days. A) Shoot K^+, B) shoot Na^+, C) root K^+, D) root Na^+, E) shoot K^+/Na^+ ratio, F) shoot K^+/Na^+ ratio, G) Shoot Cl^-, and H) Root Cl^- content. Different uppercase letters indicate statistical difference between distinct salt conditions within the same tunicamycin concentration, and lowercase letters indicate statistical difference among TM pre-treatments in the same NaCl concentration using Tukey test ($p<0.05$). Error bars represent standard error (SE) in all groups.

6.3.6 Detection of Na^+ by confocal microscopy

Confocal microscopy fluorescence technique used to examine Na^+ dynamics in leaf tissues showed higher Na^+ accumulation in the shoots, especially in the epidermal cells and within such cells (Figure 7A), and in the root hairs (Figure 7B) of NaCl-treated seedlings. However, seedlings from primed seeds under salinity decreased fluorescence emission, which was evidenced by the lower intensity green emission for shoots (7A) and roots (7B). The intensity emission of fluorescence in shoots (Figure 7C) and roots (figure 7D) was measured using Zen software. It showed lower intensity in both tissues in TM-treated seedlings under salt stress, which confirmed that TM-priming reduced Na^+ content in the apoplast.

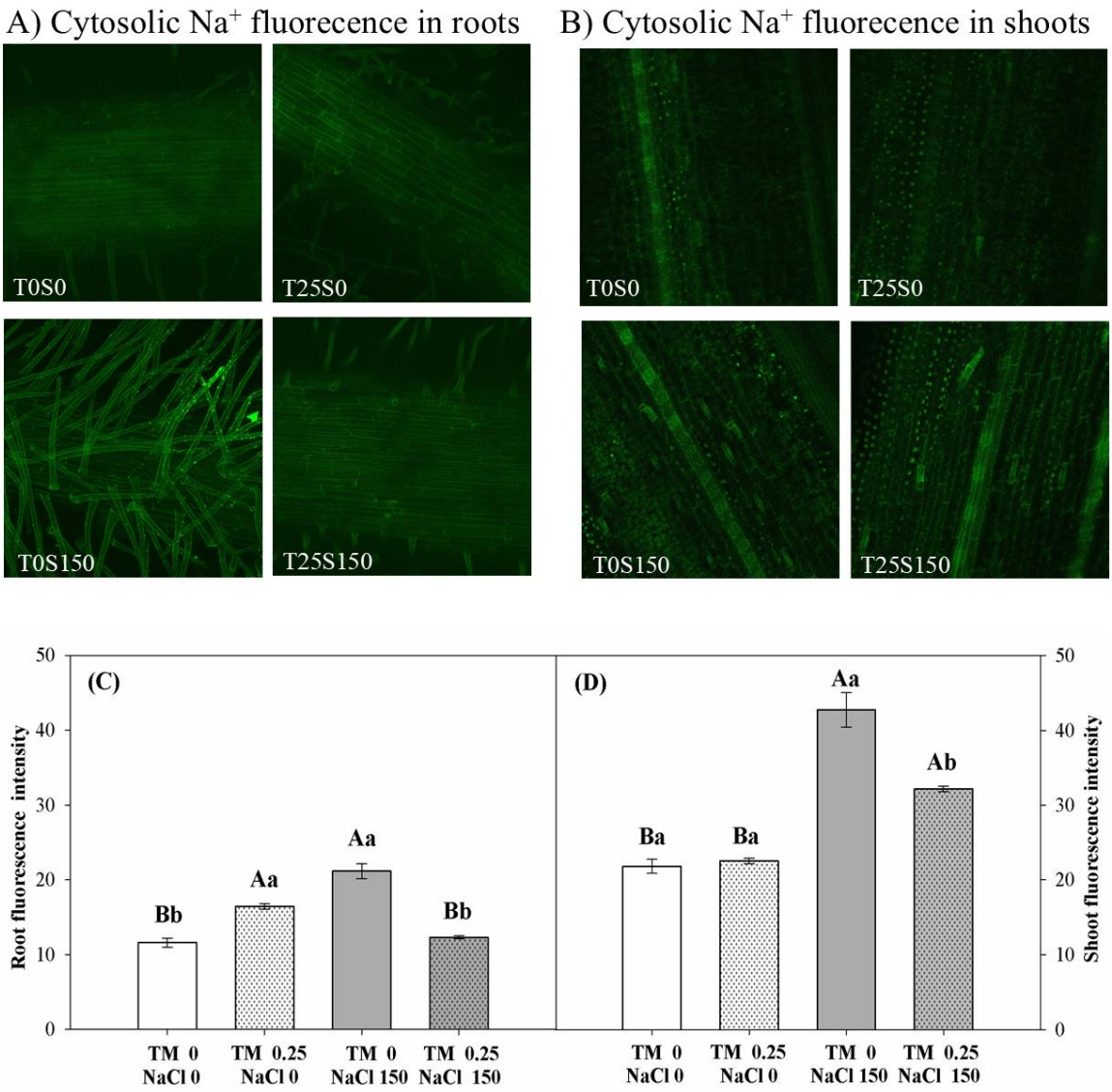

Figure 7 NaTRIUM Green-2AM Fluorescence in pre-treated seedlings with tunicamycin (0 or 0.25 µg.mL) and germinated in NaCl (0 or 150 mM) for 10 days, in roots (A) and shoots (B), the intensities of each fluorescence were estimated. Different uppercase letters indicate statistical difference between distinct salt conditions within the same tunicamycin concentration, and lowercase letters indicate statistical difference among TM pre-treatments in the same NaCl concentration using Tukey test ($p<0.05$). Error bars represent standard error (SE) in all groups.

6.3.7 Reactive oxygen species (H_2O_2 and $\bullet O_2^-$) contents and lipid peroxidation

As expected, salt stress increased H_2O_2 content in NaCl-treated seedlings compared to control groups. Interestingly, at 150 mM NaCl, TM-pre-treated seedlings showed a reduction of this ROS of 25% in shoots (Figure 8A) and 32% in roots (Figure 8B). Similarly, salinity also increased $\cdot O_2^-$ content in shoots and roots (Figure 8E and Figure 8F). However, only TM-pre-treated shoots significantly reduced their production, with a decrease of 30% when compared to TM 0 seedlings under salt stress (Figure 8E). The increased H_2O_2 and $\cdot O_2^-$ may induce malondialdehyde (MDA) production (a marker of lipid peroxidation). NaCl stress increased MDA content in both shoots and roots of salt-treated rice seedlings (Figures 8C and 8D). On the other hand, TM-pre-treated seedlings managed the decrease of this production compared to those non-pre-treated ones with 150 mM NaCl, presenting a reduction of 33% in shoots (Figure 8C) and 25% in roots (Figure 8D).

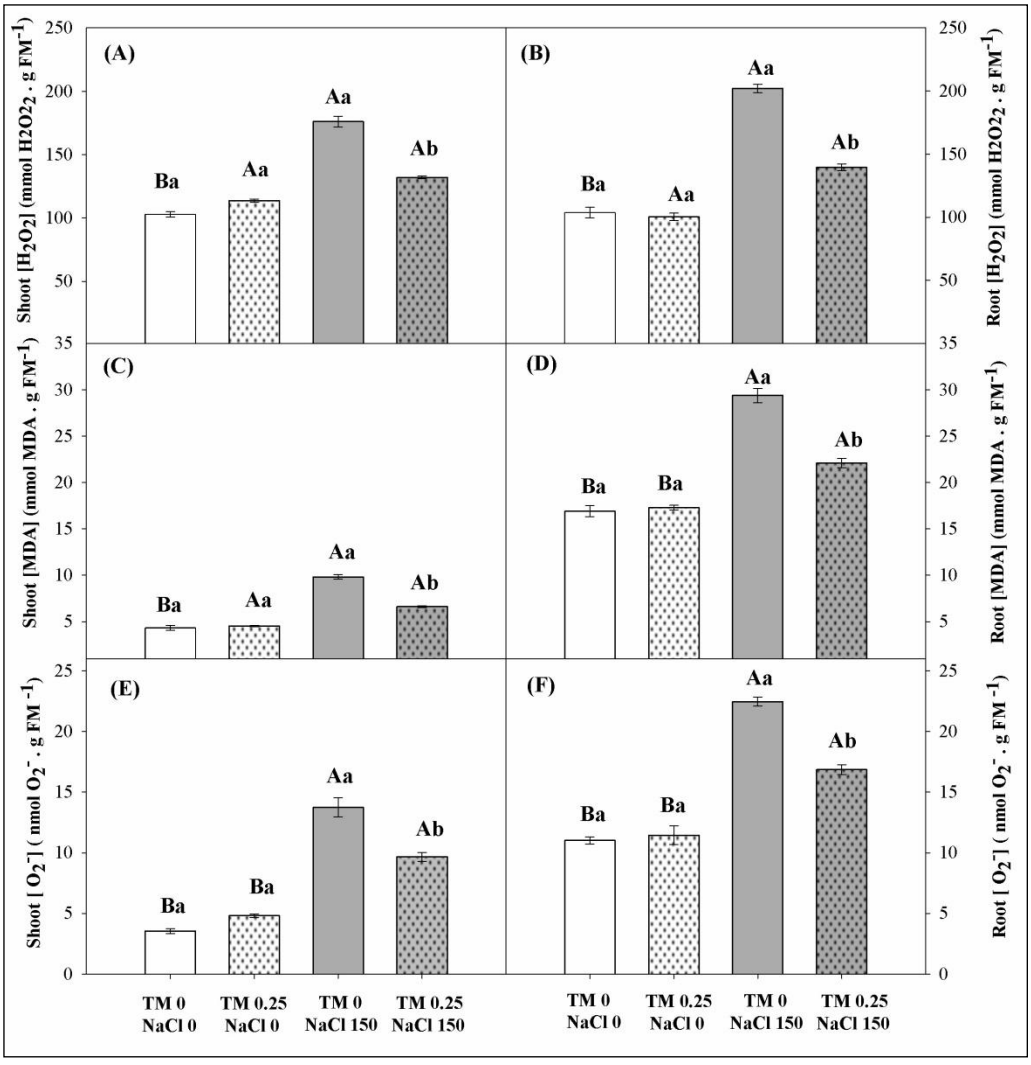

Figure 8 Reactive oxygen species and lipid peroxidation of pre-treated seeds with tunicamycin (0 or 0.25 µg.mL) and germinated in NaCl (0 or 150 mM) for 10 days. A) shoot H_2O_2 content, B) root H_2O_2 content, C), shoot MDA content, D) root MDA content, E) shoot $\cdot O_2^-$ content, and F) root $\cdot O_2^-$ content. Different uppercase letters indicate statistical difference between distinct salt conditions within the same tunicamycin concentration, and lowercase letters indicate statistical difference among TM pre-treatments in the same NaCl concentration using

Tukey test ($p<0.05$). Error bars represent standard error (SE) in all groups.

6.3.8 Antioxidant enzyme activities (SOD, CAT, APX and GPOD)

The higher its substract (H_2O_2) content, the higher CAT activity it is believed to be. As we can see, salt stress increased CAT activity in both shoots and roots of rice seedlings (Figures 9A and 9B); however, TM-conditioning, in relation to the TM 0 group, significantly augmented this enzyme activity in both shoots and roots, by 25% (Figure 9A) and 31% (Figure 9B), respectively. For Ascorbate peroxidase (APX), salt stress increased APX activity in both stressed treatments in shoots and roots (Figures 9C and 9D, respectively). However, in an interesting manner, TM-pre-treatment did not cause any significant variation in this enzyme activity. Once $\cdot O_2^-$ is the substract for SOD, it is assumed that this enzyme has its activity increased by salinity as well. As a matter of fact, NaCl augmented SOD activity in both stressed treatments in shoots and roots (Figures 9E and 9F). Nevertheless, TM-priming did induce a significant increase in SOD activity of 27% in shoots (figure 9E) and 30% in roots (Figure 9F), respectively. H_2O_2 is also substract for GPOD, so one can assume this enzyme has its activity augmented in the presence of NaCl. Salt stress did increase its activity in both NaCl treatments (Figures 9G and 9H). On the other hand, TM-primed seedlings under 150 mM NaCl displayed significantly increased activities in both shoots and roots, respectively, 26% (figure 9G), and 30% (figure 9H).

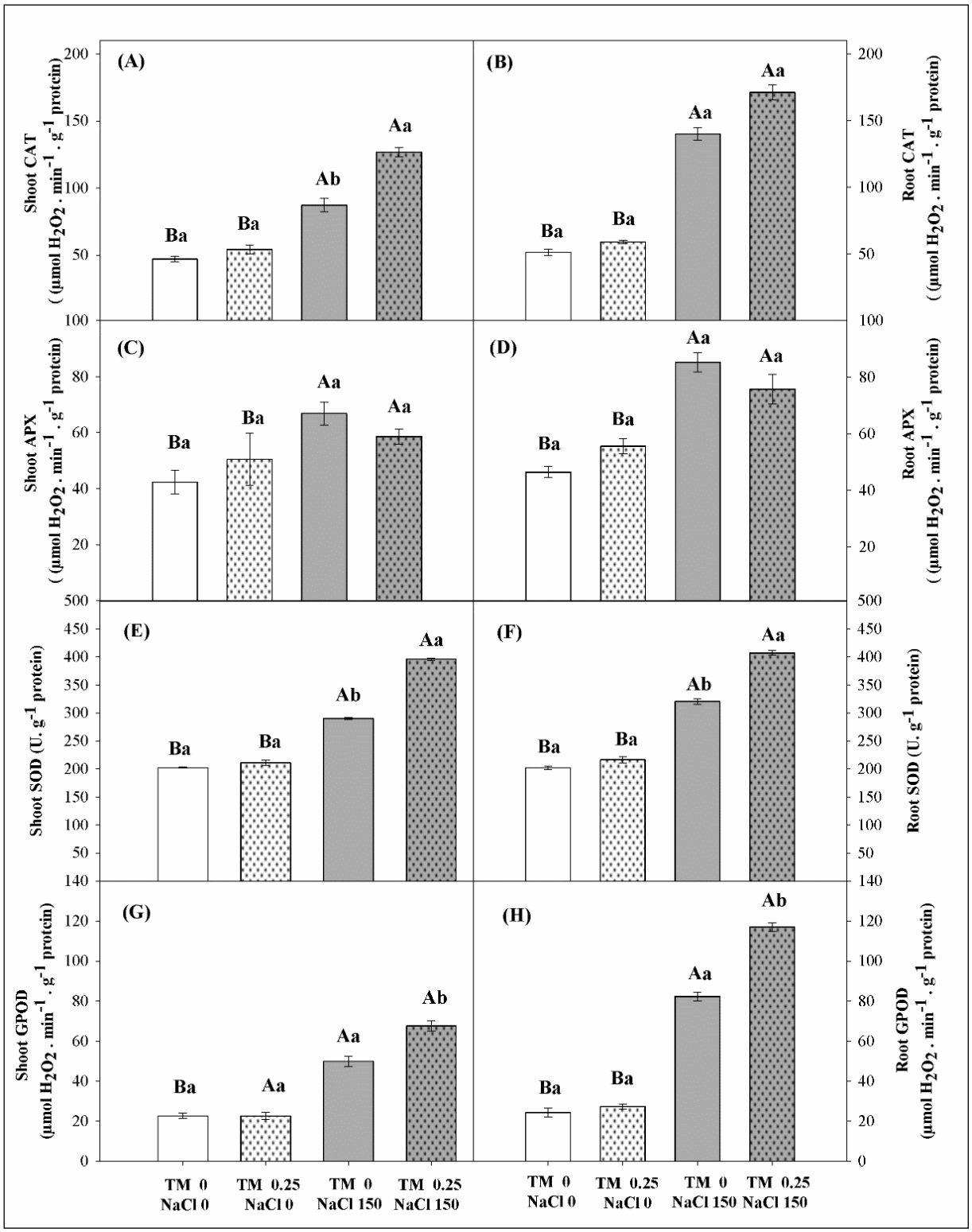

Figure 9 Antioxidant enzymatic system evaluation, of pre-treated seeds with tunicamycin (0 or 0.25 µg.mL) and germinated in NaCl (0 or 150 mM) for 10 days. A) Catalase in shoots, B) catalase in roots, C) ascorbate peroxidase in shoots, D) ascorbate peroxidase in roots. E) superoxide dismutase in shoots, F) superoxide dismutase in roots, G) guaiacol peroxidase in shoots and H) guaiacol peroxidase in roots. Different uppercase letters indicate statistical difference between distinct salt conditions within the same tunicamycin concentration, and lowercase letters indicate statistical difference among TM pre-treatments in the same NaCl concentration using Tukey test ($p<0.05$). Error bars represent standard error (SE) in all groups.

6.3.9 Gene expression analysis

In the absence of salt, TM promoted a decrease of *OsIRE1* only in the shoot, also in the absence of TM, *OsIRE1* was decreased by salinity (Figure 10A). Conversely, there were no significative changes of *OsIRE1* expression induced by TM or salinity in roots, nor *OsbZIP50* (*Atb*ZIP60 ortholog) in both shoots and roots (Figure 10B-D). On the other hand, in the presence of salt, the TM priming promoted a remarkable increase of *OsIRE1* and *OsbZIP50* in booth shoots and roots in comparison to salt without TM priming (Figure 10A-D). Similarly, the expression of *OsbZIP60* (*Atb*ZIP28 ortholog) increased in shoots of primed seed grown under salinity, however it decreased in roots (Table S5). Although we could not detect the amplification in all treatments, the expressions of *OsBiP4* and *OsBiP5* were high in shoots and roots of primed seeds grown under salinity, respectively. However, we could not find consistency in the expressions of *OsNHX1,* the expression of *OsSOS1* seems to increase under salinity, in comparison to control plants (Table S5).

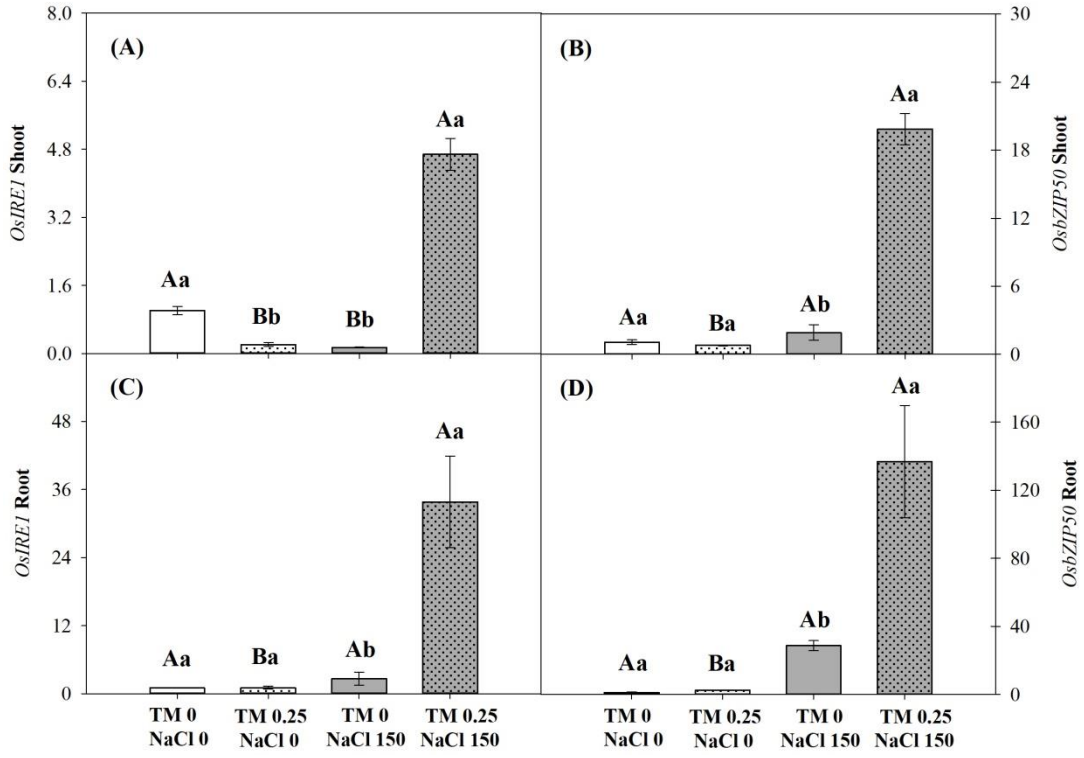

Figure 10 Relative gene expressions of pre-treated seeds with tunicamycin (0 or 0.25 µg.mL) and germinated in NaCl (0 or 150 mM) for 10 days. A-B) *OsIRE1* and *OsbZIP50* in shoots C-D) *OsIRE1* and *OsbZIP50*. Gene expressions were normalized using *OsUBQ* as a reference gene and the values expressed as $2^{-\Delta\Delta CT}$. The columns indicate the mean of three biological replicates and the bars indicate the standard deviation. Different uppercase letters indicate statistical difference between distinct salt conditions within the same tunicamycin concentration, and lowercase letters indicate statistical difference among TM pre-treatments in the same NaCl concentration using Tukey test ($p<0.05$). Error bars represent standard error (SE) in all groups.

6.3.10 Metabolomic profiling analysis

A total of 40 metabolites were identified among treatments, of which 15 were amino acids, 10 were organic acids, 11 were carbohydrates, and 4 belonged to other classes (Table 1). The classification was based on the KEGG ID or PubChem, the retention times, and mass fragmentation pattern. Among them, 36 were found in both shoots and roots, whereas 6 were exclusive to roots: the sugars mannose and sorbitol, lactic acid dimer (organic acid), n-acetyl-serine and lysine (amino acids), and urea (other compound). The only metabolite exclusive to shoots was salicylic acid.

Table 1. List of total metabolites found in rice seedlings.

METABOLITE	COMPOUND TYPE	RETENTION TIME (MIN)	COMPOUND ID	DATABASE
4 -Aminobutyric acid	Amino acid	16.57	C00334	KEGG
Alanine	Amino acid	8.39	C00041	KEGG
Asparagine	Amino acid	18.78	C00152	KEGG
beta - Alanine	Amino acid	14.91	C00099	KEGG
Glutamic acid	Amino acid	17.95	C00025	KEGG
Glycine	Amino acid	12.78	C00037	KEGG
Histidine	Amino acid	22.12	C00135	KEGG
Isoleucine	Amino acid	12.54	C00407	KEGG
Lysine	Amino acid	22.06	C00047	KEGG
N-acetyl-serine	Amino acid	17.58	65249	PubChem
O-acetyl-serine	Amino acid	14.94	C00979	KEGG
Threonine	Amino acid	14.30	C00188	KEGG
Valine	Amino acid	10.98	C00183	KEGG
Citric acid	Organic acid	20.81	C00158	KEGG
Glyceric acid	Organic acid	13.28	C00258	KEGG
Glycolic acid	Organic acid	7.73	C00160	KEGG
Lactic acid	Organic acid	7.36	C00186	KEGG
Lactic acid dimer	Organic acid	12.63	10034905	PubChem
Malic acid	Organic acid	15.98	C00149	KEGG
Oxalic acid	Organic acid	9.14	C00209	KEGG
Phosphoric acid	Organic acid	12.21	C00009	KEGG
Pyruvic acid	Organic acid	7.10	C00022	KEGG
Succinic acid	Organic acid	12.86	C00042	KEGG
Fructose	Sugar	21.61	C00095	KEGG
Galactose	Sugar	22.19	C00124	KEGG
Glucose	Sugar	21.94	C00031	KEGG
Myo-inositol	Sugar	24.23	C00137	KEGG
Maltose	Sugar	30.93	C00208	KEGG
Maltotriose	Sugar	34.10	C01835	KEGG
Mannose	Sugar	21.87	C00159	KEGG
Raffinose	Sugar	32.18	C00492	KEGG
Sorbitol	Sugar	21.73	C00764	KEGG
Sucrose	Sugar	30.03	C00089	KEGG
Putrescine	Others	17.42	C00134	KEGG
Quinic acid	Others	21.41	C00296	KEGG
Salicylic acid	Others	16.29	C00805	KEGG
Urea	Others	11.57	C00086	KEGG

To assess the differences between TM-untreated and TM-treated seedlings, multivariate analyses of the principal component (PC) of the GC-MS dataset from shoots and roots were performed separately. Concerning shoots, PC1 and PC2 accounted for 29.8% and 11.3% respectively (Figure 11A). The plot showed a complete separation of groups based on NaCl concentrations: 0 mM NaCl treatments were completely set apart from 150 mM ones, mainly influenced by PC1 component. There were overlaps between treatments with the same TM concentrations (Figure 11A). The top four positive contributions to PC1 in shoots were glyceric acid, phenylalanine, salicylic acid, and erythrose, while to PC2 were galactose, serine, asparagine and glycolic acid (Figure 11B, Table S7). The Heatmaps detailing positive or negative modulation of metabolites were generated comprising both TM (0 and 0.25 µg. mL^{-1}) and NaCl (0 and 150 mM) concentrations, in a similar manner to the PCA. Shoot heatmap analysis displayed a division between 0 mM NaCl and 150 mM NaCl treatments (Figure 11C).

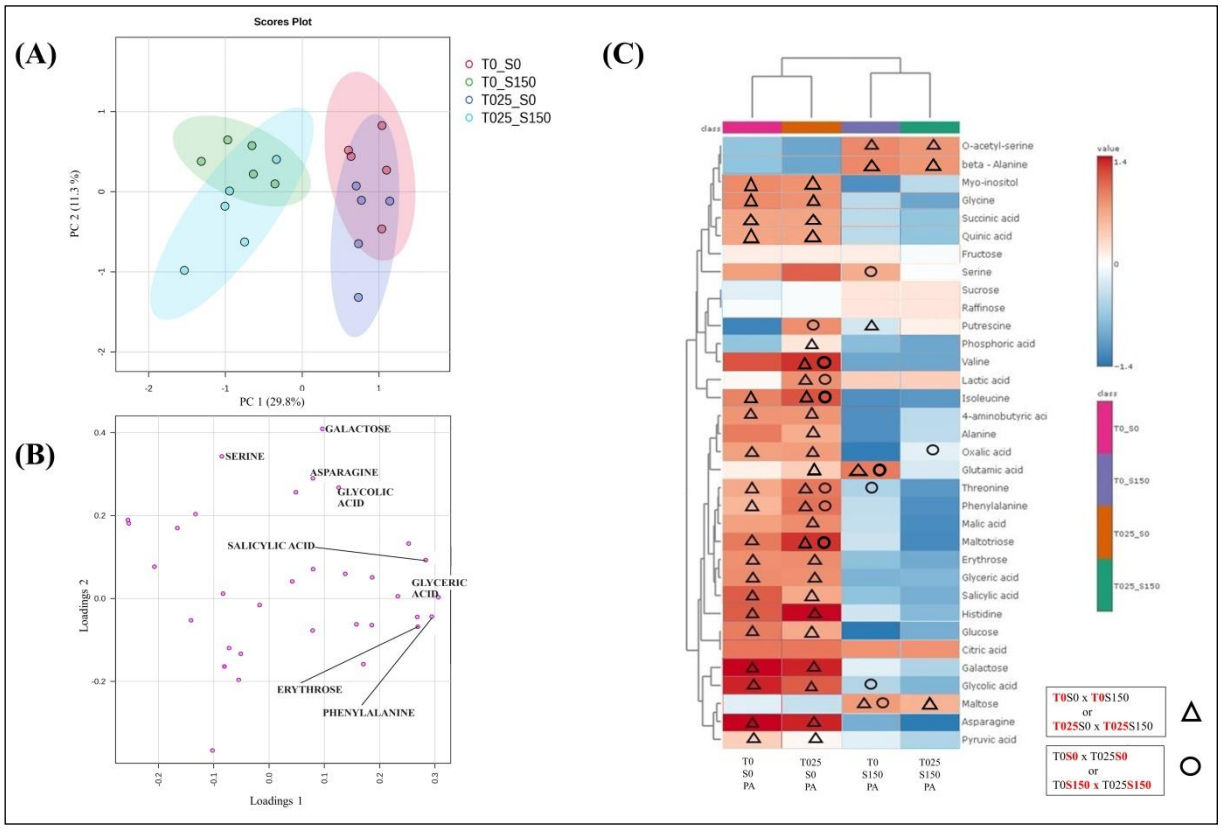

Figure 11 Multivariate analysis of metabolites found in shoots. Principal components (PC) score plot (A), PCA loading plot (B) and heatmap (C) of shoots of rice seedlings TM-primed and non-primed, grown in presence or absence of salinity. In the heatmap, triangle means statistical significance between treatments within the same TM concentration, whereas circle means significance between treatments with the same NaCl concentration based on Tukey test ($p \leq 0.05$) available on supplementary Table S7.

In general metabolites with lower abundance in control treatments (T0_S0 and T025_S0) became positively modulated by salt stress, and vice versa. Metabolites with statistical differences ($p <$

0.05) were identified among the treatments T0_S0, T025_S0, T0_S150 and T025_S150 (Supplementary table S7) and plotted in heatmap as circles and triangles. The treatment T025_S0 modulated positively eight compounds in relation to T0_S0 (see circles in T025_S0 column): serine (26 %), valine (25 %), isoleucine (26 %), threonine (24 %) and phenylalanine (25 %), maltotriose (31 %), lactic acid (67 %), and putrescine (66 %) (Figure 11C).

Comparing T0_S0 shoots to T0_S150 ones (see triangles in T0_S0 column, Figure 11C), 20 metabolites were significantly down-modulated: the amino acids glycine (57 % ↓), valine (29 % ↓), isoleucine (55 % ↓), 4-aminobutyric acid (42 % ↓), threonine (52 % ↓), phenylalanine (53 % ↓), histidine (37 % ↓), and asparagine (91 % ↓); the carbohydrates maltotriose (86 % ↓), myo-inositol (57 % ↓), erythrose (85 % ↓), glucose (27 %↓) and galactose (29 % ↓); the organic acids succinic acid (54 % ↓), oxalic acid (44 % ↓), glyceric acid (60 % ↓), glycolic acid (43 % ↓) and pyruvic acid (35 % ↓); and the secondary metabolites (others) quinic acid (41 % ↓), salicylic acid (77 % ↓, Figure 11C), while six were up-modulated by salinity (see triangles in T0_S150 column): the amino acids glutamic acid (48% ↑), o-acetylserine (38% ↑) and beta-alanine (36%); the sugar maltose (59% ↑), lactic acid (48% ↑) and putrescine (54% ↑).

Regarding T025_S150 shoots, only three metabolites were positively modulated regarding T025_S0 (see circles in T25_S150 column, Figure 11C): the amino acids O-acetylserine (44% ↑) and beta-alanine (43% ↑), and the sugar maltose (65% ↑). In relation to its saline counterpart (T0_S150, see circles in T025_S150 column), only one metabolite was significantly higher: the organic acid oxalic acid (31% ↑), whereas five had higher concentrations in T0_S150 (see circles in T0_150, Figure 11C): the amino acids glutamic acid (58% ↑), serine (71 % ↑) and threonine (59% ↑), maltose (33% ↑) and glycolic acid (18% ↑).

In roots, PC1 and PC2 accounted for 27.5% and 12.5%, respectively (Figure 12A). Similarly to shoots, the plot showed a complete separation of groups based on NaCl concentrations, particularly induced by PC1 component. Curiously, there were overlaps between treatments with the same TM concentrations mainly influenced by PC1 component. The top four positive contributions to PC1 in roots were citric acid, putrescine, o-acetylserine and glutamic acid, and to PC2 were glucose, phenylalanine, threonine, and putrescine (Figure 11B, Table S7). Heatmaps detailing positive or negative modulation of root metabolites were generated comprising both TM (0 and 0.25 µg. mL^{-1}) and NaCl (0 and 150 mM) concentrations, in a similar manner to the PCA.

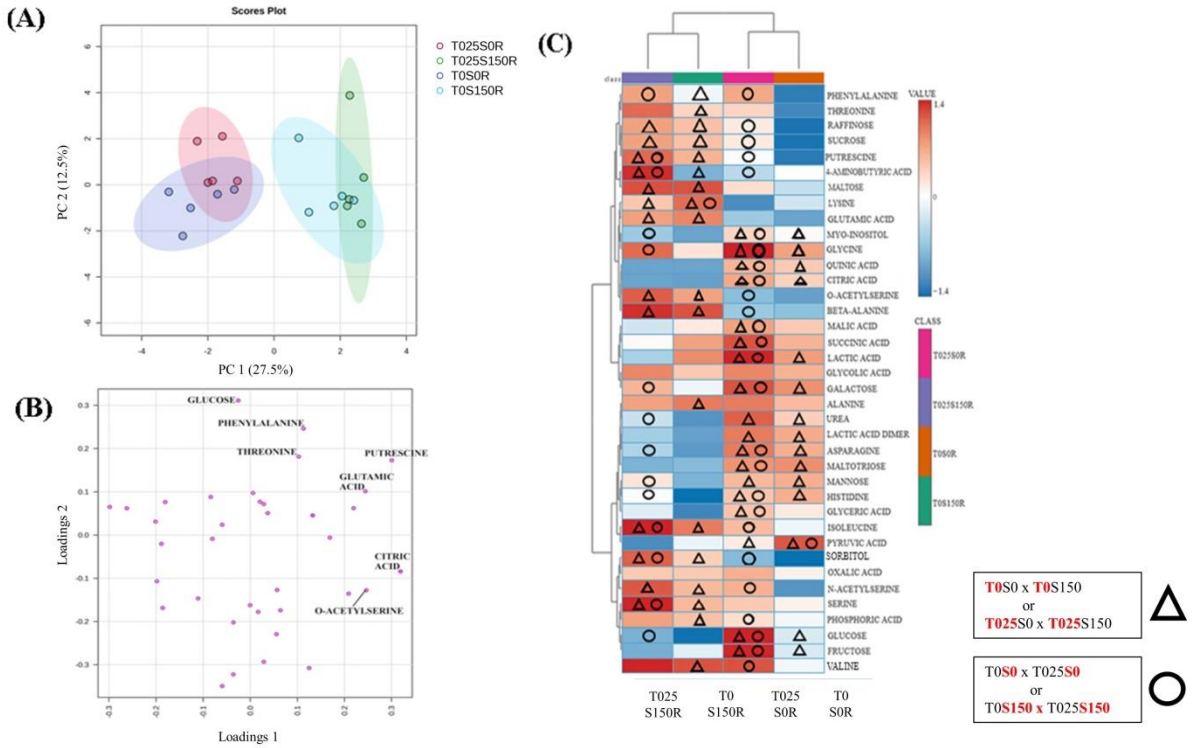

Figure 12 Multivariate analysis of metabolites found in roots. Principal components (PC) score plot (A), PCA loading plot (B) and heatmap (C) of roots of rice seedling TM-primed or not and submitted or not to salinity. In the heatmap, triangle means statistical significance between treatments within the same TM concentration, whereas circle means significance between treatments with the same NaCl concentration based on Tukey test ($p \leq 0.05$) available on supplementary Table S8.

Interestingly, in root statistical analysis (Supplementary Table S9), TM modulated positively more metabolites in both control and NaCl treatments: concerning 0 mM NaCl treatments, T025_S0 roots modulated positively 25 metabolites in comparison to its counterpart (T0_S0, see circles in T025_S0 column), being, among then, seven aminoacids: asparagine (32% ↑), beta-alanine (40% ↑), glycine (23% ↑), histidine (30% ↑), N-acetylserine (81% ↑), O-acetylserine (40% ↑), phenylalanine (71% ↑), 4-aminobutyric acid (75% ↑) and valine (61% ↑); six organic acids (citric acid (23% ↑), lactic acid (48% ↑), phosphoric acid (37% ↑), malic acid (27% ↑), glyceric acid (26% ↑) and succinic acid); seven sugars: glucose (26% ↑), galactose (24% ↑), fructose (24% ↑) maltotriose (35% ↑), raffinose (31% ↑), myo-inositol (25% ↑), sucrose (32% ↑) and sorbitol (75% ↑); and quinic acid (24% ↑) and putrescine (86% ↑) (Figure 12C).

Comparing T0_S0 roots to T0_S150 ones (see triangles in T0_S0 column), 15 metabolites were significantly down-modulated: the amino acids asparagine (66% ↓), glycine (56% ↓) and histidine (52% ↓); the organic acids citric acid (54% ↓), lactic acid (29% ↓), lactic acid dimer (81% ↓) and pyruvic acid (69% ↓); the sugars galactose (46% ↓), glucose (36% ↓) mannose (51% ↓), fructose (35% ↓) maltotriose (80% ↓) and myo-inositol (38% ↓); and secondary metabolites (others) quinic acid (54% ↓) and urea (75% ↓, figure 11C), whereas 18 were up-modulated by salinity (see triangles in T0_S150 column): the amino acids phenylalanine (51% ↑), threonine (58% ↑). 4-

aminobutyric acid (28% ↑), lysine (61% ↑), glutamic acid (84% ↑), o-acetylserine (80% ↑), beta-alanine (77% ↑), alanine (43% ↑), n-acetylserine (93% ↑), valine (60% ↑), isoleucine (46% ↑) and serine (34% ↑); the sugars raffinose (43% ↑), sucrose (40% ↑), maltose (48% ↑), sorbitol (85% ↑); phosphoric acid (66% ↑) and putrescine (94% ↑).

Concerning the heatmaps of T025_S0 roots to T025_S150 ones (see triangles in T25_S0 column), 18 metabolites were negatively modulated by salt stress: the amino acids asparagine (63% ↓), glycine (36 % ↓), and histidine (41%); the sugars fructose (43% ↓), galactose (37%), glucose (33% ↓), mannose (23%), maltotriose (88%) and myo-inositol (38%); the organic acids citric acid (58% ↓), glyceric acid (48% ↓), lactic acid (60% ↓), lactic acid dimer (87% ↓), pyruvic acid (48% ↓), malic acid (31% ↓) and succinic acid (40% ↓); and secondary metabolites (others) quinic acid (58% ↓) and urea (61% ↓), whereas 14 metabolites were positively modulated (see triangles in T025_S150 column): 4-aminobutyric acid (30% higher), lysine (64% ↑) beta-alanine (68% ↑), glutamic acid (75% ↑), N-acetylserine (67% ↑), O-acetylserine (70% ↑), isoleucine (41% ↑), serine (57% ↑), phosphoric acid (49% ↑), putrescine (70% ↑), and the sugars maltose (59% ↑), raffinose (27% ↑), sorbitol (67% ↑) and sucrose (30% ↑) (Figure 12C).

Finally, comparing both NaCl-stressed treatments, T025_S150 roots exhibited, in relation to T0_S150 (see circles in T025_S150 column), 14 positively modulated metabolites: the amino acids asparagine (36% ↑), glycine (24% ↑), histidine (43% ↑), isoleucine (23% ↑), serine (49% ↑) and phenylalanine (29% ↑); the sugars galactose (30% ↑), mannose (38% ↑), sorbitol (22% ↑), glucose (29% ↑) and myo-inositol (24% ↑); and the secondary metabolites (others) putrescine (25% ↑) and urea (36% ↑), whereas only one was higher in T0_S150: lysine, by 20% (see circles in T0_S150 column, Figure 12C).

Orthogonal partial least squares discriminant analyses (OPLS-DA) exhibited the positive and negative potential biomarkers of each TM level in the presence or of NaCl (0 mM or 150 mM. We explored the S-plot because it combines the contribution of correlation ranked from the highest to the lowest reliability values using the respective control treatment as a reference (Figure 13). Analyzing T0_S150 shoots compared to its reference (T0_S0), the top three discriminant metabolites were maltose, beta-alanine and O-acetylserine, whereas the most negative was maltotriose (Figure 12A). Concerning T025_S150 shoots in relation to its control, the top three positive metabolites were O-acetylserine, beta-alanine and maltose, whilst the most negative one was threonine (Figure 13B). Observing OPLS-DA S-plots from T0_S150 roots in comparison to its control, the top three most positive metabolites were putrescine, sorbitol and glutamic acid, whereas the most negative one was asparagine (Figure 13C). Analyzing T025_S150 roots to its control group, the top three most positive metabolites were glutamic acid, raffinose and O-acetylserine, whilst the most negative one was lactic acid dimer (figure 13D).

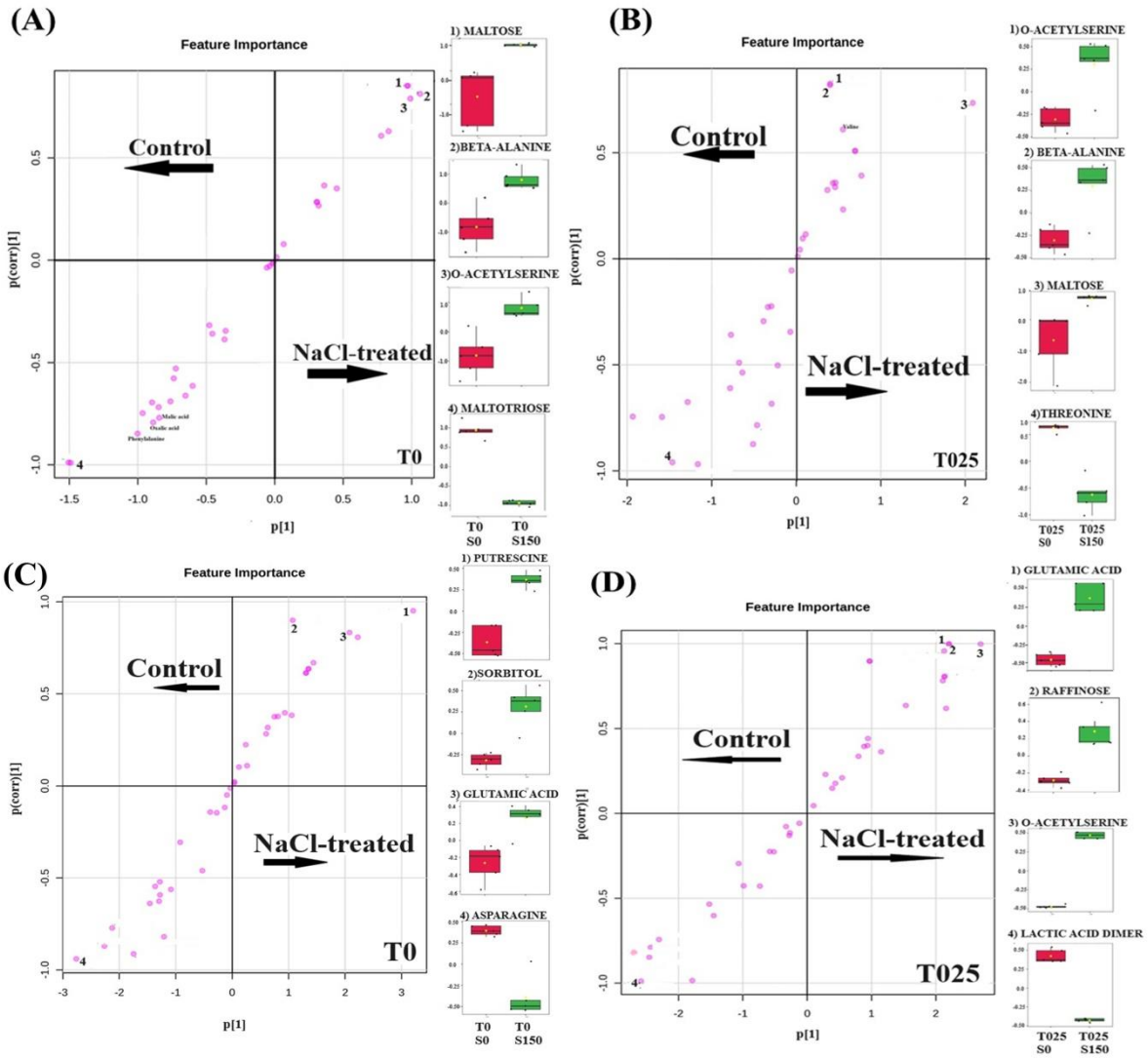

Figure 13. S-plots from orthogonal projections to latent structures discriminant analysis (OPLS-DA) shoots of rice seedlings with TM at 0 µg. mL^{-1} (A) at 0 mM NaCl (Control) or 150 mM NaCl (NaCl-treated), shoots with TM at 0.25 µg. mL^{-1} (B) at 0 mM NaCl (Control) or 150 mM NaCl (NaCl-treated), and roots with TM at 0 µg. mL^{-1} (C) at 0 mM NaCl (Control) or 150 mM NaCl (NaCl-treated), and TM at 0.25 µg. mL^{-1} (D) at 0 mM NaCl (Control) or 150 mM NaCl (NaCl-treated). The three most significant metabolites that had the major contribution to NaCl-treated (upper right corner) and Control seedlings (lower left corner) were highlighted, and their relative content were provided on the right of each plot of shoot rice seedlings.

6.4 Discussion

6.4.1 TM seed priming improves seed germination traits under salinity

To survive in a stressful environment, plants have to develop strategies and mechanisms to cope with environmental challenges, such as biotic factors (viruses and fungi) and abiotic ones (salinity); among some strategies, we can mention a more efficient antioxidant system and more

cellular energy (ATP) production (GEORGIEVA; VASSILEVA, 2023). For instance, both osmotic and ionic components triggered by salinity not only impair germination and physiological traits, but also several molecular processes, such as photosynthesis, water status, and ion balance (GANIE; MCULKIN; DEVOTO, 2024), but also generate secondary stresses, such as oxidative, ionic, and ER stresses, which can lead to inhibition of growth and crop yield (CHELE et al., 2021). Indeed, in this current work the increase of NaCl promoted a decrease in germination percentage, GSI, and SEI, as well as increased the GT, and MSET which compromised RDM, SDM, SK, and RL of seedlings after 10 DAT (Figure 1).

Particularly in seedling development, given that rice presents sensitivity to salinity in this stage (LIU et al., 2022), strategies that alleviate the deleterious effects of NaCl in plants are necessary (AYDEMIR et al., 2020). In this hand, seed priming exposes plants to a mild dose of stress, enabling them to better withstand more severe conditions (ASWATI; KALAJI; PUTHUR, 2022). This methodology not only minimizes salinity toxicity but also strengthens the defense system of crops. Here, the hydration level within the seeds is controlled by applying pre-sowing treatments, allowing specific pre-germinative metabolic processes to occur and preventing radical emergence (KHAN et al., 2022). It also helps in DNA replication responses, antioxidant function, more ATP availability, and encouraged protein biosynthesis, restoring cellular bio-membranes, and improving antioxidant enzymes (MANSOUR; ALI; SALAMA, 2019). Several studies corroborate our work with seed priming: Hidayah et al. (2022) evaluated priming rice seeds with a mixture containing 100 mM NaCl, and other salts ($CaCl_2$, and KCl) and verified that not only did the halopriming increase root and shoot dry masses, but also decreased Na^+ content in leaves and roots, and raised relative water content in primed seeds, which is in accordance with our work. Our results were corroborated by Liu et al.(2022b) as well, who also worked with rice seeds primed by salicylic acid (SA) and verified that AS increased GSI and germination potential by inducing SOD, CAT, and APX activities, therefore reducing ROS contents, and by increasing gibberellin production.

It is known that salt stress induces an overaccumulation of unfolded proteins, which leads to endoplasmic reticulum (ER) stress (PARK; PARK, 2019; REYES-IMPELLIZZERI; MORENO, 2021). Since more than one-third of plant cell proteins are synthesized and folded in this organelle, it is vital that the repair and degradation mechanisms should work well, as several N-glycosylated proteins are produced there, and most of them are enzymes, which might be associated with acclimation and tolerance (STRASSER et al., 2021). Furthermore, there is an upregulation of UPR and ER quality control genes during priming, which is maintained after recovery allowing salt-primed plants to exhibit better growth in response to future salt exposure (TIAN et al., 2019). Thus, we wondered if TM, an ER stress inductor could act as a priming to induce salt acclimation in rice.

Exogenous TM causes ER stress guiding to an ER response (MANGHWAR; LI, 2022; ASTANI et al., 2022). Indeed, this response is crucial to cell survival since ER stress increase with the time of exposition and promote a decreasing of specific genes, impairment of metabolic pathways, and

seedling growth (LIMA et al., 2022). However, lower concentrations of TM (0.5 ug.L-1) promote biomass maintenance, seedling length associated to high ER genes expression (CAVALCANTE et al., 2023). Here we noticed that seed TM priming was able to keep germination traits and seedlings growth under salt stress (Figure 1). Furthermore, these results were corroborated when we tested different doses of TM, in which 0.25 ug.L^{-1} provided best results (Figure 2). Thus, to explore how TM seed priming can prepare seedlings for severe salt conditions we set a new experimental strategy using a combination of priming and unprimed seeds in absence and presence of NaCl.

6.4.2 TM seed priming induce ER responses to mitigates negative effects of salinity in seedlings

Besides those seed germination indexes and growth parameters previously discussed (Figures 1-3), salinity severely impacted osmotic potential, chlorophyll and carotenoid contents as well as increased cell damage by electrolyte leakage (Figures 4 and 5). However, TM seed priming augmented seedling growth efficiently. It promoted the maintenance of all physiological and biochemical parameters, which is mainly due to decreased Na^+ contents in both shoots and roots promoted by TM (Figure 6), which led to less production of ROS, and allowed the increase in these parameters. Such results are in accordance with previous studies of rice seedlings treated with 0.75% (w/v) KNO_3, under 150 mM NaCl, in which seed priming not only reduced electrolyte leakage, but also increased total chlorophyll content (THEERAKULPISUT; KANAWAPEE; PANWONG, 2016). In another work, salt priming in rice seeds not only decreased electrolyte leakage and damage to the photosynthetic apparatus, but also promoted higher root growth, and ABA levels, therefore protecting against drought stress (ROSSATTO et al., 2023).

TM-priming not only increased total chlorophyll content, but also augmented carotenoid content, which might have led to more efficient photosynthetic processes and, consequently, higher initial growth, biomasses and lengths of TM-conditioned seedlings under salt stress. Certainly, photosynthesis is a vital process to plants, and most chloroplast-located proteins are produced in the ER, and then transported to chloroplasts via secretory pathway, such as carbonic anhydrase (JIAO et al., 2020). Since salinity strongly impairs photosynthesis, it is interesting that TM conditioning alleviates this condition by inducing ER and downstream responses. Although other ER genes remain to be evaluated, the downstream responses were observed here, which included the increased expression of *OsIRE1 and OsbZIP50*, a well-known branch of UPR, and the expression of *OsbZIP50* (*Atb*ZIP60 ortholog) the second branch of UPR (Figure 10). These results are in agreement with the literature, since UPR and endoplasmic reticulum-associated quality control was related to improved plant salt tolerance by seed priming and a possible mechanism of memory in plants (TIAN et al., 2019).

Salt stress leads to impairment in water balance and the quick absorption of Na^+ ions causes negative plant-water relations (BALASUBRAMANIAM et al., 2023). Sodium chloride molecules tend to dissociate into Na^+ and Cl^- ions in soil or germination/hydroponic solutions, being

quickly absorbed and accumulated in cells, and, therefore, inhibiting the uptake of other ions, such as K^+ and Ca^{2+} (ASSAHA et al., 2017; CABOT et al., 2014), which results in lower K^+/Na^+ ratios. TM-pretreatment increased this ratio in both shoots and roots in the presence of NaCl, which means it might have increased K^+ absorption and reduced Na^+ uptake (Figure 6). It has been suggested that plant survival under salt stress requires a high cytosolic K^+/Na^+ ratio in the cytoplasm (ALMEIDA; OLIVEIRA; SAIBO, 2017) , as well as the restriction of Na^+ accumulation in shoots under salt stress has been correlated with salt tolerance in rice (ALMEIDA; OLIVEIRA; SAIBO, 2017; LUTTS; KINET;BOUHARMONT, 1996).

Although chloride is considered an essential micronutrient for plants, being involved in photosynthesis and stomatal regulation (FRANCO-NAVARRO et al., 2016; SHELKE et al., 2019), in high concentrations it can lead to toxicity, bringing about several harms to the cell, such as increased ROS production, chlorophyll degradation, and reduction of chlorophyll synthesis and of actual quantum yield of PSII electron transport (SHELKE et al., 2019; TAVAKKOLI et al., 2012) . Thus, the less Cl^- available/free, the better. TM-pre-treatment might have induced Cl^- compartmentation in root vacuoles, or probably contributed to Cl^- exclusion from mesophyll cells (SHELKE et al., 2019). These results are in accordance with exogenous application of NO in rice plants under salt stress, where the authors found that NO priming decreased both root and shoot Cl^- and Na^+ contents, therefore increasing water potential (HABIB; ASHRAF, 2014). It was corroborated by confocal microscopy, which showed that T025 S150 seedlings exhibited lower cytosolic intensities of fluorescence NaTRIUM Green-2AM probe in both roots and shoots, suggesting that TM-priming helped reduce the content of this ion, which is in agreement to previous results that indicate an relation among intensity of fluorescence and sodium uptake in rice (GADELHA et al., 2021). Additionally, proteins SOS act in the mechanism of sodium exclusion, particularly in root (ALI et al., 2023), here the expression of *OsSOS1* was increased in both salt treatments which may contribute to minimizing the accumulation of sodium in roots (Supplementary Table S5) corroborating the results described above.

6.4.3 ER induction improves antioxidant system and osmoprotectants

Salinity tends to increase the production of reactive oxygen species (ROS), such as $\cdot O_2^-$ and H_2O_2, which characterizes oxidative stress (ROSSATTO et al., 2017). To cope with that extra toxicity, an efficient antioxidant system is necessary to be present or activated, which is composed of antioxidant enzymes such as SOD, CAT, APX and GPOD (Figure 8 and Figure 9), and non-enzymatic low molecular weight compounds, such as beta-alanine and maltose (Figures 10C and 11C). SOD can turn $\cdot O_2^-$,an extremely reactive ROS, into a less reactive one, H_2O_2, which will be used as substrate by CAT and GPOD, breaking it down into the non-toxic molecules H_2O and O_2 (XU et al., 2010). Not only did these enzymes have their activities increased in both shoots and roots, induced by TM, but also the ROS levels, electrolyte leakage and the lipid peroxidation decreased, which match well with the elevated growth parameters, in relation to T0S150 seedlings, revealing that TM induces an

antioxidant enzyme system that efficiently scavenges ROS, alleviating the deleterious effects of salt stress and therefore, leading to higher biomass and length. Indeed, the induction of ER response was able to reduce the negative effects of salinity decreasing ROS by enzyme activities (QUEIROZ et al., 2020). The relation among ROS and ER response have been reported, in which ER stress activates the antioxidant system to reduce ROS, and ROS activates ER response in a dynamic duo (OZGUR et al., 2018).

Our results are also in accordance with Ali et al. (2021), who tested 3 different priming agents (KNO_3, SiO_2 and SA) in rice seeds under drought conditions: mild (75% field capacity), moderate (50% FC) and severe, 25% FC, and verified an increase in SOD, CAT and APX activities under all those conditions. Swain & Rout (2020) reported slightly different results while working with SiO_2 priming in rice seeds under 100 mM NaCl: aside from CAT, GPOD, APX and SOD had their activities increased in the presence of NaCl, which lead to a lower lipid peroxidation. This was due to an efficient antioxidant enzyme system, which helped reduce ROS levels and led to increased chlorophyll content. Such results can be corroborated by Figures 4, 5, 6 and 7, and also for H_2O_2 leaf sprayed maize in the presence of NaCl, that increase in photosynthetic rate and in chlorophyll content in the primed plants (GONDIM et al., 2013).

Furthermore, TM seed priming can prepare seedlings for severe conditions, such as high salinity, by especially activating an efficient antioxidant system coupled to an increase of osmoprotectants (Figures 11 and 12, and Supplementary tables S6 and S7). For instance o-acetylserine, the direct precursor of cysteine, which generates, besides proteins, biotin, ethylene, polyamine, and GSH, which can directly or indirectly regulate the salinity stress response (VAN NGUYEN et al., 2021), beta-alanine (PARTHASARATHY; SAVKA; HUDSON, 2019) and maltose, which not only works as an osmoprotectant but also generates cellular energy (IBRAHIM; ABDELLATIF, 2016) , which could help to explain higher germination percentages, germination speed index and lower mean germination time, as well as greater lengths and dry masses. Such results are corroborated by the exogenous application of nitric oxide in rice seeds under 150 mM (TAHJIB-UL-ARIF ET AL., 2022), and primed rice seeds with H_2O_2 and submitted seedlings with 100 and 150 mM NaCl (HEMALATHA; RENUGADEVI; EEVERA, 2017).

Therefore, it is fundamental that the plant develop an efficient osmoprotective system. Not only did TM-pre-treaded seedlings displayed lower electrolyte leakage in shoots, but also showed higher osmotic potential in relation to non-TM-pretreated under salinity. One possible explanation for that is TM-pre-treatment might have increased the productions of osmoprotectants, such as beta-alanine and maltose (Figure 12C), as well as in roots the positive modulation of 4-aminobutyric acid (also known as GABA, an important molecule in plant defense to salt stress), according to Guo et al.(2023), besides the increased activities of antioxidant enzymes (Figure 9).

6.4.4 TM seed priming modulates metabolites that may contribute to salt acclimation

In an interesting manner, in both shoots and roots, salinity significantly decreased the contents of organic acids; but TM-priming, under saline conditions not only did it positively modulate amino acids and sugars, such as o-acetyserine, 4-aminobutyric acid and maltose, but also increased these contents in relation to unprimed seedlings (see circles in T025_S150 columns, in figures 11C and 12C). This decrease in organic acids might be possibly due to a shift in other metabolites, for many cellular respiration intermediates, such as pyruvic acid and succinic acid. Most amino acids might be involved in the replenishment of TCA cycle or glycolisis intermediates (glutamic acid generates α-ketoglutarate, isoleucine might generate either acetyl-CoA or succinyl-CoA), as these are being consumed for ATP synthesis (CHANDEL, 2021). Moreover, a higher content of sugars, such as sucrose, raffinose, and maltose might also be used to replenish TCA intermediates, generating more energy and leading to increased root length and lower electrolyte leakage and Na^+ accumulation.

For shoots metabolites, o-acetylserine, b-alanine, and maltose were considered key metabolites for salt response, independently of TM seed priming (Figure 13). O-acetylserine is involved in not only cysteine biosynthesis, but also ethylene, polyamines, and glutathione, important molecules involved in salt tolerance (VAN NGUYEN et al., 2021). Beta-alanine, an important osmoprotectants, and also involved in coenzyme A biosynthesis, is crucial for tricarboxylic acid cycle and fatty acid metabolisms (PARTHASARATHY; SAVKA; HUDSON, 2019); moreover, it was observed a higher content of oxalic acid (Figure 13C), an important signaling molecule in photosynthesis (LI et al., 2022). These results might have contributed to higher photosynthetic pigment contents and higher plant growth (Figure 3). Similar results were found by Gupta & De (2017) who verified that salt-tolerant rice genotypes exhibited positive modulation of beta-alanine and maltose under 150 mM NaCl. Singh et al. (2022) also reported that osmolyte accumulation (such as beta-alanine and proline) with a crosstalk with phytohormones (e.g. ethylene and ABA) led to NaCl resilience .

Concerning root metabolites, glutamic acid, raffinose and o-acetyl serine were considered key metabolites in TM-induced plants, which led to an alleviation of NaCl toxicity (Figure 13). Moreover, TM increased the modulation of other salt-responsive metabolites in roots, such as putrescine, 4-aminobutyric acid, lysine, sucrose and maltose (Figure 13C), besides increasing metabolites whose contents had been reduced by salinity, especially the amino acids phenylalanine and asparagine, and the sugars galactose, glucose and myo-inositol (Figure 12C, circles in T025_S150 columns). Glutamic acid, or its anionic form, glutamate (Glu), is a unique primary metabolite in plants: in addition to its role as an amino acid for protein synthesis, Glu is a central metabolic hub connecting carbon (C), and nitrogen (N) metabolism, also serving as an N-donor for the biosynthesis of amino acids and other N-containing compounds. Most organic N compounds synthesized in the cell either directly or indirectly receive their N from Glu (LIAO; CHUNG; HSIEH, 2022). On top of amino acid and N metabolism, Glu is a building block for essential biomolecules such as glutathione (GSH) and the cofactor folate

(LIAO; CHUNG; HSIEH, 2022). So, glutamate might have acted as carbon-donor to the TCA cycle, as some intermediates were down-modulated by salinity.

Raffinose is a trisaccharide comprised by galactose, glucose and fructose that is ubiquitous in plants and has been demonstrated to play important roles in seed desiccation tolerance/seed storability; its synthesis is catalyzed by the enzymes galactol-synthase, which adds myo-inositol to UDP-galactose, thus generating galactinol, and raffinose-synthase, which incorporates galactose units from galactinol to sucrose, producing raffinose (SENGUPTA et al., 2015) Under salt/osmotic stress, genes related to its biosynthesis, such as *AtGolS* and *AtRS* are overexpressed, which highlights the importance of this sugar to cope with NaCl (YAN et al., 2022). Here, salinity decreased galactose and myo-inositol contents, but TM significantly increased them, which might have contributed to higher raffinose contents, and therefore, more energy to deal with Na toxicity. Wanichthanarak et al.(2020), while assessing rice metabolic flux under salt stress, also reported a positive modulation of this metabolite, evidencing its role in salt tolerance.

In addition to sugars, sorbitol is a sugar alcohol produced from glucose-6-phosphate or glucose and is usually produced in leaves and transported to other tissues, such as root, via phloem (ZHANG et al., 2014). In roots, this carbohydrate has many functions: it can be metabolized to either fructose (by NAD^+-dependent sorbitol dehydrogenase) or glucose, by enzyme sorbitol oxidase (KIM et al., 2015). It has been reported that, in salt stress, sorbitol is usually accumulated, working as an osmoprotectant, playing role in the osmotic adjustment [109]. In this study, sorbitol might have replenished either glucose or fructose pools to keep glycolysis functional, besides acting as an osmoprotectant, which might have led to a higher osmotic potential, and provided energy (ATP), therefore alleviating Na^+ toxicity. Our results are in accordance with Haddadi et al.(2023), who worked with19 varieties of tef (*Eragrostis tef*), a member of Poaceae family, under 100 and 250 mM NaCl, verified that it was observed a salt-induced positive modulation of sorbitol in most varieties.

Putrescine (Put) is a polyamine (PA) derived from the amino acids arginine or ornithine, being the central product of the PA biosynthetic pathway and the most abundant PA in nature (GONZÁLEZ-HERNÁNDEZ ET AL., 2022). It is known that Put is a salt responsive metabolite, which might led to NaCl acclimation: Ghalati, Shamili & Homaei (2020) verified that Put alleviated stress not only by inducing thylakoid membrane lipid peroxidation by increasing unsaturated fatty acid content but also by upregulating ATPase, thus facilitating the Na^+ efflux. So, in this work, Put might have contributed to Na^+ efflux, which induced higher germination parameters. In addition to the classic role of synthesis of intracellular proteins, in case of energy deficiency, lysine (Lys) and isoleucine (Ile) catabolism flux directly into the tricarboxylic acid (TCA) cycle, when high expression of genes related to the biosynthesis of these amino acids are linked to high TCA intermediates (YANG; ZHAO; LIU, 2020). So, in this scenario, Lys and Ile might have contributed to replenishing TCA intermediates to keep energy production high, to cope with salt stress. These results are in accordance with Rajkumari et al .(2023), who also noticed that the concentration of these amino acids and putrescine increased in

salt-tolerant rice genotypes, which matches with TM-primed metabolites.

It is also worth to point out that, although TM-priming does seem like a promising approach for salt acclimation in plants, it presents some limitations: TM is usually a costly antibiotic, especially when purchased in small quantities (SIGMA-ALDRICH, 2019), and studies have shown that TM inhibits the first step of bacterial cell wall teichoic acid biosynthesis, but is also toxic to mammalian cells (PRICE et al., 2017). Therefore, analogs with lower price and toxicity are required. Price et al. (2019) reported that TM analogs TunR1 and TunR2 significantly reduced TM toxicity in eukariotic cells such as insect larvae cells, not allowing these compounds to perform a binding interaction between the urydil group and the Phe249 residue of PNPT (polyribonucleotide nucleotidyltransferase 1), while maintaing TUN relative antibiotic activity. TunR1 and TunR2 are TM analogs derived from TM catalytic hydrogenation: while TunR1 presents hydrogenation in the fatty acid region, TunR2, in addition to the saturated fatty acid chain of TunR1, has a reduced double bond in the uracyl group (HERING et al., 2020). Therefore, these coumpounds might act as substitutes for future TM-primings.

6.5 Conclusion

Overall, TM priming alleviated the deleterious effects of salt stress in 10 DAT rice seedlings which induced higher biomasses and physiological parameters but also might have helped pump Na^+ out of the cell. It activates unfolded protein response by the expression of *OsIRE1 and OsbZIP50* that may contribute to an efficient antioxidant system and the positive modulation of primary metabolites (e.g maltose, glutamic acid, o-acetylserine and lysine) involved in energy synthesis or production of intermediates of TCA cycle (such as malic acid and succinic acid). Besides, the unprecedented TM priming reveals to be a promising approach for salt stress acclimation. However, more studies are necessary for a better comprehension of the theme and its relation to ER response, especially with TM analogs in order to reduce TM toxicity and biomagnification in the enrivonment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions statement

FDBO carried out all the research, data acquisition and wrote the manuscript. FLPC and IRSC helped in metabolomics analysis; IMCP and IACC helped in confocal experiments, MSA and EG-F revised the manuscript, HHC designed the research concept, data acquisition and wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

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6.6 SUPPLEMENTARY DATA

Figure S1 Seedlings phenotype

Supplementary table S1: NaCl Screening I – 0 µg. mL TM

		Mean square									
Source of Variation	D.F.	PG**	ABS*	GSI**	MGT *	SESI**	MSET**	RDM**	SDM**	SL**	RL**
Treatment	4	1115.20	548.30	193.70	13.99	849.48	16.27	943.56	2381.35	36.55	28.09
Error	15	15.20	25.66	1.37	0.20	1.92	0.21	5.09	7.16	0.02	0.02
C.V. %		5.23	10.28	11.38	8.76	6.70	8.74	9.71	9.97	5.37	4.50

* Significant with $p < 0.05$; ** significant with $p < 0.01$; non-significant (ns) with $p > 0.05$.

PG: percentage of germination; ABS: Abaffy, Broyden and Spedicato algorithm; GSI: germination speed index; MGT: mean germination time; SESI: shoot elongation speed index; MSET: mean shoot elongation time; RDM: root dry mass; SDM: shoot dry mass; SL: shoot length; RL: root length.

Supplementary table S2: NaCl Screening II – 0.50 µg. mL TM

		Mean square									
Source of Variation	D.F.	PG**	ABS**	GSI**	MGT*	SESI^{ns}	MSET**	RDM*	SDM**	SL**	RL**
Treatment	4	376.70	483.80	174.60	15.17	718.66	13.67	923.69	1799.42	30.85	21.19
Error	15	15.33	17.33	0.75	0.15	1.65	0.15	9.41	7.99	0.02	0.02
C.V. %		4.63	8.13	7.69	7.64	5.62	7.92	9.35	7.58	5.26	3.84

* Significant with $p < 0.05$; ** significant with $p < 0.01$; non-significant (ns) with $p > 0.05$.

PG: percentage of germination; ABS: Abaffy, Broyden and Spedicato algorithm; GSI: germination speed index; MGT: mean germination time; SESI: shoot elongation speed index; MSET: mean shoot elongation time; RDM: root dry mass; SDM: shoot dry mass; SL: shoot length; RL: root length.

Supplementary table S3: TM Screening – 150 mM NaCl

		Mean square									
Source of Variation	D.F.	PG**	ABS^{ns}	GSI*	MGT*	SESI^{ns}	MSET^{ns}	RDM*	SDM*	SL^{ns}	RL^{ns}
Treatment	4	53.80	82.7	4.68	0.65	24.82	1.30	61.00	115.44	3.99	0.68
Error	15	5.53	26.2	2.18	0.03	2.15	0.07	2.92	9.18	0.03	0.04
C.V. %		2.66	11.75	6.30	4.95	10.24	4.61	18.33	23.06	11.15	15.14

* Significant with $p < 0.05$; ** significant with $p < 0.01$; non-significant (ns) with $p > 0.05$.

PG: percentage of germination; ABS: Abaffy, Broyden and Spedicato algorithm; GSI: germination speed index; MGT: mean germination time; SESI: shoot elongation speed index; MSET: mean shoot elongation time; RDM: root dry mass; SDM: shoot dry mass; SL: shoot length; RL: root length.

Supplementary table S4: Loading scores of metabolites found shoots and roots ranked by principal component 1 and 2 values.

Shoots				Roots			
Metabolite	**PC1**	**Metabolite**	**PC2**	**Metabolite**	**PC1**	**Metabolite**	**PC2**
Glyceric acid	0.31	Galactose	0.41	Putrescine	0.35	Threonine	0.26
Phenylalanine	0.29	Serine	0.34	Glutamic_acid	0.25	Glucose	0.22
Salicylic acid	0.28	Asparagine	0.29	O_acetylserine	0.25	Phenylalanine	0.21
Erythrose	0.27	Glycolic acid	0.27	Citric_acid	0.23	Putrescine	0.18
Threonine	0.27	Maltose	0.26	Quinic_acid	0.23	N_acetyl_serine	0.15
Maltotriose	0.25	Myo-inositol	0.20	beta_alanine	0.21	Asparagine	0.12
γ-Aminobutyric ac	0.23	O-acetyl-serine	0.19	Glycine	0.18	Maltotriose	0.09
Malic acid	0.19	beta - Alanine	0.18	Raffinose	0.16	Lactic_acid_dimer	0.09
Oxalic acid	0.19	Succinic acid	0.17	Sucrose	0.16	γ-Aminobutyric ac	0.08
Glutamic acid	0.17	Maltotriose	0.13	Threonine	0.15	Valine	0.08
Alanine	0.16	Salicylic acid	0.09	myo_inositol	0.14	Glutamic_acid	0.07
Glucose	0.14	Glycine	0.08	Lysine	0.11	Glyceric_acid	0.06
Glycolic acid	0.13	Histidine	0.07	Phenylalanine	0.10	Sucrose	0.05
Galactose	0.10	Glucose	0.06	Galactose	0.08	Raffinose	0.05
Histidine	0.08	Malic acid	0.05	Succinic_acid	0.06	Fructose	0.05
Asparagine	0.08	Pyruvic acid	0.04	N_acetyl_serine	0.06	Urea	0.05
Citric acid	0.08	Fructose	0.01	Malic_Acid	0.05	Glycolic_acid	0.03
Maltose	0.05	γ-Aminobutyric ac	0.01	Glycolic_acid	0.04	Phosphoric_acid	0.02
Pyruvic acid	0.04	Glyceric acid	0.00	Valine	0.03	Histidine	0.01
Lactic acid	-0.02	Lactic acid	-0.02	Maltose	0.02	myo_inositol	-0.01
Phosphoric acid	-0.05	Phenylalanine	-0.04	Lactic_acid	0.02	Glycine	-0.01
Putrescine	-0.05	Threonine	-0.04	γ-Aminobutyric ac	0.01	Citric_acid	-0.04
Quinic acid	-0.07	Isoleucine	-0.05	Sorbitol	0.00	Quinic_acid	-0.04
Sucrose	-0.08	Alanine	-0.06	Glucose	-0.02	Isoleucine	-0.07
Raffinose	-0.08	Oxalic acid	-0.06	Serine	-0.03	beta_alanine	-0.07
Fructose	-0.08	Erythrose	-0.07	Mannose	-0.05	Pyruvic_acid	-0.08
Serine	-0.09	Citric acid	-0.08	Alanine	-0.07	O_acetylserine	-0.09
Valine	-0.10	Quinic acid	-0.12	Glyceric_acid	-0.08	Malic_Acid	-0.14
Myo-inositol	-0.13	Phosphoric acid	-0.13	Histidine	-0.08	Lactic_acid	-0.15
Isoleucine	-0.14	Glutamic acid	-0.16	Fructose	-0.09	Serine	-0.19
Succinic acid	-0.17	Sucrose	-0.16	Isoleucine	-0.14	Oxalic_acid	-0.21
Glycine	-0.21	Raffinose	-0.16	Oxalic_acid	-0.14	Mannose	-0.22
beta - Alanine	-0.25	Putrescine	-0.20	Urea	-0.17	Alanine	-0.23
O-acetyl-serine	-0.26	Valine	-0.37	Pyruvic_acid	-0.21	Galactose	-0.24
				Phosphoric_acid	-0.23	Maltose	-0.27
				Lactica_acid_dimer	-0.25	Lysine	-0.28
				Maltotriose	-0.29	Succinic_acid	-0.31
				Asparagine	-0.30	Sorbitol	-0.41

Supplementary table S5: Shoot metabolites statistics

Different uppercase letters indicate statistical difference between pre-treatment in the same TM concentration, and lowercase letters indicate statistical difference among same NaCl concentration and the same pre-treatment using Tukey test ($p \leq 0.05$).

	T0S0			T025S0			T0S150			T025S150		
Amino acids	**mean**	**SD**	**T**	**mean**	**SD**	**T**	**mean**	**SD**	**T**	**mean**	**SD**	**T**
Alanine	4,218	0,723	Aa	4,751	1,054	Aa	1,565	0,383	ABa	2,165	0,356	Ba
Beta-Alanine	0,559	0,111	Ba	0,512	0,057	Ba	0,902	0,087	Aa	0,900	0,188	Aa
Asparagine	29,021	3,603	Aa	33,577	4,456	Aa	2,507	0,762	Ba	0,937	0,566	Ba
Glycine	11,990	1,034	Aa	13,243	0,636	Aa	5,134	0,154	Ba	5,643	1,199	Ba
Histidine	0,219	0,018	Aa	0,238	0,011	Aa	0,138	0,006	Ba	0,122	0,027	Ba
Isoleucine	13,337	1,943	Ab	18,009	1,305	Aa	5,994	0,378	Ba	3,217	1,220	Ba
Phenylalanine	0,631	0,168	Ab	0,839	0,091	Aa	0,299	0,040	Ba	0,178	0,041	Ba
Glutamic acid	5,380	1,286	Ba	6,387	0,595	Aa	10,373	1,113	Aa	4,373	1,167	Bb
Serine	9,594	1,975	Ab	12,156	1,384	Aa	8,799	1,260	ABa	2,545	1,536	Bb
O-acetyl-serine	0,559	0,111	Ba	0,508	0,051	Ba	0,902	0,087	Aa	0,900	0,188	Aa
Threonine	3,927	0,737	Ab	5,137	0,497	Aa	1,908	0,210	Ba	0,781	0,358	Bb
Valine	19,129	3,695	Ab	25,382	1,767	Aa	13,664	0,880	Ba	8,748	2,534	Ba
γ-Aminobutyric ac	52,494	5,006	Aa	51,939	3,191	Aa	30,823	1,311	Ba	33,469	6,648	Ba
Carbohydrates												
Erythrose	2,584	0,349	Aa	2,634	0,114	Aa	0,392	0,031	Ba	0,498	0,105	Ba
Fructose	140,207	40,748	Aa	164,346	13,226	Aa	156,198	4,978	Aa	147,880	29,693	Aa
Galactose	99,078	7,260	Aa	102,272	4,684	Aa	79,886	2,989	Ba	71,680	16,081	Ba
Glucose	55,974	4,186	Aa	58,630	2,897	Aa	51,206	1,637	Ba	44,970	9,461	Ba
Myo-inositol	10,660	0,986	Aa	10,441	0,573	Aa	4,642	0,119	Ba	4,684	0,945	Ba
Maltose	42,262	4,890	Ba	34,554	1,035	Ba	103,464	8,178	Aa	69,187	13,271	Ab
Maltotriose	6,821	1,351	Ab	9,832	1,508	Aa	0,997	0,063	Ba	0,740	0,190	Ba
Raffinose	136,630	2,181	Aa	144,471	7,590	Aa	174,304	5,503	Aa	148,934	34,105	Aa
Sucrose	136,692	2,106	Aa	144,471	7,590	Aa	174,304	5,503	Aa	148,934	34,105	Aa
Organic acids												
Citric acid	7,89084	0,66	Aa	7,79882	3,208	Aa	7,8641	0,1532	Aa	7,03412	0,848	Aa
Glyceric acid	2,116	0,2489	Aa	2,08318	0,26	Aa	0,858	0,0287	Ba	0,9144	0,199	Ba
Glycolic acid	0,10957	0,0169	Aa	0,08225	0,01	Aa	0,0627	0,0036	Ba	0,0527	0,007	Bb
Lactic acid	2,38115	0,6596	Bb	7,1731	1,217	Aa	4,6181	1,0099	Aa	4,16938	0,879	Ba
Malic acid	7,64787	0,8964	Aa	7,8284	0,88	Aa	6,0948	0,3402	Aa	5,3847	1,204	Ba
Oxalic acid	6,47107	0,6692	Aa	6,48408	0,373	Aa	3,0886	1,1105	Bb	4,88615	2,042	Ba
Phosphoric acid	146,448	17,474	Aa	151,591	12,58	Aa	140,6	7,1065	Aa	111,536	19,82	Ba
Pyruvic acid	0,25332	0,048	Aa	0,24704	0,041	Aa	0,1643	0,0179	Ba	0,1433	0,034	Ba
Succinic acid	13,5529	1,2106	Aa	12,9936	0,494	Aa	6,1857	0,221	Ba	5,83469	1,245	Ba
Others												
Quinic acid	20,4224	2,0251	Aa	19,784	0,541	Aa	12,081	0,6995	Ba	11,2361	2,558	Ba
Salicylic acid	3,25778	0,4655	Aa	3,13677	0,158	Aa	0,7578	0,0412	Ba	0,6843	0,157	Ba
Putrescine	0,39419	0,0783	Bb	1,15541	0,114	Aa	0,8681	0,3654	Aa	1,04939	0,148	Aa

Supplementary table S6: Root metabolites statistics

Different uppercase letters indicate statistical difference between pre-treatment in the same TM concentration, and lowercase letters indicate statistical difference among same NaCl concentration and the same pre-treatment using Tukey test ($p \leq 0.05$).

	T0S0			T025S0			T0S150			T025S150		
Amino acids	**mean**	**SD**	**T**	**mean**	**SD**	**T**	**mean**	**SD**	**T**	**mean**	**SD**	**T**
Alanine	4,339	2,229	Ba	3,016	0,384	Aa	2,448	0,732	Aa	3,885	0,683	Ab
Valine	9,162	2,807	Bb	23,453	5,100	Aa	22,814	5,397	Aab	25,527	4,213	Aa
Isoleucine	5,785	2,532	Bb	15,691	4,548	Ba	12,544	3,524	Ab	16,263	2,503	Aa
Glycine	8,262	0,515	Ab	12,758	0,447	Aa	6,868	1,427	Ab	9,077	0,768	Ba
Serine	3,226	1,657	Ba	4,103	2,852	Ba	4,916	2,339	Ab	9,711	2,802	Aa
Threonine	1,005	0,528	Bb	2,410	0,823	Aa	2,368	0,845	Aab	2,926	1,025	Aab
O-acetylserine	0,764	0,093	Bb	1,279	0,082	Ba	3,914	0,902	Aa	4,239	0,270	Aa
Beta-alanine	0,764	0,093	Bb	1,279	0,082	Ba	3,919	0,902	Aa	4,239	0,270	Aa
4-Aminobutyric ac	28,599	3,623	Bb	41,581	2,226	Ba	39,811	8,679	Ab	58,582	11,792	Aa
N-acetyl-serine	0,212	0,137	Bb	1,172	0,137	Ba	2,916	1,196	Aa	3,591	0,594	Aa
Glutamic-acid	0,941	0,496	Ba	1,095	0,143	Ba	5,740	2,278	Aa	4,532	1,300	Aa
Phenylalanine	0,328	0,224	Bb	1,125	0,141	Aa	0,673	0,342	Ab	0,927	0,363	Aa
Asparagine	0,168	0,033	Ab	0,297	0,080	Aa	0,058	0,012	Bb	0,091	0,012	Ba
Lysine	0,140	0,016	Ba	0,104	0,017	Ba	0,360	0,039	Aa	0,290	0,021	Ab
Histidine	0,184	0,031	Ab	0,261	0,013	Aa	0,087	0,021	Bb	0,153	0,015	Ba
Carbohydrates												
Fructose	176,913	18,779	Ab	232,884	10,760	Aa	115,555	17,173	Ba	131,918	9,557	Ba
Sorbitol	25,686	8,588	Bb	66,643	3,810	Ba	160,692	3,746	Ab	204,111	6,188	Aa
Mannose	93,559	12,896	Aa	88,851	10,247	Aa	45,158	7,725	Bb	68,516	14,136	Ba
Galactose	93,559	12,896	Ab	122,222	7,167	Aa	49,758	8,976	Bb	71,516	15,481	Ba
Glucose	52,903	7,574	Ab	70,764	3,763	Aa	33,626	5,477	Bb	47,426	9,742	Ba
myo-inositol	6,947	0,960	Ab	11,172	0,687	Aa	4,348	0,941	Bb	5,687	2,130	Ba
Maltose	51,628	4,818	Ba	43,288	0,756	Ba	99,307	21,206	Aa	104,848	18,342	Aa
Raffinose	120,220	5,547	Bb	176,111	5,871	Ba	212,635	23,814	Aa	241,238	25,542	Aa
Sucrose	120,220	5,547	Bb	176,127	5,864	Ba	212,635	23,814	Aa	241,238	25,542	Aa
Maltotriose	1,675	0,167	Ab	2,562	0,350	Aa	0,334	0,080	Ba	0,300	0,046	Ba
Organic acids												
Pyruvic-acid	0,903	0,192	Aa	0,548	0,030	Ab	0,276	0,073	Ba	0,280	0,107	Ba
Lactic-acid	16,295	1,987	Ab	22,133	1,545	Aa	6,424	1,069	Ba	7,954	3,790	Ba
Glycolic-acid	0,108	0,009	Aa	0,104	0,007	Aa	0,089	0,024	Aa	0,117	0,010	Aa
Oxalic-acid	7,222	1,941	Aa	8,320	2,329	Aa	6,370	2,163	Aa	7,459	4,788	Aa
Phosphoric-acid	37,951	8,403	Bb	59,776	3,533	Ba	112,117	18,432	Aa	117,193	7,524	Aa
Succinic-acid	6,663	1,035	ABb	10,025	0,336	Aa	7,441	1,519	ABa	5,930	2,942	Ba
Glyceric-acid	1,640	0,256	Ab	2,228	0,068	Aa	0,880	0,203	Aa	1,154	0,075	Ba
Malic-Acid	19,877	3,787	Ab	26,966	2,179	Aa	19,172	4,228	Aa	18,526	1,466	Ba
Lactic-acid-d	2,358	0,676	Aa	3,407	0,652	Aa	0,444	0,105	Ba	0,458	0,022	Ba
Citric-acid	16,020	2,866	Ab	20,409	0,866	Aa	7,303	1,660	Ba	8,502	0,334	Ba
Others												
Quinic-acid	16,020	2,866	Ab	20,409	0,866	Aa	7,303	1,660	Ba	8,502	0,334	Ba
Putrescine	0,181	0,084	Ba	1,288	0,133	Ba	3,150	1,329	Ab	4,216	0,495	Aa
Urea	0,688	0,082	Aa	0,692	0,059	Aa	0,170	0,048	Bb	0,268	0,025	Ba

7 CONSIDERAÇÕES FINAIS

O condicionamento fisiológico de sementes com TM aliviou os efeitos deletérios da salinidade tanto nos índices de germinação quanto nos parâmetros de crescimento de plântulas de arroz aos 10 DAT. Possivelmente isto ocorreu, principalmente, por meio da ativação do sistema de defesa antioxidante que diminuiu o conteúdo de espécies reativas de oxigênio e pela modulação positiva de metabólitos primários e responsivos ao estresse, que não só produziram energia de forma eficiente como também auxiliaram na exclusão do íon da célula, conforme foi confirmado via fluorescência por microscopia confocal. Embora os eventos fisiológicos, bioquímicos e metabólitos tenham sidos investigados, a relação deles com a resposta do RE ainda precisa ser investigada, seja por meio da expressão de genes relacionados à essa organela, seja pela atividade das enzimas dessas vias *downstreans*, ou ainda pela influência de fitormônios vegetais, como ABA e etileno. De modo geral, o *priming* das sementes com TM, além de inédito, demonstra ser uma abordagem promissora para a aclimatação ao estresse salino; contudo, tais estudos e análises se mostram necessários para um aprofundamento da compreensão do tema.

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