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INSTITUTO DE AGRONOMIA
PROGRAMA DE PÓS-GRADUAÇÃO EM AGRONOMIA – CIÊNCIA DO SOLO

TESE

Contribuição de Variações no Perfil de Metilação do DNA e Alterações Metabólicas e Fisiológicas para a Adaptação de Plantas de Arroz (*Oryza sativa* L.) a Sistemas de Cultivo com Baixo Teor de Nitrogênio

Erinaldo Gomes Pereira

2022

UNIVERSIDADE FEDERAL RURAL DO RIO DE JANEIRO
INSTITUTO DE AGRONOMIA
PROGRAMA DE PÓS-GRADUAÇÃO EM AGRONOMIA
CIÊNCIA DO SOLO

CONTRIBUIÇÃO DE VARIAÇÕES NO PERFIL DE METILAÇÃO DO DNA E ALTERAÇÕES METABÓLICAS E FISIOLÓGICAS PARA A ADAPTAÇÃO DE PLANTAS DE ARROZ (*ORYZA SATIVA* L.) A SISTEMAS DE CULTIVO COM BAIXO TEOR DE NITROGÊNIO

ERINALDO GOMES PEREIRA

Sob a Orientação do professor
Manlio Silvestre Fernandes

e Coorientação do Professor
Leandro Azevedo Santos

Tese submetida como requisito parcial para obtenção do grau de **Doutor,** no Programa de Pós-Graduação em Agronomia - Ciência do Solo, Área de Concentração em Fertilidade do Solo e Nutrição de Plantas.

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TESE APROVADA EM: 25/11/2022.

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Manlio Silvestre Fernandes. Ph.D. UFRRJ
(Orientador)

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Marcia Soares Vidal. Dra. Embrapa Agrobiologia

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À minha família e aos bons amigos pelo apoio ao longo dessa caminhada.

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BIOGRAFIA

Erinaldo Gomes Pereira, natural de Minas Novas-MG, cursou o ensino básico no Colégio Estadual José Bento Nogueira e o ensino fundamental no Colégio Estadual Presidente Costa e Silva, ambos em sua cidade Natal. No ano de 2009, ingressou no curso de Técnico Agrícola com Habilitação em Agropecuária concomitante ao ensino médio, no Instituto Federal de Educação, Ciência e Tecnologia – Minas Gerais – Campus São João Evangelista. Em 2012, ingressou no curso de graduação em Agronomia da UFRRJ. Durante a graduação foi estagiário durante três anos no Laboratório de Fertilidade de Solos sob a orientação do professor Dr. Luiz Rodrigues Freire. Foi bolsista de iniciação científica do CNPq por quatro anos no Laboratório de Nutrição Mineral de Plantas, sob orientação do professor Manlio Silvestre Fernandes, trabalhando com o estudo de cultivares de arroz tradicionais do Maranhão e manipulação genética de plantas de arroz. Em março de 2017, ingressou no Mestrado pelo Programa de Pós Graduação em Agronomia - Ciência do Solo - UFRRJ sob orientação do professor Manlio Silvestre Fernandes, onde desenvolveu a dissertação intitulada "Caracterização Funcional do Transportador OsAAP1 e Avaliação de Mecanismos Associados à Eficiência de Uso de Nitrogênio, Utilizando o Sistema CRISPR-Cas 9". Em 2019 ingressou no Doutorado pelo Programa de Pós Graduação em Agronomia - Ciência do Solo-UFRRJ.

RESUMO GERAL

PEREIRA, Erinaldo Gomes. **Contribuição de variações no perfil de metilação do DNA e alterações metabólicas e fisiológicas para adaptação de plantas de arroz (*Oryza sativa* L.) a sistemas de cultivo com baixo teor de nitrogênio.** 2022. 80f. Tese (Doutorado em Agronomia – Ciência do Solo). Instituto de Agronomia, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ. 2022.

Deficiência por nitrogênio (N) é um estresse abiótico a que plantas de arroz estão susceptíveis ao longo do ciclo de cultivo, devido sobretudo à grande demanda por N apresentada por essa cultura e, também, pela fácil perda desse nutriente no solo. A metilação do DNA é um mecanismo epigenético que afeta o desempenho das plantas frente a diversos estresses ambientais. Os objetivos dessa pesquisa foram: 1) verificar se plantas de arroz submetidas ao estresse pelo baixo fornecimento de N modificam o perfil de metilação do DNA; 2) verificar possíveis alterações no perfil de metilação do DNA, modificações morfo-fisiológicas, metabólicas, e expressão gênica em plantas de arroz submetidas a diferentes ciclos de cultivo com baixo N ; 3) estabelecer relações entre modificações na metilação do DNA e alterações morfo-fisiológicas e metabólicas; 4) determinar se a exposição a ciclos de cultivo com baixo N resulta em plantas melhor adaptadas a essa condição. Para isso, as variedades de arroz Esmeralda (melhorada), Manteiga e Piauí (tradicionais do estado do Maranhão), foram submetidas aos seguintes tratamentos: Controle – plantas cultivadas com N-suficiente (60 kg N ha^{-1}) durante três ciclos de cultivo sucessivos; NS1 – plantas cultivadas com baixo N (10 kg N ha^{-1}) somente no último ciclo de cultivo (primeira exposição ao estresse); NS2 – plantas cultivadas com baixo N no primeiro e terceiro ciclo de cultivo (estresse intermitente); NS3 – plantas cultivadas com baixo N durante três ciclos sucessivos (estresse recorrente). Foi verificado em plantas da variedade Esmeralda uma redução na metilação total do DNA cultivadas com baixo N em comparação ao controle. Plantas submetidas aos tratamentos de baixo N apresentaram maior redução no número de bandas hemi-metiladas em detrimento de bandas totalmente metiladas. A redução mais severa no número de bandas hemi-metiladas foi verificada em plantas submetidas ao tratamento NS2, o que foi acompanhado por um aumento mais expressivo no número de bandas totalmente metiladas. Essa modificação no padrão de metilação do DNA observado em NS2 foi seguido por uma redução na produção de grãos, na eficiência fotossintética e nos parâmetros relacionados a eficiência de uso de N. As variedades Manteiga e Piauí apresentaram um aumento expressivo no número total de bandas metiladas quando submetidas aos tratamentos de estresse por baixo N em comparação ao controle. Esse resultado deveu-se, sobretudo, ao forte aumento no número de bandas totalmente metiladas. Plantas submetidas aos tratamentos NS2 e NS3 tiveram aumento mais expressivo no número de bandas totalmente metiladas, e uma redução no número de bandas hemi-metiladas. Essa modificação no padrão de metilação foi acompanhada por maior rendimento de grãos, melhor eficiência fotossintética, aumento na eficiência de uso de N e alterações na expressão gênica. Os resultados mostram que a exposição ao baixo N altera o padrão de metilação do DNA, e que o tipo e a intensidade dessa alteração dependem tanto da duração do estresse quanto das variedades em estudo. As alterações no padrão de metilação DNA são acompanhadas por modificações morfo-fisiológicas, metabólicas e na expressão gênica. A exposição ao estresse intermitente e recorrente melhora o desempenho das variedades Manteiga e Piauí, enquanto o estresse intermitente é prejudicial a variedade Esmeralda.

Palavras chave: Arroz. Nitrogênio. Estresse. Epigenética. Metilação. Desmetilação.

GENERAL ABSTRACT

PEREIRA, Erinaldo Gomes. **Contribution of variations in DNA methylation profile and metabolic and physiological changes to adaptation of rice (*Oryza sativa* L.) plants to low-nitrogen cropping systems.** 2022. 80p. Thesis (Doctor in Agronomy – Soil Science). Instituto de Agronomia, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ. 2022.

Nitrogen (N) deficiency is an abiotic stress to which rice plants are susceptible to throughout the crop cycle, mainly due to the high demand for N presented by this crop and also because of the easy loss of this nutrient in the soil. DNA methylation is an epigenetic mechanism that affects plant performance in the face of various environmental stresses. The objectives of this research were: 1) to verify if rice plants submitted to low N stress modify their DNA methylation profile; 2) to verify possible alterations in the DNA methylation profile, morpho-physiological, metabolic changes, and gene expression in rice plants submitted to different crop cycles with low N; 3) to establish relationships between DNA methylation modifications and morpho-physiological and metabolic changes; 4) to determine if exposure to low N cycles results in plants better adapted to this condition. For this, the rice varieties Esmeralda (improved), Manteiga and Piauí (traditional from Maranhão state), were subjected to the following treatments: Control - plants grown with N-sufficient (60 kg N ha^{-1}) for three successive crop cycles; NS1 - plants grown with low N (10 kg N ha^{-1}) only in the last crop cycle (first stress exposure); NS2 - plants grown with low N in the first and third crop cycle (intermittent stress); NS3 - plants grown with low N for three successive cycles (recurrent stress). A reduction in total DNA methylation was verified in plants of the Esmeralda variety grown with low N compared to the control. Plants submitted to the low N treatments showed a greater reduction in the number of hemi-methylated bands at the expense of fully methylated bands. The most severe reduction in the number of hemi-methylated bands was seen in plants submitted to the NS2 treatment, which was accompanied by a more significant increase in the number of fully methylated bands. This modification in the DNA methylation pattern observed in NS2 was followed by a reduction in grain yield, photosynthetic efficiency, and N use efficiency related parameters. The varieties Manteiga and Piauí showed a significant increase in the total number of methylated bands when subjected to the low N stress treatments compared to the control. This result was mainly due to a strong increase in the number of fully methylated bands. Plants subjected to the NS2 and NS3 treatments had a more significant increase in the number of fully methylated bands, and a decrease in the number of hemi-methylated bands. This modification in methylation pattern was accompanied by higher grain yield, improved photosynthetic efficiency, increased N use efficiency, and changes in gene expression. The results show that exposure to low N alters the DNA methylation pattern, and that the type and intensity of this alteration depend on both the duration of stress and the varieties under study. Changes in the DNA methylation pattern are accompanied by morpho-physiological, metabolic, and gene expression modifications. Exposure to intermittent and recurrent stress improves the performance of the varieties Manteiga and Piauí, whereas intermittent stress is detrimental to the variety Esmeralda.

Key words: Rice. Nitrogen. Stress. Epigenetic. Methylation. Demethylation.

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Dio.RC energy flux dissipated as heat per RC**; Tro.RC** maximum capture rate per RC**; REo.RC** reduction flux of electrons in the final electron acceptor in photosystem I (PSI); **φ(Po)** photochemical maximum quantum yield**; φ(Eo)** quantum yield of electron transport from Quinone A (Q_A-) to the electron acceptor intersystem**; Piabs** partial photosynthetic performance index; **NT** Number of tillers per plant; **SDW** Shoot dry weight; **TWFG** Total weight of filled grains**; FGN** Filled grains number**; NUpE** N uptake efficiency; **NUtE** N utilization efficiency; **NUE** N use efficiency........................20

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ABBREVIATION LIST

NS1 - N-stress 1

NS2 - N-stress 2

NS3 - N-stress 3

MSAPs - Methylation-sensitive amplified polymorphisms

Dio/RC - Energy flux dissipated as heat per reaction center (RC)

ABS/RC - Absorption flux per RC

REo/RC - Reduction flux of electrons in the final electron acceptor in photosystem I (PSI)

φ(Po) - Photochemical maximum quantum yield

φ(Eo) - Quantum yield of electron transport from quinone A (QA-) to the electron acceptor intersystem

φ(Ro) - Quantum yield of electron transport from QA- to the final PSI electron aceptor

Piabs - Partial photosynthetic performance index

Pitotal - Total photosynthetic performance index

DAE - Days after emergency

HMR - Hemi-methylated ratio

FMR - Fully-methylated ratio

SDW - Shoot dry weight

TWFG - Total weight of filled grains

FGN - Filled grains number

NUpE - N uptake efficiency

NUtE - N utilization efficiency

NUE - N use efficiency

DRM - Domains rearranged methylases

METs - Methyltransferases

CMT2 - Chromomethylase DNA methyltransferase 2

DNMT - DNA (cytosine-5)-methyltransferase

ROS - Repressor of silencing

DML3 - Demeter-like 3

AAPs - Amino acid permeases

GS1.1 - Glutamine synthetase 1.1

Fe-GOGAT - Ferredoxin-dependent glutamate synthase

NADH-GOGAT 2 - NADH-dependent glutamate synthase

GDH - Glutamate dehydrogenase

NUpE - Nitrogen uptake efficiency

NUtE - Nitrogen utilization efficiency

NUE - Nitrogen use efficiency

NRE - Nitrogen remobilization efficiency

NHI - N harvest index

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1 GENERAL INTRODUCTION

As sessile organisms, plants are constantly challenged by various environmental stresses, requiring mechanisms that allow for an adjustment in their growth and development (SECCO et al., 2017). In addition to the signaling pathways of plants to major environmental stresses already unraveled in the past decade (ZHU, 2016), recent studies have demonstrated the participation of epigenetic mechanisms in response to multiple abiotic stresses (CHANG et al., 2020; DO AMARAL et al., 2020; FERREIRA et al., 2015; MAGER and LUDEWIG, 2018; WU et al., 2020).

Epigenetics is defined as the change in gene expression that occurs without changes in DNA sequence (IWASAKI and PASZKOWSKI, 2014). Chemical modification of DNA (methylation), post-translational modifications of histones (methylation, acetylation, sumoylation, phosphorylation, among others), and regulatory RNAs (non-coding RNAs), are epigenetic mechanisms that together make up the plant epigenome and determine chromatin conformation and, consequently, the greater or lesser accessibility of DNA (KUMAR, 2019). The diversity and flexibility of these mechanisms contribute to plant adaptation to environmental stresses (SECCO et al., 2017). Epigenetic marks can be lost once the stress event ceases, maintained within the same generation, or even passed on to the next generation, thus giving rise to an "epigenetic memory" to a stress event (AVRAMOVA, 2015; DING et al., 2012; IWASAKI and PASZKOWSKI, 2014).

Methylation of the fifth carbon of the cytosine residue (5m-C) is one of the most studied epigenetic marks in plants and mammals (SAZE et al., 2012), showing strong influence on chromatin structure. In eukaryotes, gene expression is highly influenced by chromatin structure. Gene expression is active in less condensed chromatin (euchromatin) and inactivated in compacted chromatin (heterochromatin) (SAZE et al., 2012). In addition to influencing gene expression through increased or decreased chromatin compaction, DNA methylation can also alter the expression of certain genes by preventing transcription factors from coupling to the promoter region (ZHAO and ZHOU, 2012).

Nitrogen (N), when present at low level in the soil, can be considered as a potential stress factor for crops given the great demand that plants have for this macronutrient (FAGODIYA et al., 2020). Leaching and volatilization promote the reduction of N levels in the soil and, consequently, the lower availability of this nutrient (FAGODIYA et al., 2020). Increasing N levels via mineral nitrogen fertilization is a clear, but costly and sometimes environmentally incorrect alternative (LEE, 2021). Increasing the plants' resistance to low N and/or varying soil N levels is an alternative to be considered in order to reduce losses in crop development and production, as well as a more sustainable agriculture.

In this context, the thesis was built with the objective of initially addressing in the first chapter possible alterations in the DNA methylation profile in the Esmeralda rice variety in response to different crop cycles with low N. Then, to investigate morpho-physiological, metabolic and N use efficiency modifications. Finally, correlations were established between these modifications and the alterations in the methylation profile. In the second chapter, two traditional rice varieties, Manteiga and Piauí, were also submitted to different crop cycles with low N. Morpho-physiological, metabolic, N use efficiency and DNA methylation profiles were analyzed. In addition, this chapter analyzed the expression of genes related to N metabolism, as well as the expression of enzymes responsible for the process of DNA methylation and demethylation.

2 CHAPTER I

DIFFERENT LOW-NITROGEN-STRESS REGIMES RESULT IN DISTINCT DNA-METHYLATION PATTERNS, METABOLIC PROFILES, AND MORPHO-PHYSIOLOGICAL CHANGES IN RICE

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2.1 RESUMO

Metilação e desmetilação do DNA são respostas epigenéticas a estresses abióticos. Os objetivos desse estudo foram: i) identificar alterações no padrão de metilação do DNA entre plantas de arroz cultivadas com N suficiente e baixo nível de N; ii) observar se o condicionamento ao N-stress promovia alterações do padrão de metilação; iii) estabelecer possíveis relações entre alterações na metilação e modificações fenotípicas, fisiológicas e metabólicas na planta. Os tratamentos de estresse por N aplicados foram: plantas expostas ao estresse somente em uma geração (NS1), plantas expostas ao estresse por duas (NS2 – estresse intermitente) e três (NS3 – estresse recorrente) gerações. O estresse abiótico foi obtido por meio do fornecimento de uma adubação equivalente a apenas 10 kg N ha^{-1}. Como controle foram utilizadas plantas cultivadas com 60 kg N ha^{-1}. Quando comparado ao controle, foi observado que o N stress induziu uma redução da metilação total do DNA. A maior redução foi verificada nas bandas hemi-metiladas. NS1 e NS3 tiveram uma redução semelhante no total de bandas hemi-metiladas e bandas totalmente metiladas. Também foi observado nas plantas sob os tratamentos NS1 e NS3 características fenotípicas e eficiência fotossintética semelhantes. Por outro lado, o tratamento NS2 aumentou o número de bandas totalmente metiladas, o que foi acompanhado por uma forte redução no rendimento de grãos e na eficiência fotossintética. Foi possível verificar que o estresse por baixo N estimula modificações na metilação do DNA e que a duração estresse por baixo N interfere no padrão de metilação das plantas. Estresse por baixo N também afetou as respostas fenotípicas, fisiológicas e metabólicas das plantas.

2.2 ABSTRACT

DNA methylation and demethylation are epigenetic responses to abiotic stresses. The aims of this study were to i) identify alterations in the DNA methylation patterns among rice plants grown with sufficient and low nitrogen (N) levels; ii) observe whether conditioning to N-stress promoted alterations in methylation patterns; and iii) search for possible relationships among methylation alterations and phenotypic, physiological, and metabolic changes.

The N-stress treatments applied were as follows: plants were exposed to N-stress for only one generation (NS1), plants were exposed to stress for two (NS2 – intermittent stress) and three (NS3 – recurrent stress) generations. N stress was created by supplying fertilizer amounts that were equivalent to only 10 kg N ha^{-1}. As a control, plants grown with 60 kg N ha^{-1} were used.

When compared to the control, it was observed that N stress led to a reduction in total DNA methylation. The greatest reduction was observed in the hemi-methylated bands. NS1 and NS3 had similar reductions in the total hemi-methylated and fully methylated bands. Similar phenotypic characteristics and photosynthetic efficiencies were also observed in plants from the NS1 and NS3 treatments. On the other hand, the NS2 treatment increased the number of fully methylated bands, which was followed by a strong reduction in grain yield and photosynthetic efficiency. It was found that N stress stimulates changes in DNA methylation and that the duration of N stress conditioning interferes with the methylation patterns of plants. N stress also affected the phenotypic, physiological, and metabolic responses of plants.

2.3 INTRODUCTION

DNA methylation and histone chemical modifications are the most studied epigenetic mechanisms in plants (KARACA et al., 2019). Methylation is characterized by the addition of a methyl group to carbon 5 of cytosine, which forms 5-methylcytosine (5mC) (YAISH et al. 2018). It can occur in the gene body or promoter region. Methylation in the promoter region is usually associated with repression of promoter activity and may result in chromatin silencing by physically impeding transcription factor coupling or by recruiting chromatin repressor proteins (ZHAO and ZHOU, 2012). On the other hand, low methylation levels in the gene body may stimulate gene expression (YAISH et al. 2018). DNA naturally contains several methylated regions, which together make up the epigenome of each species. This epigenome is flexible and can be modified depending on environmental stimuli. Abiotic stresses such as salinity DO AMARAL et al., 2020), drought (NEVES et al., 2017), cold (SHAN et al., 2013), and nutritional deficiency (KOU et al., 2011; SECCO et al., 2015) are stimuli that can induce changes in the epigenome. The intensity and duration of stress are determinants for the addition or loss of epigenetic marks (SRIKANT and DROST, 2021).

Nitrogen (N) is one of the nutrients that are most important to cultivated species. This nutrient is contained in a series of fundamental biomolecules, such as amino acids, proteins, and nucleic acids, in addition to hormones, chlorophyll, and membranes (SOUZA and FERNANDES, 2018) and participates in several essential physiological processes, including photosynthesis. Soils usually do not provide sufficient N to support crop growth, which makes it necessary to apply mineral nitrogen fertilizers (RANJAN and YADAV, 2019). Approximately 30 to 50% of the total N applied to soil is absorbed by plants, and the rest is lost through processes such as leaching, denitrification, and volatilization (NAHER et al., 2020). Nitrogen deficiency that is caused either by poor supply through fertilization or low acquisition efficiency can be experienced sporadically, during the cultivation cycle, or recurrently, over generations and constitutes a stress factor for plants. Exposure to N stress can stimulate epigenetic changes in DNA, which can be transferred to following generations through "epigenetic memory". Over the years, several studies have focused on understanding the memory mechanism for stress events in different cultivated species (WANG et al., 2011; NEVES et al., 2017; DO AMARAL et al., 2020; FAN et al., 2021). However, whether and how this memory benefits rice plants in poor-N environments still needs to be further investigated.

Rice is a cereal grown around the world and is part of the daily diet of more than half the world's population (CARCEA, 2021). As a result, rice production ensures long-term food security, especially in developing countries (RANJAN and YADAV, 2019). In addition to being a rich source of carbohydrates (75-80%) and vitamins, rice also contains proteins (7-10%) that are easily digested and have high biological value (VERMA and SRIVASTAV, 2020). Nitrogen fertilization is essential for achieving satisfactory production; thus, N is a limiting factor in the development of rice crops (RANJAN and YADAV, 2019). Poor-N conditions, whether due to insufficient input, soil losses, or low absorption efficiency, can be experienced by this crop during the growing season. Thus, rice crops may experience fluctuations in N availability throughout the growing cycle, which can lead to reductions in growth (PEREIRA et al., 2021), productivity, and changes in the nutritional composition of rice grains (CHENG et al., 2021). Although the effects of poor N availability are widely discussed and known (GUO et al., 2017; CHENG et al., 2021), studies that seek to address the epigenetic impacts of this abiotic stress, as well as the interactions among epigenetic, physiological, and metabolic stresses, are still scarce (KOU et al., 2011), especially studies that seek to simulate real field conditions (WU et al., 2020; FAN et al., 2021). This

information is essential to begin to understand the possible adaptive mechanisms of rice plants facing different N stresses.

The objectives of this study were to determine whether subjecting rice plants to different cycles of low-N conditions would promote changes in DNA methylation patterns and to understand the possible phenotypic, physiological, and metabolic changes.

2.4 MATERIAL AND METHODS

2.4.1 Plant material and n-stress treatment

Seeds of rice (*Oryza sativa* L.) cv. Esmeralda were obtained from the germplasm bank of the Brazilian Agricultural Research Corporation (Embrapa). The Esmeralda cultivar belongs to the japonica subspecies. It originated from a simple cross, which was conducted by Embrapa, between the CNAx4909-68-MM2-PY2 strain and BRS Primavera cultivar, and both are predominantly Japanese. This cross is an upland rice with good resistance to abiotic stresses, such as less favorable soils or climate conditions (DE CASTRO et al., 2014). Uniform seeds were germinated in plastic pots (0.7 L) under a layer of distilled water. Five days after germination, uniform seedlings were separated and transferred to pots (8 L) containing soil collected from the A horizon of a *Planossolo* and were grown in a greenhouse (photoperiod and natural irradiance; temperature 28–32 °C) at the Federal Rural University of Rio de Janeiro (UFRRJ). Two plants were placed in each pot. Before the beginning of the experiment, a chemical analysis of the soil was performed (appendix – Table 7), and a need to correct the potassium levels was identified (FREIRE et al., 2013). Therefore, fertilization with potassium sulfate (K_2SO_4) equivalent to 60 kg of K_2O per hectare was carried out. The initial N concentration in the soil was equivalent to 30.3 kg N ha^{-1}. Treatments that imposed N stress were started fifteen days after the transfer of plants to pots.

To impose N stress, the plants were divided into four groups, which were organized in a completely randomized design (DIC), and three cultivation cycles were carried out with variations in the N supply (appendix – Fig. 13). The first cultivation cycle started in December 2019, when the plants were subjected to two N regimes: i) plants in groups 1 and 2 received fertilization that was equivalent to 60 kg N ha^{-1} (e.g., sufficient N); ii) 3 and 4 received fertilization that was equivalent 10 kg N ha^{-1} (e.g., N stress). At the end of the first cycle, seeds were collected and used to conduct the second cycle, which started in April 2020. In the second cycle, plants in groups 1, 2, and 3 received sufficient N, while the plants in group 4 received low levels of N. At the end of the second cycle, seeds were collected and used to conduct the third cycle, which started in September 2020. In this cycle, the plants from group 1 were cultivated with sufficient N, while the plants from groups 2, 3, and 4 received low levels of N. The seeds that were obtained from each group were used for planting in the same group in the following cycle (appendix – Fig. 13). Group 1 was the control I and consisted of plants that were not subjected to N stress. They were used as a control for the parameters analyzed in this study, and thus represented the maximum potential of this cultivar under standard growing conditions; group 2 (NS1) contains plants that were exposed to N stress only in the third cycle; group 3 (NS2) contains plants that were exposed to N-stress during cycles 1 and 3, which represents intermittent exposure to N-stress; group 3 (NS3) plants were exposed to N stress during each of the three cultivation cycles, which represents recurrent N-stress. For cycle three, eight replicates were conducted, with two plants per pot. Four replications were collected at anthesis period to determine the total nitrogen contents, metabolite profiles, and methylation patterns. The other replications were conducted until the end of the cycle three to determine N use and phenotypic parameters, and proteins in grains. Photosynthetic parameters were measured throughout cycle three. All data shown in this study refer to cycle three.

2.4.2 DNA extraction

Total genomic DNA was extracted according to the CTAB method described by MURRAY and THOMPSON (1981). Approximately 0.5 g of leaf samples collected at anthesis were used. DNA quality was verified on agarose gel (1.5%) and used a spectrophotometer (Thermo Scientific NanoDrop 2000c). DNA samples were stored at -80 °C.

2.4.3 Methylation-sensitive amplification polymorphism assay

The methylation profiles were determined by using the methylation-sensitive amplification polymorphism assay (MSAP) technique as described by TANG et al. (2014). This technique is based on the sensitivity difference of two restriction enzymes (e.g., HpaII and MspI) to DNA methylation. Both enzymes recognize the 5´-CCGG-3´ tetranucleotide sequence. The enzyme EcoRI of a frequent cut and is not sensitive to methylated regions is used together with HpaII and Msp1 to cleave sequences that have fully methylated internal cytosines. The two enzymes cleave unmethylated sequences (KOU et al., 2011). The EcoRI enzyme, which is frequently cut and lacks sensitivity to methylated regions, is used together with HpaII and MspI. This technique has the advantage of being relatively easy to perform and it is also has possible to detect large numbers of polymorphisms with this technique (TANG et al., 2014). To assess the methylation differences among treatments, one pair of pre-selective primers and 12 pairs of selective primers (appendix - Table 8) were used. The fragments were resolved using polyacrylamide gel electrophoresis (6%) and were visualized by silver staining (KOU et al. 2011).

2.4.4 Phenotypic characterization and total N determination

At the end of the third cultivation cycle, counting and determination of the average length of the tillers were carried out. Then, the seeds were collected, weighed, and stored. The vegetative parts were cut, dried and placed in an oven with air circulation at 65 °C for 3 days. Next, the materials were weighed and crushed in a mill, and 0.2-g samples were obtained to determine the total nitrogen contents. The same procedure was performed for the plants collected during anthesis. One hundred seeds from each repetition were separated to determine the total N contents. These seeds were dehulled and crushed, and 0.2-g quantities from each sample were used. The total N values were determined according to TEDESCO et al. (1995).

2.4.5 Photosynthetic parameter associated with chlorophyll *a* fluorescence

The transient fluorescence levels of chlorophyll “a” were measured in the middle third of the more expanded leaf. A Handy PEA fluorimeter (Plant Efficiency Analyzer, Hansatech Instruments Ltd, UK) was used. Dark adaptation was performed with appropriate clips for 40 minutes in the morning period, starting at 07:00 am. The chlorophyll “a” fluorescence levels were then measured. Measurements were taken at 14 and 29 (initial growth phase), 41 (mid-cycle), 64 and 68 (anthesis), 75 and 83 (grain filling) days after emergence (DAE), and on days 14, 29, and 41 DAE, the fluorescence levels were measured in the youngest fully expanded leaves and from 64 DAE in the flag leaves. Fluorescence emissions were induced in small 4-mm diameter circles by using pulses of saturating light (3 $mmol^{-2}$ s-1). The fluorescence values that were obtained after applying light pulses were used to calculate the parameters established by the JIP Test according to TSIMILLI-MICHAEL (2020) and TSIMILLI-MICHAEL and STRASSER (2008). The plotted JIP test parameters represent the averages of eight measurements per treatment.

2.4.6 Parameters related to n-use efficiency

The parameters related to N use efficiency were determined according to COELHO et al. (2016). Therefore, the following formulas were used: i) N uptake efficiency (NUpE): total N of the aerial part (including panicles)/N supplied; ii) N utilization efficiency (NUtE): weight of full grains/N supplied; iii) N use efficiency (NUE): full-grain weight/aerial Part N content; iv) N remobilization efficiency (NRE): N remobilization to grains/total N in anthesis; and v) N harvest index (NHI): N in grain/total N in straw at maturity.

2.4.7 Protein fractions in grains

Protein fractions were measured in brown rice grains. Sequential protein extractions were conducted according to the procedures described by DOLL and ANDERSEN (1981) and TURLEY and CHING (1986). To extract the albumin + globulin fraction, a saline solution (2.9% NaCl+0.002% Na-EDTA) was used. Prolamin extractions were performed by adding an alcohol solution (50% isopropanol, 41 mM boric acid, 0.6% 2-mercaptoethanol) to the saline extraction residue. An alkaline solution (0.48% boric acid + 0.4% NaOH) was added to the alcohol extraction residue for glutelin extraction. Protein fractions were quantified according to (BRADFORD, 1976).

2.4.8 Metabolite profiling by GC/MS

All samples were collected from the middle third of the flag leaves of rice plants at the beginning of anthesis (64 DAE). The samples were macerated in liquid nitrogen, and 0.250 g of completely pulverized material was set aside. Then, an appropriate extraction buffer (LISEC et al., 2006) and aliquot of Ribitol (internal standard) were added. The metabolites were analyzed with a GC/MS QP-2010 Plus instrument (Shimadzu, Japan). A Factor Four/VF-5 ms column (30 x 0.25 x 0.25) was used. The operating conditions were ionization at 70 eV, mass range of 40–800 m/z, column oven temperature of 70°C, injection temperature of 230°C, injection mode split, and column flow of 1 mL/min helium gas. The retention indexes were calculated based on the retention indexes of two series of n-alkanes (e.g., C8-C20 and C21-C40). Metabolites were identified by comparing the retention indexes and mass spectra with the Develonutri Reference Book database (HALKET and FRASER, 2010). The data were initially normalized based on the ribitol content present in each sample, and a spreadsheet containing the concentrations of each identified metabolite was then created. This spreadsheet was exported to the MetaboAnalyst web-based platform (http://www.metaboanalyst.ca/). Filters based on standard deviations were used to automatically remove low-quality data. Then, the data were normalized by their sums, transformed using a base-10 logarithmic scale, and converted using the Pareto scale method. Data were analyzed using cluster analysis by using Orthogonal Partial Least Square-Discriminant Analysis (OrthoPLS-DA) to differentiate the treatments by the formation of groups. One-way analysis of variance (ANOVA) was used to identify metabolites with different abundances.

2.4.9 Statistical analysis

Data normality and homoscedasticity were determined according to Lilliefors and Cochran tests, respectively. Then, the F test ($p < 0.05$) was applied for the analysis of variance, and the Scott–Knott test was used for comparisons of means. All the statistical analysis was performed in R software (R Core Team 2019), except for OrthoPLS-DA performed in the MetaboAnalyst web-based platform (http://www.metaboanalyst.ca/).

2.5 RESULTS

2.5.1 DNA methylation global patterns

The differences in the cytosine methylation patterns at 5´mCCGG sites among plants grown with sufficient N (control) and plants subjected to different levels of N stress (e.g., NS1, NS2, and NS3), as well as the differences among stress levels, were determined through MSAP. In total, 640 bands were amplified in all treatments and in the control (Table 1). Therefore, 12 primer combinations were used (appendix - Table 8). The fragments were classified into four types according to the presence (1) or absence (0) of bands for the EcoRI/HpaII and EcoRI/Msp1 enzyme combinations. Type I: unmethylated bands for the two enzyme combinations; Type II: hemi-methylated bands present only for the EcoRI/HpaII combination; Type III: presence of bands for the EcoRI/Msp1 combination; Type IV: absence of bands for the two enzyme combinations. Type III and IV represent complete methylation (Tang et al. 2014). The total methylation in the DNA of the control plants was 40.78%, while for the NS1, NS2, and NS3 plants, these values were 34.53, 37.19, and 34.84%, respectively. These results demonstrate that rice (cv. Esmeralda) plants subjected to N stress have lower DNA methylation rates regardless of the frequency of N stress (Table 1).

A further reduction in the proportion of hemi-methylated bands was observed. Hemi-methylation indicated the methylation level of the single strain in double strain DNA. A difference of up to 6.1% was observed between NS2 and control for hemi-methyl bands. On the other hand, smaller reductions in the numbers of fully methylated bands were observed, with reductions of 1.71 and 1.09% for NS1 and NS3, respectively. However, plants that were conditioned to two cycles of N stress (NS2) showed a 2.5% increase in the number of fully methylated bands. NS2 resulted in a smaller reduction in total DNA methylation, particularly due to the increased number of fully methylated bands at the expense a reduced number of hemi-methylated bands. This shows that the fully methylated bands were upregulated in plants conditioned to two alternating cycles of N stress. Meanwhile, the hemi-methylated bands were downregulated under these same conditions. Plants subjected to the NS1 treatment showed greater reductions in the total number of methylated bands, which were mainly due to greater reductions in the number of fully methylated bands.

Table 1. Global DNA methylation patterns in flag leaves of rice plants (cv. Esmeralda) subjected to distinct N-stress regimes: NS1 (two successive N-sufficient cycles + one N-stress cycle); NS2 (one N-stress cycle + one N-sufficient cycle + one N-stress cycle); NS3 (three successive N-stress cycles). Control: (three successive N sufficient cycles).

MSAP band type[a]	Control	NS1	NS2	NS3
I	379	419	402	417
II	126	97	87	95
III	112	94	96	94
IV	23	30	55	34
Total amplified bands	640	640	640	640
Total methylated bands[b]	261	221	238	223
Fully methylated bands[c]	135	124	151	128
MSAP %[d]	40.78	34.53	37.19	34.84
Hemi-methylated ratio (%)[e]	19.69	15.16	13.59	14.84
Fully-methylated ratio (%)[f]	21.09	19.38	23.59	20.00

In which: ***MSAP*** Methylation Sensitive Amplification Polymorphism.

aType I is the presence of bands in both EcoRI/HpaII and EcoRI/MspI enzymes and indicates the absence of methylation; type II are bands generated with EcoRI/HpaII enzymes but not with EcoRI/MspI; type III are bands generated with EcoRI/MspI enzymes but not with EcoRI/HpaII; and type IV represents the absence of bands in both enzyme combinations. bThe total methylated bands is the sum of the type II, III and IV bands. cFully methylated bands are the type III + IV bands. dMSAP is the ratio between the total methylated bands (II + III + IV) and the total amplified bands. eRatio of hemi-methylated bands is the ratio between the total of hemi-methylated bands (II) and the total of amplified bands. fThe ratio of fully methylated bands is the ratio between fully methylated bands (III + IV) and the total amplified bands.

2.5.2 N stress induced methylation and demethylation changes

Based on the global methylation patterns described above, a more detailed analysis of the methylation and demethylation changes that occurred in the DNA of plants conditioned to N stress was performed (Table 2). Therefore, the bands were categorized based on three patterns. A: bands that did not experience any changes in methylation patterns, which included classes A1 – A4; B: bands that were demethylated, which included classes B1 – B6; and C: represents the addition of methyl (CH3) in cytosines, which is represented by classes C1 – C6. In total, 16 classes were defined. Pattern A, without alterations, was numerically superior to the others, which corresponded to 79.69% of the amplified bands (in the NS1 treatment), followed by patterns B (demethylation) and C (methylation). Plants subjected to the NS3 treatment had greater changes in DNA demethylation, as shown by the higher frequency of demethylation (15.31%), than for the NS1 and NS2 treatments. On the other hand, plants subjected to the NS1 treatment had smaller changes in methylation, particularly due to the lower methylation frequency (7.50%). The NS2 treatment promoted intermediate behavior regarding the frequency of unaltered bands (76.56%). On the other hand, plants conditioned to two cycles of N stress (NS2) had the lowest demethylation frequency (11.41%) and the highest methylation frequency (12.03%).

Table 2. Classification of different methylation profiles in flag leaves of rice plants (cv. Esmeralda) subjected to distinct N-stress regimes: NS1 (two successive N-sufficient cycles + one N-stress cycle); NS2 (one N-stress cycle + one N-sufficient cycle + one N-stress cycle); NS3 (three successive N-stress cycles). Control: (three successive N sufficient cycles).

Classes	Banding pattern				Nº of sites (Frequency of methylation changes)		
	Control		Stress (N)				
	HpaII	MspI	HpaII	MspI	NS1	NS2	NS3
A No change					510 (79.69%)	490 (76.56%)	478 (74.69%)
A1	1	1	1	1	353	340	342
A2	1	0	1	0	72	62	62
A3	0	1	0	1	76	74	70
A4	0	0	0	0	9	14	4
B Demethylation					82 (12.81%)	73 (11.41%)	98 (15.31%)
B1	1	0	1	1	47	44	52
B2	0	1	1	1	19	17	21
B3	0	0	1	1	0	1	2
B4	0	1	1	0	2	3	6
B5	0	0	1	0	8	2	9
B6	0	0	0	1	6	6	8
C Methylation					48 (7.50%)	77 (12.03%)	64 (10%)
C1	1	1	1	0	15	20	18
C2	1	1	0	1	9	15	15
C3	1	0	0	1	3	1	1
C4	1	1	0	0	2	4	4
C5	1	0	0	0	4	19	11
C6	0	1	0	0	15	18	15

Scores 1 or 0 represent the presence or absence of bands, respectively.

2.5.3 N-stress effects on growth and production

As expected, N stress promoted significant phenotypic changes ($p < 0.05$). All plants subjected to N stress, when compared to the control, showed reductions in shoot dry weights (Fig. 1E) and grain yields (Fig. 1 F and G). The NS2 treatment promoted the most severe reduction in grain production, which differed significantly ($p < 0.05$) from both the control, NS1 and NS3 treatments (Figs. F and G). Plants conditioned to NS1 had similar tillering to the control and superior tillering ($p < 0.05$) to the NS2 and NS3 treatments (Fig. 1D). The average tiller lengths (Fig. 1C) and weights of 100 full grains (Fig. 1H) did not show any significant differences.

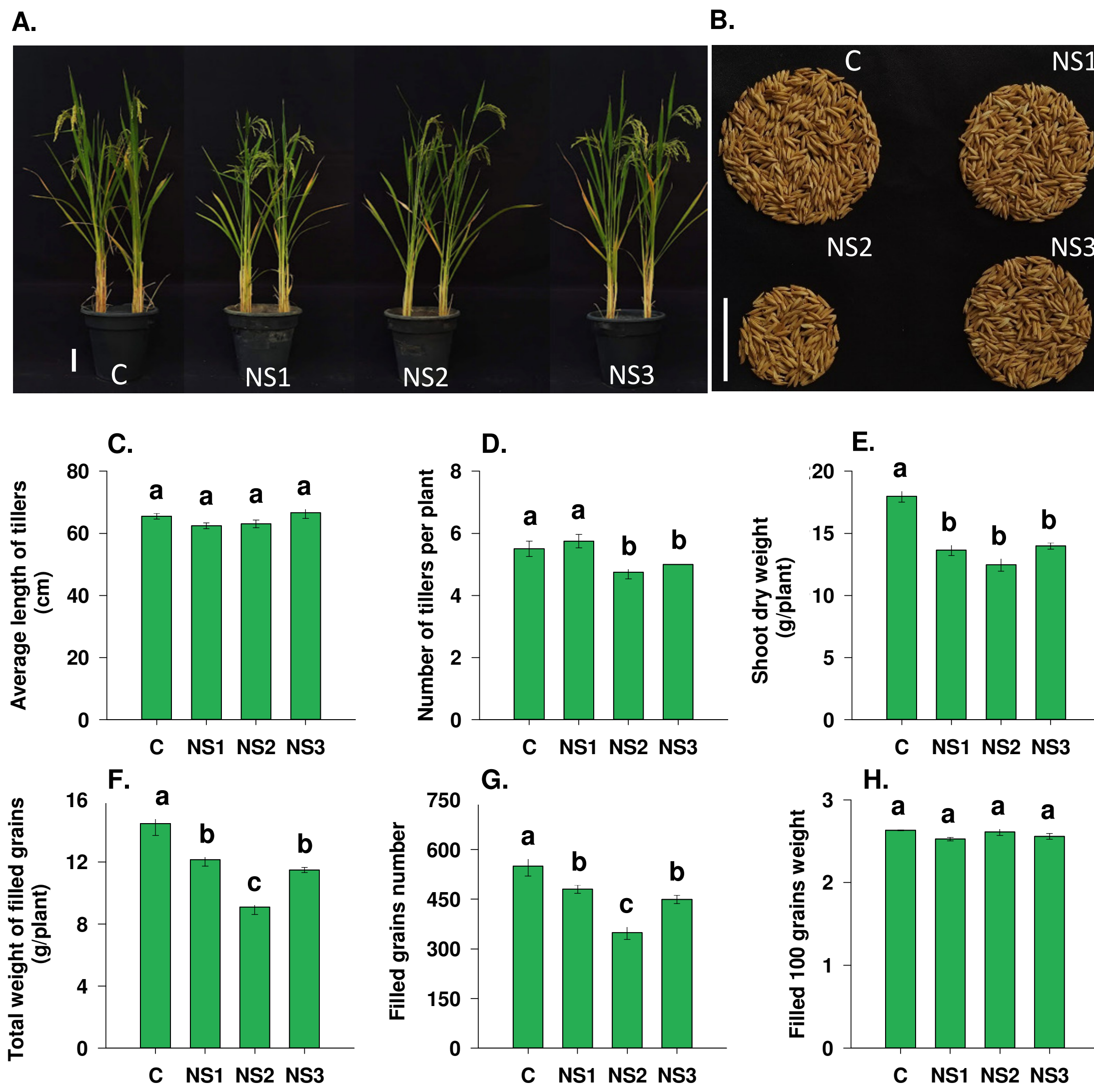

Figure 1. Phenotypic characterizations of rice plants (cv. Esmeralda) subjected to distinct N-stress regimes: NS1 (two successive N-sufficient cycles + one N-stress cycle); NS2 (one N-stress cycle + one N-sufficient cycle + one N-stress cycle); NS3 (three successive N-stress cycles). Control: (three successive N sufficient cycles). The results refer to the last cultivation cycle, in which NS1, NS2, and NS3 received fertilization amounts equivalent to 10 kg N ha-1, while the control plants received fertilization amounts equivalent to 60 kg N ha^{-1}. Scale bars, 10 cm in (A) and 5 cm in (B). Data represent the average of four biological replicates, and error bars represent standard error (SE). Different lowercase

letters between treatments within a column indicate statistical differences by the Scott-Knott test ($p < 0.05$).

2.5.4 Photosynthetic efficiency

Rice plants, when conditioned to different cycles of N stress, showed significant differences (appendix - Table 9) with respect to the photosynthetic parameters that were related to energy flux per reaction center (e.g., ABS/RC, Dio/RC, TRo/RC, and Reo/RC), quantum yields (e.g., (φ(Po), φ(Eo) and φ(Ro)) and performance of the photosynthetic apparatus (e.g., Piabs and Pitotal), which were obtained from the JIP test (Fig. 2). The values of the photosynthetic parameters were normalized by using the control (plants grown with sufficient N) as a reference. Thus, values smaller than those of the control indicated reductions in the photosynthetic capacity of plants conditioned to N stress. On the other hand, similar values indicated the maintenance of photosynthetic capacity.

Plants subjected to the NS1 treatment exhibited photosynthetic performances that were similar to plants in the control treatment during the initial growth phase (e.g., 14 and 29 DAE); anthesis (e.g., 64 to 68 DAE); and during the initial grain filling phase (e.g., 75 DAE) (Fig. 2). At 14 and 29 DAE (Fig. 2A and B), this behavior was evidenced by the maximum quantum yield of PSII (φ(Po)), by the quantum yield of electron transport (φ(Eo)), and by the partial (Piabs) and total photosynthetic performance index (Pitotal), respectively. During anthesis (Fig. 2D and E), the plants in the NS1 treatment showed several photosynthetic parameters that were similar to the control, particularly in the length of the antenna complex (ABS/RC), loss of energy by heat (Dio/RC), and energy flux trapped by RC (TRo/RC), as well as for φ(Po), Piabs and Pitotal. During the initial phase of grain filling (Fig. 2F), plants in the NS1 treatment had similar φ(Eo) and Pitotal values compared to the control treatment.

Subjecting plants to two stress cycles (NS2) reduced their photosynthetic efficiencies when compared to the control during nearly all phases of the crop cycle. The φ(Eo) values for NS2 were similar to the control in the early development (Fig. 2A) and grain filling stages (Fig. 2F) and for the Pitotal values during initial grain filling stage (Fig. 2F). During anthesis (Fig. 2D and E), the plants had Dio/RC values, a parameter related to energy loss by heat, and TRo/RC values that were significantly higher (appendix - Table 9) than the control and NS1 and NS3 treatments. This behavior was followed by a reduction in the maximum quantum yield of PSII.

Plants subjected to the NS3 treatment behaved similarly to the control during the early development (Fig. 2B), mid-cycle (Fig. 2C), and anthesis stages (Fig. 2D and E). At 29 DAE, only the total photosynthetic performance (Pitotal) was similar between plants in NS3 and the control groups (Fig. 2B). On the other hand, at anthesis, similar values were observed between the NS3 and control groups for most analyzed parameters, especially ABS/RC, Dio/RC, φ(Po), and Piabs (Fig. 2D and E).

A comparison of treatments NS1, NS2, and NS3 showed that plants in the NS1 and NS3 exhibited similar photochemical efficiencies over the longest growing cycle (Fig. 2 and appendix - Table 9). This behavior exhibited by NS1 and NS3 plants was also, for most of the cycles, similar to the control. On the other hand, NS2 was different from the other treatments (e.g., NS1 and NS3), as well as from the control, which showed lower photosynthetic efficiencies for plants subjected to two intermittent N stress cycles (NS2).

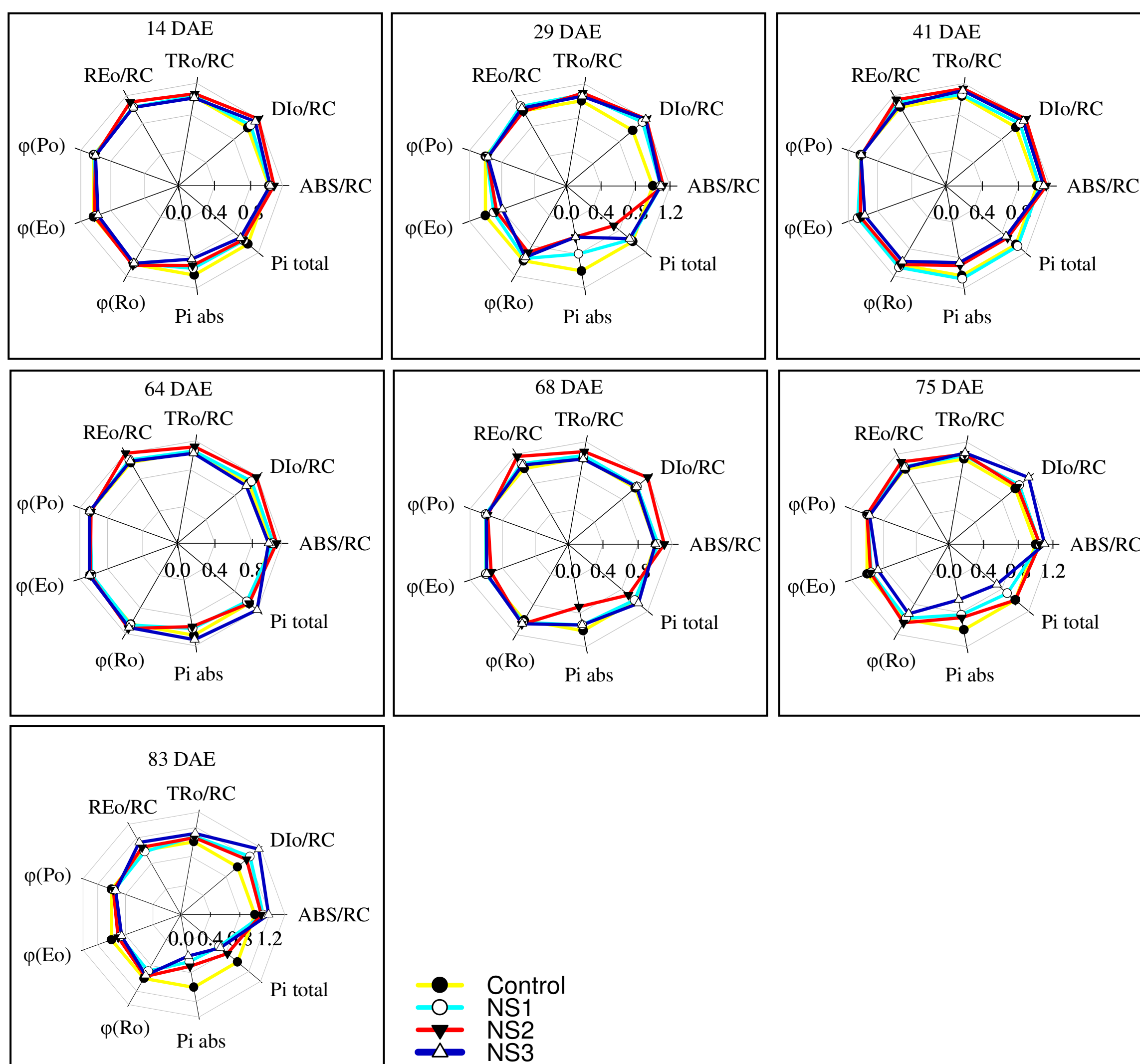

Figure 2. Photosynthetic parameters obtained from the chlorophyll "a" transient fluorescence levels in leaves of rice plants (cv. Esmeralda) subjected to distinct N-stress regimes: NS1 (two successive N-sufficient cycles + one N-stress cycle); NS2 (one N-stress cycle + one N-sufficient cycle + one N-stress cycle); NS3 (three successive N-stress cycles). Control: (three successive N sufficient cycles). The parameters were calculated using the JIP test and represent: i) energy fluxes ***Dio/RC*** energy flux dissipated as heat per reaction center (RC), ***ABS/RC*** RC absorption flux, ***TRo/RC*** maximum capture rate per RC, and ***REo/RC*** reduction flux of electrons in the final electron acceptor in photosystem I (PSI); ii) productivity ***φ(Po)*** photochemical maximum quantum yield, ***φ(Eo)*** quantum yield of electron transport from quinone A (Q_A-) to the electron acceptor intersystem, and ***φ(R_o)*** quantum yield of electron transport from Q_A- to the final PSI electron acceptor; iii) performance ***Piabs*** partial photosynthetic performance index and ***Pitotal*** total photosynthetic performance index. Yellow, light blue, red, and dark blue lines correspond to control, NS1, NS2, and NS3 treatments, respectively. ***DAE*** days after emergency.

2.5.4 N use and absorption

The treatments applied in this experiment promoted differences in those parameters associated with N use (Table 3), except for the remobilization efficiency (NRE) and N harvest index (NHI). The control plants exhibited higher uptake efficiency (NUpE), utilization (NUtE), and use (NUE) of N. Plants subjected to one and three stress cycles by N (e.g., NS1 and NS3) exhibited higher NUpE and NUE ($p < 0.05$) values than plants subjected to two alternating stress cycles (NS2), which showed the harmful effects of intermittent N stress on N absorption and use, which was similar to that observed for the photosynthetic efficiency parameters (Fig. 2).

Table 3. Parameters related to the nitrogen utilization of rice plants (cv. Esmeralda) subjected to distinct N-stress regimes: NS1 (two successive N-sufficient cycles + one N-stress cycle); NS2 (one N-stress cycle + one N-sufficient cycle + one N-stress cycle); NS3 (three successive N-stress cycles). Control: (three successive N sufficient cycles).

Treatments	NUpE	NUtE	NUE	NRE	NHI
Control	2.25±0.08a	87.70±7.67a	152.29±16.15a	0.22±0.10ns	1.65±0.14ns
NS1	1.74±0.11b	73.64±2.95b	127.74±8.70b	0.21±0.02	2.20±0.30
NS2	1.40±0.08c	68.16±3.86b	95.66±10.06c	0.28±0.08	1.76±0.29
NS3	1.63±0.06b	74.34±1.18b	120.79±3.37b	0.17±0.05	1.85±0.19

In which: ***NUpE*** N uptake efficiency; ***NUtE*** N utilization efficiency; ***NUE*** N use efficiency; ***NRE*** N remobilization efficiency; ***NHI*** N harvest index. Data represent the average ± SD of four biological replicates. Different lowercase letters between treatments within a column indicate statistical differences by the Scott-Knott test ($p < 0.05$). ***ns*** no significant difference.

2.5.5 Total protein and fractions in grains

The levels of total protein and protein fractions in the grains were significantly higher ($p < 0.05$) in plants subjected to cultivation with sufficient N (control) and did not differ among the applied treatments (Table 4). On the other hand, the prolamin contents in the grains of plants subjected to NS2 were higher ($p < 0.05$) than those in treatments NS1 and NS3 and were equal to the control treatment.

Table 4. Total protein contents and protein fractions in grains of rice plants (cv. Esmeralda) subjected to distinct N-stress regimes: NS1 (two successive N-sufficient cycles + one N-stress cycle); NS2 (one N-stress cycle + one N-sufficient cycle + one N-stress cycle); NS3 (three successive N-stress cycles). Control: (three successive N sufficient cycles).

Treatments	TP	Glu	Glob + Alb	Prol
	mg.g^{-1}			
Control	63.01±3.28a	35.66±2.90a	18.38±3.15a	4.04±0.30a
NS1	55.32±1.07b	22.69±2.07b	11.30±2.06b	3.17±0.88b
NS2	55.39±1.42b	25.07±1.86b	12.32±1.63b	3.70±0.16a
NS3	51.81±2.26b	21.71±1.14b	13.04±2.32b	2.57±0.40b

In which: ***TP*** Total protein; ***Glu*** Glutelin; ***Glob + Alb*** Globulins + Albumins; ***Prol*** Prolamins. Data represent the average ± SD of four biological replicates. Different lowercase letters

between treatments within a column indicate statistical differences by the Scott-Knott test ($p < 0.05$).

2.5.6 Metabolic profiles

To explore the metabolic changes caused by different N-stress cycles, an analysis of the metabolic profiles was conducted based on an established GC–MS protocol (LISEC et al., 2006) in flag leaves that were collected at the beginning of anthesis (64 DAE). Thirty-four compounds were identified, of which 12 were organic acids, 9 were sugars, 7 were amino acids and derivatives, and 6 were polyalcohols (appendix - Table 10).

Based on OrthoPLS-DA, which was used to facilitate our understanding of the alteration patterns in metabolic profiles by forming distinct groups, the formation of four groups was observed (Fig. 3). The control treatment differed strongly from the N-stress treatments. Differences were also observed among the N-stress treatments.

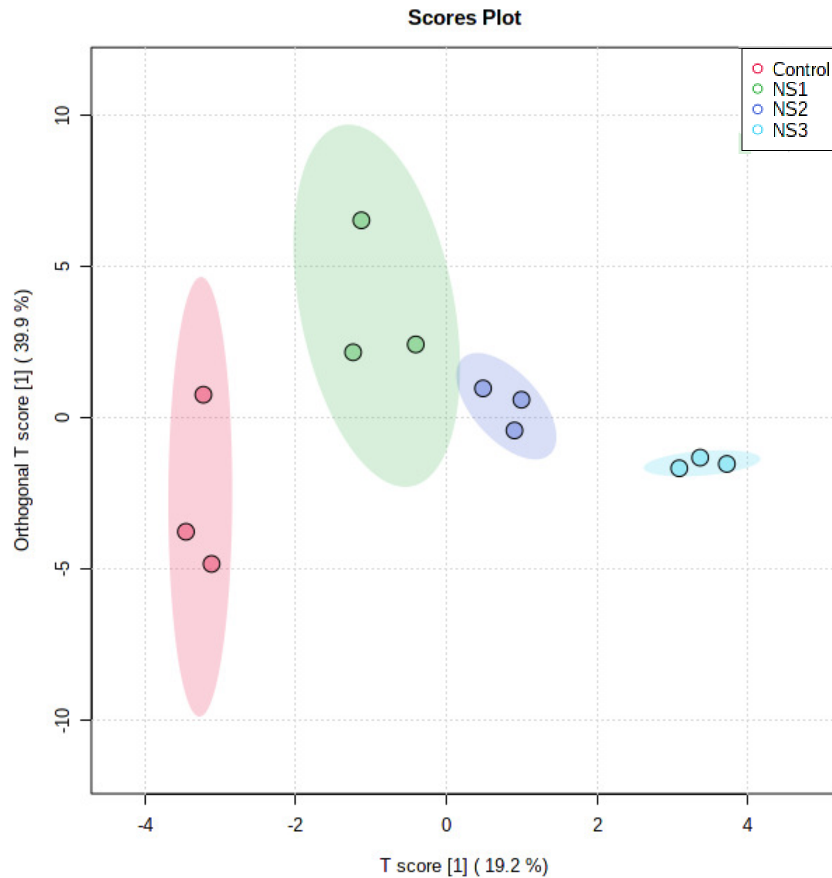

Figure 3. Cluster analysis by using orthogonal partial least squares-discriminant analysis (OrthoPLS-DA) of the metabolites identified in the flag leaves of rice plants (cv. Esmeralda) subjected to distinct N-stress regimes: NS1 (two successive N-sufficient cycles + one N-stress cycle); NS2 (one N-stress cycle + one N-sufficient cycle + one N-stress cycle); NS3 (three successive N-stress cycles). Control: (three successive N sufficient cycles). Only metabolites regulated differently by ANOVA test ($p < 0.05$) were used. Raw data be found in appendix – Table 10. Red, green, dark blue, and light blue colors correspond to control, NS1, NS2, and NS3 treatments, respectively.

ANOVA and the Scott–Knott test were used to identify the metabolites that experienced differential regulation. The contents of 11 metabolites were significantly altered (Fig. 4). Citric acid and isocitric acid were upregulated in all N-stressed plants, while alanine was downregulated. A comparison of the plants that were subjected to N stress (e.g., NS1, NS2, and NS3) showed that intermittent stress (NS2) and recurrent stress (NS3) promoted upregulation of lactic acid, oxalic acid, hexyl alcohol, palmitic acid, fructose, glucose, and sucrose. Plants subjected to only one N stress event (NS1) showed upregulation of erythronic acid.

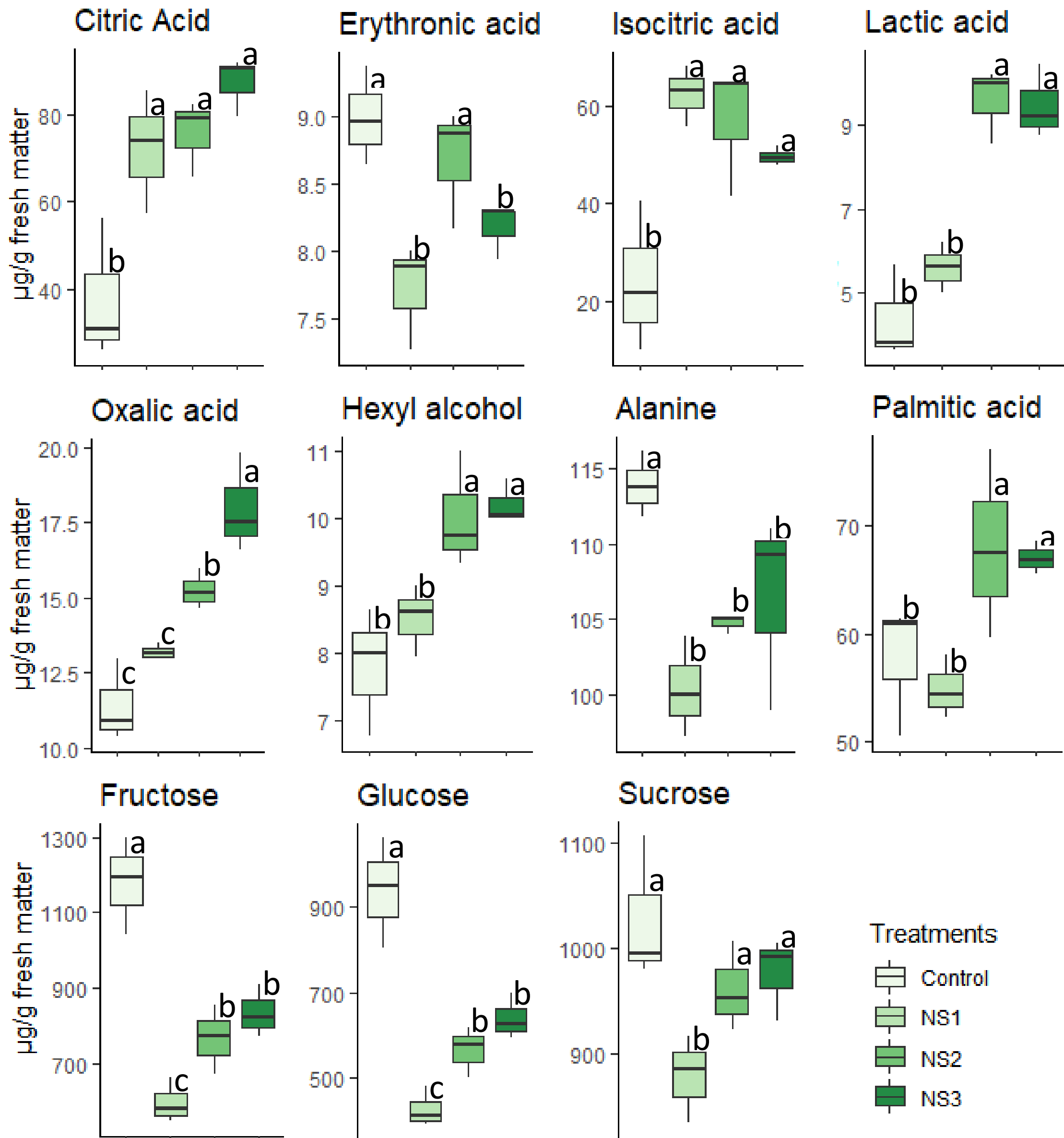

Figure 4. Metabolite contents in the flag leaves of rice plants (cv. Esmeralda) subjected to distinct N-stress regimes: NS1 (two successive N-sufficient cycles + one N-stress cycle); NS2 (one N-stress cycle + one N-sufficient cycle + one N-stress cycle); NS3 (three successive N-stress cycles). Control: (three successive N sufficient cycles). Data represent the average of three biological replicates, and error bars represent standard error (SE). Different lowercase letters between treatments within a column indicate statistical differences by the Scott-Knott test ($p < 0.05$).

2.5.7 Correlation analysis

A strong correlation was observed between the ratio of hemi-methylated and fully-methylated bands with photosynthetic (Fig. 5A), agronomic (Fig. 5C), and N use (Fig.5D) parameters. Overall, hemi-methylated bands showed positive correlation, while fully-methylated bands showed negative correlation with most morphophysiological parameters. No significant correlation was observed between methylation pattern and metabolites (Fig. 5B).

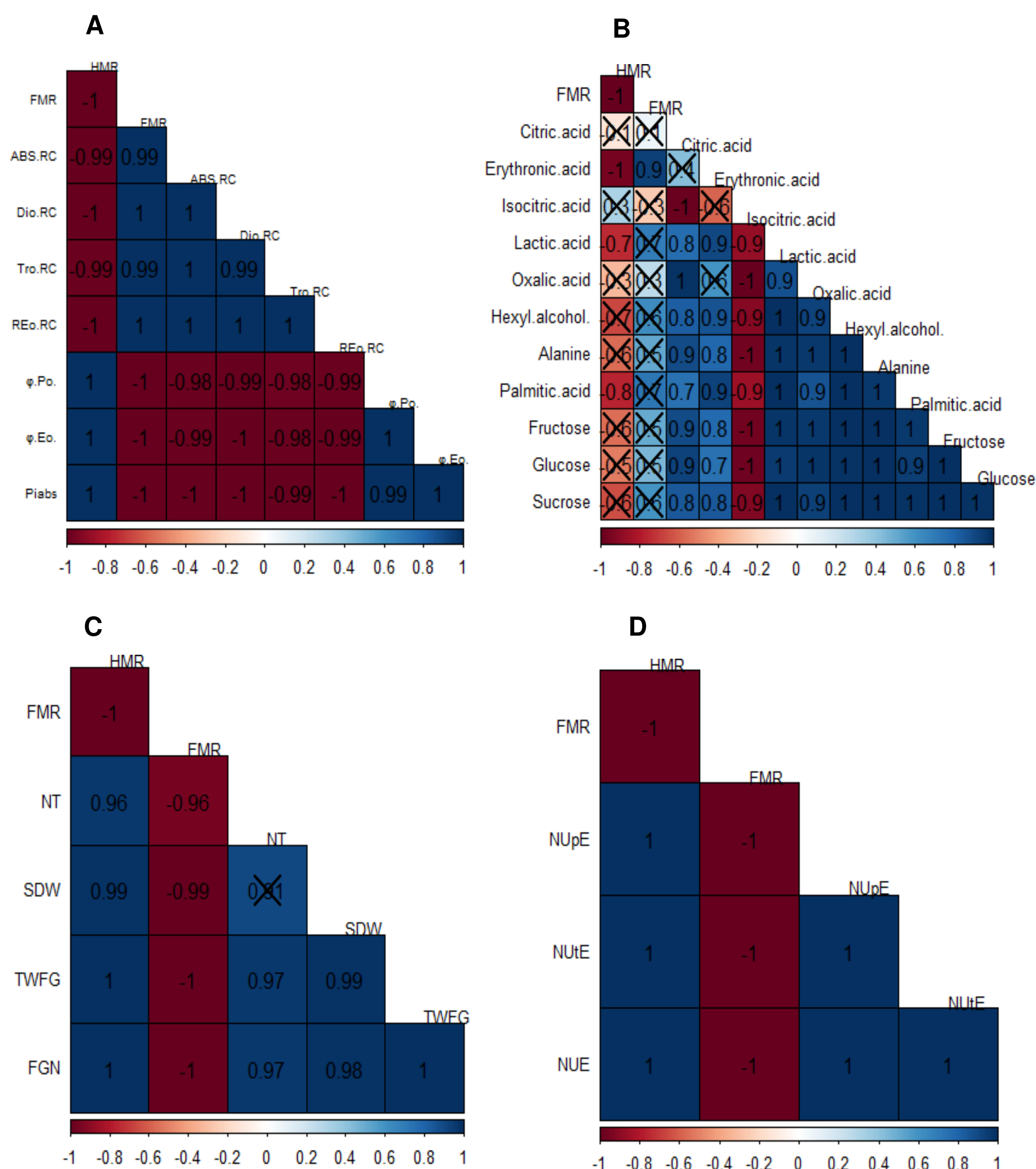

Figure 5. Pearson correlation between ratio of hemi-methylated and fully-methylated bands, and photosynthetic (A), metabolite (B), agronomic (C), and N use (D) parameters in rice plants (cv. Esmeralda) subjected to distinct N-stress regimes. ***HMR*** Hemi-methylated ratio**;** ***FMR*** Fully-methylated ratio**;** ***ABS.RC*** absorption flux per reaction center (RC)**;** ***Dio.RC*** energy flux dissipated as heat per RC**;** ***Tro.RC*** maximum capture rate per RC**;** **REo.RC** reduction flux of electrons in the final electron acceptor in photosystem I (PSI); ***φ(Po)*** photochemical maximum quantum yield**;** ***φ(Eo)*** quantum yield of electron transport from Quinone A (Q_A-) to the electron acceptor intersystem**;** ***Piabs*** partial photosynthetic performance index; ***NT*** Number of tillers per plant; ***SDW*** Shoot dry weight; ***TWFG*** Total weight of filled grains**;** ***FGN*** Filled grains number**;** ***NUpE*** N uptake efficiency; ***NUtE*** N utilization efficiency; ***NUE*** N use efficiency.

2.6 DISCUSSION

In comparison to the control group, plants subjected to N stress (e.g., NS1, NS2, and NS3) showed reductions in grain yields (Fig. 1), reductions in the values of parameters related to N use (Table 3) and reductions in protein contents in grains (Table 4). Significant changes were also observed in the metabolic profiles among the N-stress treatments and the control (Fig. 4). This behavior was expected, as it shows the efficiency of N-stress imposition throughout the crop cycles. The lower biomass production amounts, grain yields, grain protein levels, and N use efficiencies reported in this study are consistent with the effects of low N supplies that have already been reported in the literature, and these results indicate the importance of nitrogen to plants (HSIEH et al., 2018; XIONG et al., 2018; PEREIRA et al., 2021). Likewise, the reductions in sucrose and glucose concentrations can be viewed as direct consequences of lower N supplies, since nitrogen and carbon metabolism are strongly related. On the other hand, the increases in citric and isocitric acid concentrations may have contributed to the maintenance of important biological processes, such as energy production and antioxidant defense, as was also suggested by MATSUNAMI et al. (2020).

In general, plants conditioned to 1 and 3 cycles of N stress (e.g., NS1 and NS3, respectively) were more similar in terms of their changes in global DNA methylation patterns, phenotypic characteristics, and photosynthetic parameters (Table 1, Figs. 1 and 2). As they are sessile organisms, plants are subjected to a series of environmental stresses on a daily basis. Stresses such as drought, cold, salinity, and nutritional deficiency have already been described as abiotic factors that stimulate changes in DNA methylation (ZHAO and ZHOU, 2012). DO AMARAL et al. (2020) found that exposure to saline conditions decreased the overall DNA methylation patterns in salinity-tolerant rice plants. Similar behaviors were observed after exposure of maize plants to cold stress (SHAN et al., 2013). Nitrogen deficiency conditions also were indicated to be an abiotic factor capable of stimulating a 14.6% reduction in the methylation content of maize plants (MAGER and LUDEWIG 2018). However, rice plants did not undergo significant changes in methylation patterns when exposed to N deficiency (KOU et al., 2011). It was observed in this study that N stress promoted alterations in DNA methylation patterns (Tables 1 and 2). Furthermore, it was shown that the intensity and type of alteration (e.g., methylation/demethylation) were directly dependent on the number of cycles of adaptation to N stress. Adaptation to 1 and 3 stress cycles (e.g., NS1 and NS3, respectively) resulted in greater reductions in the total numbers of methylated bands (6.25% and 5.94%, respectively), which was mainly due to greater reductions in the numbers of hemi-methylated bands (e.g., 4.53% and 4.85%, respectively), which were followed by reductions of 1.71% and 1.09%, respectively, in the numbers of fully methylated bands (Table 1). NS2 promoted a reduction in the number of hemi-methylated bands (6.1%), but this reduction was followed by an increase in the number of fully methylated bands (2.59%). Analysis of the methylation profiles (Table 2) enabled the identification of some very interesting facts: i) it was observed that the NS1 and NS3 treatments stimulated higher DNA demethylation frequencies (e.g., 12.81% and 15.31%, respectively), while the NS2 treatment promoted a higher methylation frequency (e.g., 12.03%); ii) the first exposure to N stress stimulated a lower methylation frequency of 7.50%; iii) successive cycles of N stress (NS3) promoted the maintenance and intensification of the demethylation process, as shown by the 2.5% increase in the NS3 group compared to the NS1 group. Although the results point to the emergence of “n "epigenetic mem”ry" to N stress, it is not yet possible for us to make this statement based only on the data obtained in this paper; iv) exposure to intermittent N stress (NS2) did not result in increases in the demethylation process as were observed in the NS3 group, which allows us to speculate that the regular supply of N between N-stress cycles was sufficient to

alter the epigenome of the NS2 plants, which showed increases in methylation frequency. Epigenetic memory is possible through the transgenerational passage of chemical marks (FORESTAN et al., 2020). Through this process, it is possible to create epialleles, which can cause a plant to be more or less tolerant to a given stress event. The ability to transmit epigenetic marks and, consequently, create this memory is inherent to each species and depends on the frequency of exposure to a stressful situation (LI et al., 2021). For example, adaptive traits acquired by rice plants conditioned to N stress were followed by epigenetic inheritance of the cytosine methylation pattern (Kou et al. 2011). FORESTAN et al. (2020) demonstrated the presence of stress memory in maize plants that were subjected to drought stress followed by a recovery period, and in addition, three categories of stress memory genes were identified: transcriptional memory genes, candidate genes for stress memory, and delayed memory genes. On the other hand, SECCO et al. (2015) found no evidence of transgenerational inheritance of acquired methylation changes in rice plants stressed by poor phosphorus supplies.

DNA demethylation and methylation are directly related to gene activation or repression (TANG et al. 2014). Demethylation allows specific regions of the gene, such as promoter regions, to become more accessible to proteins that are responsible for the transcription process. On the other hand, methylation, which can occur both in gene bodies and in promoter regions, can cause chromatin silencing (ZHAO and ZHOU, 2012). Although we have not yet explored the patterns of methylation change in each gene, which would allow us to establish a relationship among methylation changes with the expressions of specific genes and the associated physiological and metabolic processes, it is reasonable to infer that the changes in the overall methylation patterns observed in this study certainly caused changes in gene expression, given the relationship between cytosine methylation and gene expression (BOYKO and KOVALCHUK., 2008; DO AMARAL et al., 2020; MAGER AND LUDEWIG., 2018; SECCO et al., 2015). The metabolic, phenotypic, and N use changes, as well as the protein contents in the grains obtained in this study, were certainly caused by changes in gene expression that were due to differential methylation induced by N stress.

The contrasting behaviors in the methylation patterns exhibited by plants subjected to intermittent N stress (NS2) with respect to the NS1 and NS3 were also observed in the phenotypic and photosynthetic parameters. Plants conditioned to NS2 had lower photosynthetic efficiencies, while NS1 and NS3 plants maintained photosynthetic efficiencies that were similar to those of the control plants (Fig. 2). This reduced photosynthetic efficiency observed in NS2 was more intense during anthesis (Figs. 2D and E), which may have caused lower availabilities of metabolites in the following stages, such as during grain filling, which would explain the lower grain yields (Fig. 1F and G). The analysis of photosynthetic efficiency using the measurement of chlorophyll "a" fluorescence is based on how plants use absorbed energy. This energy may be used for photosynthetic reactions dissipated by heat and/or be re-emitted as fluorescence (KALAJI et al., 2017). These three forms occur simultaneously and in different proportions, and environmental imbalances such as abiotic stresses can alter these proportions. Although chlorophyll "a" fluorescence represents only 0.5–10% of the absorbed energy, its intensity is inversely proportional to the fraction of energy used in photosynthesis. Thus, it can be used to estimate the photosynthetic efficiency (KALAJI et al., 2017). It can also be used to monitor regulatory processes that affect the PSII antenna complex, as chlorophyll a fluorescence also exhibits an inverse behavior of energy dissipation in the form of heat (KRAUSE and WEIS, 1991). The increases in absorption flux (ABS/RC) and energy trapping (TRo/RC) that were observed in the NS2 plants during anthesis (Figs. 2D and E), which were followed by greater heat energy dissipation (Dio/RC), resulted in lower PSII quantum efficiencies (φ(Po)) and, consequently, reductions in the photosynthetic performance index (Piabs). The rise in energy dissipation through heat, as

observed in NS2 plants during anthesis (Figs. 2D and E), serves as an indicator of the loss of photosynthetic efficiency, as it impacts the stability of the thylakoid membrane (KALAJI et al. 2017). The increased energy dissipation in the form of heat has been identified in plants subjected to stress conditions. (DE CARVALHO et al. 2021), for example, reported a substantial increase in heat energy dissipation in Digitaria insularis plants after herbicide application, which suggested a severe reduction in the photosynthetic capacity of this weed and, consequently, its effective control. Another good indicator for decreased photosynthetic efficiencies in plants is the φ(Po) parameter, as it provides the maximum PSII quantum yield. The reductions in φ(Po) observed in NS2 plants are not consistent with the increased energy dissipation in the form of heat and reinforces the reductions in photosynthetic efficiency found in these plants, particularly during anthesis (Figs. 2D and E). Reductions in φ(Po) were also observed in rice plants subjected to cadmium (MA et al., 2016) and nitrogen (HUANG et al., 2004) stresses, as well as in watermelon plants grown under water stress conditions (MALAMBANE et al. 2021).

The metabolic profiles of plants subjected to N stress were affected. Ortho-PLSDA analysis, which was conducted using the 34 compounds identified by GC–MS, showed the formation of four distinct groups, which displayed the metabolic differences that existed among control plants and plants subjected to N stress, as well as among the different N stress levels (Fig. 3). The comparison in plants that underwent only one N-stress cycle (NS1) with plants submitted to two or three cycles (NS2 or NS3, respectively) showed that an increase in the number of N stress cycles stimulated greater changes in metabolite concentrations (Fig. 4). Although NS2 and NS3 presented similar metabolic alterations, it can be inferred that for NS2, these alterations were not beneficial because of the decreased photosynthetic efficiency (Fig. 2) and grain yields (Fig. 1) that were previously described. On the other hand, the different patterns of metabolic alterations exhibited by NS1 and NS3 plants were equally beneficial. Thus, the metabolic alterations were directly influenced by the number of cycles of conditioning to N-stress.

Finally, a correlation analysis showed significant linkage between methylation patterns and morphophysiological variables, further explaining the differences between N-stress treatments. It was found that fully methylated bands correlate negatively and hemi-methylated bands correlate positively with most morphophysiological variables (Figs. 4A, C, and D). In NS2 a higher percentage of fully methylated bands and a lower percentage of hemi-methylated bands were observed (Table 1), explaining the lower behavior of NS2 compared to the other N-stress treatments.

2.7 CONCLUSIONS

We showed in this study that N stress induces both DNA demethylation and methylation in proportions that depend on the stress regimes to which plants are subjected. The demethylation/methylation intensities depend on the stresses experienced by rice plants. Plants that recurrently experience N stress (NS3) have higher frequencies of DNA demethylation/methylation than plants that experience N stress for the first time (NS1), which suggests the occurrence of progressive changes in the epigenome of plants subjected to recurring N stress. On the other hand, intermittent N stress (NS2) promotes a methylation pattern that is distinct from the NS1 and NS3 plants, which was found to be harmful to rice plants.

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3 CHAPTER II

CONDITIONING TO LOW-NITROGEN REGIMES RESULTS IN RICE PLANTS BETTER ADAPTED TO LOW-NITROGEN STRESS

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3.1 RESUMO

Deficiência de nitrogênio (N) é um estresse abiótico ao qual as plantas de arroz estão susceptíveis. O objetivo desse estudo foi investigar se a exposição de plantas de arroz, var. Manteiga e Piauí, a ciclos completos de cultivo com baixo estresse por baixo N resulta em plantas melhor adaptadas a essa condição. As plantas foram cultivadas durante três ciclos de acordo com as seguintes condições: controle, N-suficiente (60 Kg N ha^{-1}) durante os três ciclos; NS1, exposição ao estresse por N (10 Kg N ha^{-1}) somente no último ciclo; NS2 (estresse intermitente), exposição ao N-stress durante o primeiro e terceiro ciclo; e NS3 (estresse recorrente), exposição ao N-stress durante os três ciclos. Plantas submetidas aos tratamentos NS2 e NS3 apresentaram aumento da razão de bandas totalmente metiladas e diminuição da razão de bandas hemi-metiladas. Essa modificação foi mais significante nos tratamentos NS2 e NS3 do que em NS1, os quais apresentaram maior eficiência de uso de N, eficiência fotossintética, rendimento e qualidade dos grãos. Os dados obtidos nesse estudo demonstram que a exposição de plantas de arroz ao estresse intermitente e recorrente por N promove alterações moleculares, fisiológicas e metabólicas. Essas alterações em conjunto melhoram a adaptação ao estresse por N e promovem aumento no rendimento e a qualidade dos grãos.

3.2 ABSTRACT

Nitrogen (N) deficiency is abiotic stress to which rice plants may be frequently exposed. The aim was to investigate whether the exposure of rice plants to complete cultivation cycles with low-N stress results in plants better adapted to this condition. The plants were grown for three cycles under the following conditions: control, sufficient N (60 kg N ha^{-1}) in all three cycles; NS1, exposed to N stress (10 kg N ha^{-1}) only in the third cycle; NS2 (intermittent stress), exposed to N stress in the first and third cycles; and NS3 (recurrent stress), exposed to N stress in all three cycles. Plants subjected to the N stress treatments increased the ratio of fully methylated bands and decreased the ratio of hemimethylated bands. This change was more significant in the NS2 and NS3 than in the NS1 treatment, which showed greater N use efficiency, photosynthetic efficiency, and grain yield and quality. The data obtained in this study show that exposure of rice plants to intermittent and recurrent N stress (i.e., the NS2 and NS3 treatments, respectively) promotes molecular, physiological, and metabolic changes, which together improve N-stress adaptation and result in greater grain yield and quality compared to that in NS1.

3.3 INTRODUCTION

Rice is a staple food for more than half of the world's population, especially for people living in poverty (FAN et al., 2021). Rice cultivation requires a greater input of nitrogen fertilizers than other crops (ZHAO et al., 2018b). However, this high demand for nitrogen (N), combined with the low efficiency of the nitrogen supply and, sometimes, with an insufficient supply of N, can result in abiotic stress and cause severe reductions in the growth of rice and grain yield (KOU et al., 2011; XIONG et al., 2018).

With the green revolution, the intensive use of fertilizers began to be adopted as a way to increase the productivity of agricultural crops (KHUSH, 2001). However, this process has always been expensive and environmentally harmful (FAGODIYA et al., 2020; LIU et al., 2021). From 1966 to 2016, the global consumption of nitrogen fertilizers increased approximately fifty-fold (ZHANG et al., 2020). In a future scenario in which the effects of climate change are intensified, studies point to a reduction in the internal efficiency of N use (iENU) in rice crops, which will increase the need for N absorption and incentives for the use of nitrogen fertilizers (YULONG et al., 2021). Thus, it is necessary to search for viable strategies to circumvent the imminent increase in the amounts of nitrogenous mineral fertilizers applied to the soil.

Abiotic stresses may be intermittent, recurrent or transient. Studies have shown that successive stresses can prepare plants for adverse future conditions (NEVES et al., 2017; SECCO et al., 2015). Adaptation to stress can occur through the storage of information about the stimulus, thus generating a memory of stress (KOU et al., 2011; SANTANA-VIEIRA et al., 2016; ZHENG et al., 2017). This memory is associated with biochemical and physiological changes and molecular processes, such as the addition or removal of epigenetic markers (KOU et al., 2011; ZHAO and ZHOU, 2012).

Epigenetic modifications play an important role in the tolerance of living organisms to environmental stresses (BAULCOMBE and DEAN, 2014; KUMAR, 2019; WU et al., 2020). Different epigenetic markers are acquired during a stress event, which may be transient, disappear after stress, or be inherited by subsequent generations, often resulting in phenotypic variations (KUMAR, 2018). DNA methylation at the fifth cytosine (5m'C) is a well-documented epigenetic marker in plants, being found in the context of the CG, CHG and CHH sequences, where H is A, C or T (BHATTARAI et al., 2021). Methyltransferase activity is required for the addition of a methyl group (CH_3) to DNA, while the removal of CH_3 is mediated by demethylases (LI et al., 2021a). The position in which epigenetic markers, such as methylation, are added to the genes directly influences gene activation or repression and, consequently, the behavior of plants under specific stresses (CHANG et al., 2020).

Despite relatively recent advances, the positive effects of successive N-stress events are still poorly studied for rice crops. KOU et al. (2011) showed that the progeny of rice plants that underwent stress experiences due to low N supply were more tolerant to the same stress, suggesting the transgenerational inheritance of an acquired trait. WU et al. (2020) demonstrated the possibility of more sustainable rice production via nitrogen-responsive chromatin. The hypothesis addressed by this study is that rice plants conditioned to low N supply during entire cultivation cycles present molecular, physiological and metabolic changes, which together contribute to the better performance of these plants when subjected again to low N conditions. The objectives of this study were to determine possible changes in the overall profile of DNA methylation, morphophysiological and metabolic parameters, gene expression, N use efficiency, and quality and yield of rice, induced by different N stress treatments.

3.4 MATERIAL AND METHODS

3.4.1 Plant materials and experimental design

Rice seeds (*Oryza sativa* L.) of the varieties Manteiga and Piauí, two traditional rice varieties from the state of Maranhão, Brazil, were obtained from the germplasm bank of the Laboratory of Plant Mineral Nutrition (LNMP) of the Federal Rural University of Rio de Janeiro (Universidade Federal Rural do Rio de Janeiro (UFRRJ)). The reason for using two genotypes was to increase the robustness of the study. Both varieties have already been widely studied by our research group and show some contrasting morphophysiological characteristics (FERREIRA et al., 2020; PEREIRA et al., 2022, 2021).

Seeds were disinfected with 2.5% sodium hypochlorite (v/v) for 1 hour, washed three times with distilled water and placed in plastic pots (0.7 L) containing distilled water to germinate. Five days after germination (DAG), the seedlings were transferred to pots (8 L) containing soil collected from the A horizon of a Planosol and grown in a greenhouse (photoperiod and natural irradiance; temperature 28-32 °C). Before the beginning of the experiment, chemical analysis of the soil was performed, and the need to correct the potassium content was observed (appendix – Table 7). This correction was performed according to FREIRE et al. (2013) through fertilization with potassium sulfate (K_2SO_4). At 10 DAG, the plants were separated into four groups, arranged according to the completely randomized experimental design (DIC), with two plants per pot and eight replicates per treatment: the plants in Group 1 were grown with sufficient N (60 kg N ha^{-1}) for three complete crop cycles; the plants in Group 2 were grown with sufficient N during the first and second cycles and subjected to N stress due to low N (10 kg N ha^{-1}) in the third cycle; the plants in Group 3 were subjected to N stress in the first and third cycles, with cultivation under sufficient N in the second cycle; and the plants in Group 4 were subjected to N stress during all three cultivation cycles. Thus, Group 1 represented the control treatment, i.e., plants that were not subjected to N stress; Group 2 represented the NS1 treatment, with plants undergoing N stress only once; Group 3 represented the NS2 treatment, with plants undergoing intermittent N stress; and Group 4 represented the NS3 treatment, with plants undergoing recurrent N stress (appendix – Fig. 13).

Sufficient N was established by applying 60 kg N ha^{-1} urea equivalent. To create N stress conditions, the N concentration applied was 10 kg N ha^{-1}. Seeds collected at the end of the first cultivation cycle were used to conduct the second cycle, and seeds collected at the end of the second cycle were used to conduct the third cycle. All the data shown in this article were collected from the third cultivation cycle. At 79 days after emergence (DAE), plants from four replicates were collected for analysis of the overall DNA methylation profile, total nitrogen content, metabolic profile and gene expression. The other four replicates were maintained until the end of the cycle to determine the variables described below.

3.4.2 Phenotypic characterization

Plants were harvested at the end of the third cultivation cycle, and the number and average length of tillers, the shoot dry weight, the number and total weight of full grains, and the weight of 100 full grains per plant were determined.

3.4.3 Proteins in the grains

The sequential extraction of protein from the grains was performed according to Doll & ANDERSEN (1981) and TURLEY and CHING (1986). The albumin + globulin fractions were extracted with a saline solution (2.9% NaCl + 0.002% Na-EDTA). Next, the prolamine fraction was extracted by adding alcohol solution (50% isopropanol, 41 mM boric acid, 0.6% 2-mercaptoethanol) to the salt extraction residue. Finally, the glutelin fraction was extracted by adding an alkaline solution (0.48% boric acid + 0.4% NaOH) to the residue of the alcoholic solution. The protein fractions were quantified according to BRADFORD (1976).

3.4.4 DNA extraction and analysis of DNA methylation-sensitive amplified polymorphisms (*MSAPs*)

For analysis of the methylation profile, at 79 DAE, samples of flag leaves were collected from plants of four replicates. These samples were then mixed to provide a pool for MSAP analysis. Next, genomic DNA was extracted according to the CTAB method described by MURRAY and THOMPSON (1981). The quality of the DNA was verified in agarose gel (1.5%) and in the NanoDrop 2000c spectrophotometer (Thermo Scientific).

The overall methylation profile was determined using the MSAP technique according to TANG et al. (2014), with modifications. This technique is based on the difference in sensitivity that the Msp I and Hpa II restriction enzymes have to methylated DNA. The two enzymes recognize the 5´-CCGG-3´ tetranucleotide sequence. However, HpaII cleaves the hemi-methylated sequence (only one methylated strand), while Msp I is active when the internal cytosines are methylated in one strand or both strands. Thus, hemimethylated external cytokines and fully methylated internal cytosines at the 5´-CCGG-3´ sites can be unequivocally distinguished by Hpa II and Msp I, respectively TANG et al. (2014). Two combinations of enzymes were used for MSAP analysis: Eco RI/Hpa II and Eco RI/Msp I. Information about the adapters, preamplification primers and selective amplification are provided in appendix – Table 8.

A total of 12 primer combinations were used for selective amplification of the fragments. The PCR products of the MSAP fragments were separated on a 6% acrylamide gel and visualized by silver staining KOU et al. (2011). These fragments were classified into four types according to their presence or absence in the acrylamide gel: Type I, bands present for both combinations of enzymes (Eco RI/Hpa II and Eco RI/Msp I); Type II, bands present only for the Eco RI/Hpa II combination; Type III, bands present only for Eco RI/Msp I; Type IV, the absence of bands for both combinations of enzymes (Eco RI/Hpa II and Eco RI/Msp I). Type I bands indicate the absence of methylation; Type II bands represent the hemimethylated state of the 5´-CCGG-3´ site in only one of the DNA strands; Type III bands represent complete methylation of the internal cytosine in both strands; Type IV bands represent complete methylation of the external cytosines in both strands (SHAN et al., 2013).

3.4.5 Real-time quantitative RT–PCR (qRT–PCR) analysis

Flag leaves collected 79 DAE were used for qRT–PCR analysis. Total RNA was extracted according to GAO et al. (2001) using NTES buffer (0.2 mM Tris-HCl pH 8.0; 25 mM EDTA pH 8.0; 0.3 mM NaCl; and 2% SDS). The quantification of total RNA was performed with a Nanodrop 2000c spectrophotometer (Thermo Scientific), and the quality was assessed using the A260/230 and A260/A280 ratios and visualization in a 1% agarose gel. DNase treatment and cDNA synthesis were performed using DNase I (Sigma–Aldrich) and a High-capacity RNA-to-cDNA Kit (Thermo Scientific), respectively, according to the

manufacturer's instructions. qRT–PCR was performed in the StepOnePlus Real-Time PCR System (Applied Biosystems) using the SYBR Green PCR Master Mix Kit (Applied Biosystems, Inc.). *OsAct1* and *OsEf1-α* were used as endogenous controls. The primers used in this study are listed in appendix – Table 11. Relative gene expression was calculated according to LIVAK and SCHMITTGEN (2001).

3.4.6 Total N and N use parameters

The total N content was determined in the shoots of plants collected in the reproductive (79 DAE) phase, as well in shoots and grains collected in the ripening (108 DAE) phase. The plant material and the dehulled grains were dried in an oven with forced air circulation at 65 °C for 3 days. Then, this material was ground, separating a fraction of 0.2 grams per sample, and the total N content was determined according to the Kjeldahl method (TEDESCO et al. 1995). After obtaining the total N content, the parameters related to the use of N were quantified according to COELHO et al. (2016). The following formulas were used: i) N uptake efficiency (NUpE): total N of the aerial part (including panicles)/N supplied; ii) N utilization efficiency (NUtE): weight of full grains/N supplied; iii) N use efficiency (NUE): full grain weight/aerial part N content; iv) N remobilization efficiency (NRE): N remobilization to grains/total N in anthesis; v) N harvest index (NHI): N in grains/total N in the straw at maturity.

3.4.7 Analysis of chlorophyll *a* fluorescence

Chlorophyll a transient fluorescence was measured in dark-adapted green leaves using a HandyPEA fluorimeter (Plant Efficiency Analyzer, Hansatech Instruments Ltd, UK). The measurements were performed at the vegetative (29, 44, 56 DAE), reproductive (79 DAE), and ripening (83 and 90 DAE) phases. Eight measurements were performed per treatment. In the vegetative phase, fluorescence was measured in the middle third of the most expanded leaves. In the reproductive and ripening phases, the measurements were performed in the middle third of the flag leaves. Adaptation to dark was always performed in the morning for 40 minutes with appropriate clips. The emission of fluorescence was induced by the exposure of a leaf area with a diameter of 4 mm to a pulse of saturating light at 3 mmol-2 s-1. From the transient fluorescence curve obtained after the saturating light pulse, the fluorescence intensities determined at 50 μs (initial fluorescence - F0), 100, 300 μs, 2 (FJ) and 30 (FI) ms and maximum fluorescence (FM) were used to calculate the photosynthetic parameters established by the JIP test (TSIMILLI-MICHAEL, 2020; TSIMILLI-MICHAEL and STRASSER, 2008).

3.4.8 Metabolome analysis

The metabolite contents were obtained from flag leaves collected in the reproductive phase (79 DAE). After collection, the leaves were frozen in liquid nitrogen and stored at -80 °C until analysis. The extraction was performed according to LISEC et al. (2006), with minor modifications, by adding the appropriate buffer and ribitol (internal standard) to 250 mg of tissue macerated in liquid nitrogen. Metabolites were analyzed with a GC/MS QP-2010 Plus instrument (Shimadzu, Japan). A Factor Four/VF-5 ms column (30 x 0.25 x 0.25) was used. The equipment operating conditions were ionization at 70 eV, mass range of 40-800 m/z, column oven temperature of 70 °C, injection temperature of 230 °C, injection mode split, and column flow of 1 mL/min helium gas. The retention index was calculated based on the retention index of two series of n-alkanes (C8-C 20 and C21-C40). Chromatograms were exported from the GC–MS solution (version 4.42) to R software. Peak detection, retention

time alignment and library matching were performed using the Target Search R package (CUADROS-INOSTROZA et al., 2009). The semi-quantitative evaluation of GC-chromatograms was obtained by normalizing the intensity of each peak by factors obtained from the intensity of the internal standard in each sample. To analyze the metabolic pathways affected by the N-stress treatments, the metabolites identified using Target Search software were subjected to analysis of variance (ANOVA) and the Scott-Nott test. Then, a spreadsheet was created of the differentially abundant metabolites ($p < 0.05$). This spreadsheet was exported to the MetaboAnalyst web-based platform (http://www.metaboanalyst.ca/), where the metabolic pathways were analyzed.

3.4.9 Statistical analyses

The normality and homoscedasticity of the data were determined according to the Lilliefors and Cochran tests, respectively. Next, ANOVA was applied using the F test ($p < 0.05$), and the means were compared using the Scott–Knott test. Statistical analyses were performed in R software (R CORE TEAM, 2019). Bar graphs were made in SigmaPlot. Heatmaps and pie charts were obtained in R software and Excel 2016, respectively.

3.5 RESULTS

3.5.1 Phenotypic parameters and grain production

The N-stress treatments promoted significant changes in the growth and grain production of the studied varieties (Fig. 6). Plants of the Manteiga variety that were subjected to the N-stress treatments showed a significant reduction in shoot dry weight and lower tillering compared to those in the control treatment, except NS3, which did not show a significant reduction in the number of tillers (Figs. 6A, B). Plants of the Piauí variety that were subjected to N-stress treatments also showed a significant reduction in shoot dry weight when compared to that of the control (Fig. 6A). This reduction was more pronounced in plants subjected to NS1, accompanied by a lower average tiller length (Fig. 6B). Regarding grain production, plants of the varieties Manteiga and Piauí subjected to the NS2 and NS3 treatments had grain production values significantly higher than plants subjected to the NS1 treatment and similar to those of plants in the control treatment (Fig. 6E). This pattern was also observed for the number of full grains of the Manteiga variety (Fig. 6D).

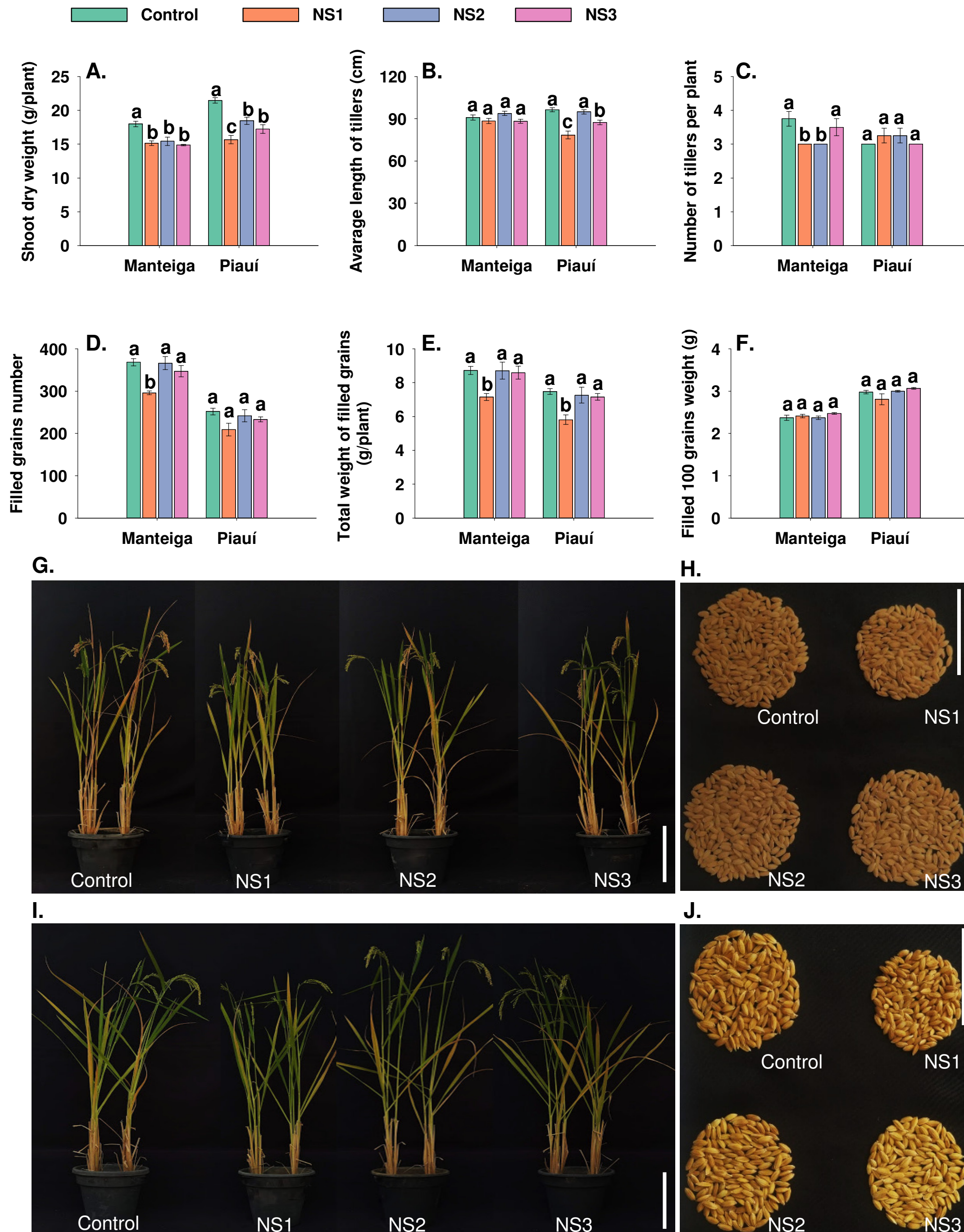

Figure 6. Phenotypic characterization of the varieties Manteiga and Piauí subjected to N-stress regimes. NS1 (1st and 2nd cycles: sufficient N; 3rd cycle: N stress); NS2 (1st cycle: N stress; 2nd cycle: sufficient N; and 3rd cycle: N stress); NS3 (1st, 2nd and 3rd cycle: N stress). C - control: (1st, 2nd and 3rd cycle: sufficient N). The results refer to the third cultivation cycle; NS1, NS2, and NS3 received fertilizer equivalent to 10 kg N ha^{-1}, while control plants received fertilizer equivalent to 60 kg N ha^{-1}. Figures G and H refer to the Manteiga variety, while Figures I and J refer to the Piauí variety. The data represent the averages of four biological replicates. The error bars represent the standard errors (SEs). Different lowercase letters indicate significant differences according to the Scott–Knott test ($p < 0.05$) between treatments for each variety separately. Scale bars: 30 cm in (G, I) and 15 cm in (H, J).

3.5.2 Proteins in grains

There were no significant changes in total protein content between the control and the N-stress treatments, except for the Manteiga variety in the NS2 treatment (Fig. 7A, B). On the other hand, for both varieties, the glutelin content was significantly higher in the grains of plants subjected to the NS2 treatment than in grains of plants subjected to the NS1 and NS3 treatments. Compared to the NS1 treatment, a higher prolamine content was observed in the grains of the Manteiga variety among plants subjected to the NS2 and NS3 treatments (Fig. 7A) and of the Piauí variety among plants subjected to the NS2 treatment (Fig. 7B). Plants of the Piauí variety subjected to the NS3 treatment had a higher globulin + albumin content than those subjected to the NS1 and NS2 treatments.

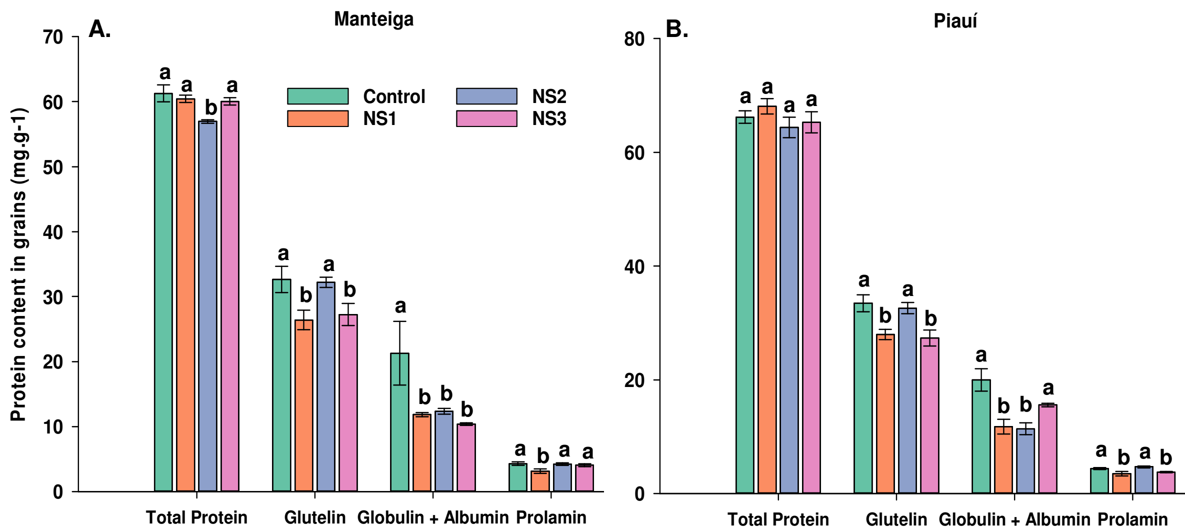

Figure 7. Total protein and protein fractions in rice grains of the varieties Manteiga (A) and Piauí (B) subjected to N-stress regimes. NS1 (1st and 2nd cycles: sufficient N; 3rd cycle: N stress); NS2 (1st cycle: N stress; 2nd cycle: sufficient N; and 3rd cycle: N stress); NS3 (1st, 2nd and 3rd cycle: N stress). C - control: (1st, 2nd and 3rd cycle: sufficient N). The results refer to the third cultivation cycle; NS1, NS2, and NS3 received fertilizer equivalent to 10 kg N ha^{-1}, while control plants received fertilizer equivalent to 60 kg N ha^{-1}. The data represent the averages of four biological replicates. The error bars represent the standard errors (SEs). Different lowercase letters indicate significant differences according to the Scott–Knott test ($p < 0.05$) between treatments.

3.5.3 MSAP analysis

Using 12 combinations of selective primers, it was possible to amplify a total of 639 and 682 bands in the Manteiga and Piauí varieties, respectively (Table 5). A total of 237, 272, 278 and 263 methylated bands were identified in the Manteiga variety for the control, NS1, NS2 and NS3 treatments, respectively. In the Piauí variety, a total of 192, 232, 284 and 276 methylated bands were identified for the control, NS1, NS2 and NS3 treatments, respectively. These results demonstrate that N stress induced an increase in DNA methylation, and this increase was more intense in plants subjected to intermittent N stress (i.e., the NS2 treatment).

Regarding the hemimethylated band ratio, treatments NS2 and NS3 caused a greater reduction in the frequency of these bands in both varieties compared to the control or NS1. Interestingly, the NS1 treatment induced an increase in the number of hemimethylated bands in the Piauí variety, unlike the Manteiga variety, for which there was a reduction.

The N-stress treatments provided an increase in the ratio of fully methylated bands, both in the Manteiga variety and in the Piauí variety. This increase was more intense in treatments NS2 and NS3, which suggests that intermittent or recurrent N stress induces greater hypermethylation of DNA, especially in the Piauí variety.

Table 5. Global DNA methylation patterns in flag leaves of rice plants (varieties Manteiga and Piauí) subjected to N-stress regimes. NS1 (1st and 2nd cycles: sufficient N; 3rd cycle: N stress); NS2 (1st cycle: N stress; 2nd cycle: sufficient N; and 3rd cycle: N stress); NS3 (1st, 2nd and 3rd cycle: N stress). C - control: (1st, 2nd and 3rd cycle: sufficient N). The results refer to the third cultivation cycle; NS1, NS2, and NS3 received fertilizer equivalent to 10 kg N ha^{-1}, while control plants received fertilizer equivalent to 60 kg N ha^{-1}.

MSAP band type	Manteiga				Piauí			
	Control	NS1	NS2	NS3	Control	NS1	NS2	NS3
I	402	367	361	376	490	450	398	406
II	119	111	93	96	130	148	76	68
III	66	101	127	106	46	58	146	134
IV	52	60	58	61	16	26	62	74
Total amplified bands[a]	639	639	639	639	682	682	682	682
Total methylated bands[b]	237	272	278	263	192	232	284	276
Fully methylated bands[c]	118	161	185	167	62	84	208	208
MSAP %[d]	37.09	42.57	43.51	41.16	28.15	34.02	41.64	40.47
Hemi-methylated ratio (%)[e]	18.62	17.37	14.55	15.02	19.06	21.70	11.14	9.97
Fully methylated ratio (%)[f]	18.47	25.20	28.95	26.13	9.09	12.32	30.50	30.50

Type I is unmethylated bands; Type II is hemi-methylated bands; Type III and IV are fully methylated bands. [a]Total amplified bands = I + II + III + IV; [b]Total methylated bands = II + III + IV; [c]Fully methylated bands = III + IV; [d]MSAP (%) = (II + III + IV) / Total amplified bands; [e]Hemi methylated ratio (%) = [(II) / (I + II + III + IV)] × 100 %; [f]Fully methylated ratio (%) = [(III + IV) / (I + II + III + IV)] × 100 %.

3.5.4 DNA methylation patterns

The banding patterns were evaluated to identify changes in cytosine methylation under N-stress conditions compared to the control. Sixteen different band patterns were identified between the control and the N-stress treatments (Table 6). Classes A1-A4 indicate no change in the methylation pattern between the control and N-stress treatments. Classes B1-B6 indicate cytosine demethylation patterns, while classes C1-C6 indicate cytosine methylation patterns. In the Manteiga variety, 82.03%, 72.66% and 72.34% of the bands remained unchanged when subjected to treatments NS1, NS2 and NS3, respectively. The percentage of bands with unchanged methylation profiles in the Piauí variety was 82.99%, 72.33% and 71.26% for treatments NS1, NS2 and NS3, respectively. These results clearly indicate a deeper change in the methylation profile in the treatments in which the plants were subjected to more cycles of N-stress, i.e., NS2 and NS3. Regarding the distribution of demethylated bands between the N-stress treatments, contrasting behaviors were observed between the varieties. A higher frequency of demethylation was observed in the Manteiga variety when subjected to the NS2 and NS3 treatments, whereas in the Piauí variety, there was a slight reduction in these same treatments. Regarding the distribution of methylated bands, both varieties had a higher frequency of methylation in the NS2 and NS3 treatments than in the NS1 treatment. However, the increase in the frequency of methylation was more significant in the Piauí variety. As observed in Table 5, this result indicates that in both varieties, the DNA methylation profile was more significantly altered when plants were exposed to a greater number of N-stress cycles. However, the pattern of changes in the methylation profile revealed small differences between the varieties. The Manteiga variety showed an increase in the frequencies of demethylation and methylation in the NS2 and NS3 treatments, while the Piauí variety showed a slight reduction in the frequency of demethylation and a more significant increase in the frequency of methylation in the NS2 and NS3 treatments than the Manteiga variety.

Table 6. Different patterns of DNA methylation induced by N-stress regimes in rice plants (varieties Manteiga and Piauí). NS1 (1st and 2nd cycles: sufficient N; 3rd cycle: N stress); NS2 (1st cycle: N stress; 2nd cycle: sufficient N; and 3rd cycle: N stress); NS3 (1st, 2nd and 3rd cycle: N stress). C - control: (1st, 2nd and 3rd cycle: sufficient N). The results refer to the third cultivation cycle; NS1, NS2, and NS3 received fertilizer equivalent to 10 kg N ha^{-1}, while control plants received fertilizer equivalent to 60 kg N ha^{-1}.

Classes	Banding pattern				Nº of sites					
	Control		N stress		Manteiga			Piauí		
	MspI	HpaII	MspI	HpaII	NS1	NS2	NS3	NS1	NS2	NS3
A – No change										
A1	1	1	1	1	351	334	339	422	386	392
A2	0	1	0	1	87	69	65	96	62	52
A3	1	0	1	0	54	50	41	38	40	38
A4	0	0	0	0	33	12	18	10	8	4
Total					525	465	463	566	496	486
Frequency (%)					82.03	72.66	72.34	82.99	72.33	71.26
B - Demethylation										
B1	0	1	1	1	14	23	32	28	12	12
B2	1	0	1	1	1	0	2	0	0	2
B3	0	0	1	1	1	4	3	0	0	0
B4	1	0	0	1	1	0	1	0	0	0
B5	0	0	0	1	4	7	9	2	6	8
B6	0	0	1	0	14	29	22	4	2	4
Total					35	63	69	34	20	26
Frequency (%)					5.47	9.84	10.78	4.99	2.93	3.81

Be continued...

Table 6 - Continuation

Classes	Banding pattern				Nº of sites					
	Control		N stress		Manteiga			Piauí		
	MspI	HpaII	MspI	HpaII	NS1	NS2	NS3	NS1	NS2	NS3
C - Methylation										
C1	1	1	0	1	19	17	21	50	8	8
C2	1	1	1	0	32	44	38	12	88	66
C3	0	1	1	0	1	4	5	4	16	26
C4	1	1	0	0	0	7	4	6	8	24
C5	0	1	0	0	17	23	17	2	40	40
C6	1	0	0	0	10	16	22	8	6	6
Total					79	111	107	82	166	170
Frequency (%)					12.34	17.34	16.72	12.02	24.34	24.93

Frequency of methylation changes in rice plants. 1 and 0 represents the presence and absence of bands, respectively.

3.5.5 Gene expression

A gene expression analysis was performed to verify the effect of N-stress treatments on the expression of genes encoding DNA methyltransferases (*OsDRM1a*, *OsDRM1b*, *OsDRM2*, *OsDRM3*, *OsMET1.1*, *OsMET1.2*, *OsCMT2*, *OsDNMT2*), DNA glycosylase/lyase (*OsDML3a*, *OsROS1a*, *OsROS1b*), amino acid transporters (*OsAAP1*, *OsAAP4*, *OsAAP6*) and enzymes related to nitrogen metabolism (*OsGS1.1*, *OsFe-GOGAT*, *OsNADH-GOGAT-2*, *OsGDH1*, *OsGDH2*). The level of expression of some genes varied according to the N-stress treatments applied (Figs. 8 and 9). The NS2 and NS3 treatments promoted an increase in *OsMET1.2* expression (Fig. 8E) and a reduction in *OsCMT2* expression (Fig. 8F) in the Manteiga variety. Only the NS3 treatment promoted a significant reduction in the expression of *OsROS1b* (Fig. 8J). The NS2 and NS3 treatments, compared to the NS1 treatment, reduced the expression of *OsGS1.1* (Fig. 8N) and *OsGDH2* (Fig. 8R), which was accompanied by increased expression of the amino acid transporter *OsAAP4* in the NS2 treatment (Fig. 8L). Plants of the Piauí variety subjected to the NS2 and NS3 treatments showed lower expression of *OsDRM1b* (Fig. 8B). Similar behavior was observed for *OsDRM1a* in the NS3 treatment (Fig. 9A). Interestingly, higher expression of *OsROS1b* (Fig. 9J) and *OsDML3a* (Fig. 9K) was observed in the NS1 treatment, demonstrating that in the evaluated period, this treatment altered only the expression of DNA glycosylase/lyase, thus not influencing the expression of DNA methyltransferases. All the N stress treatments resulted in higher *OsGDH1* expression (Fig. 9R). On the other hand, only the NS2 treatment induced a significant increase in the expression of the amino acid transporters *OsAAP4* (Fig. 9M) and *OsAAP6* (Fig. 9N).

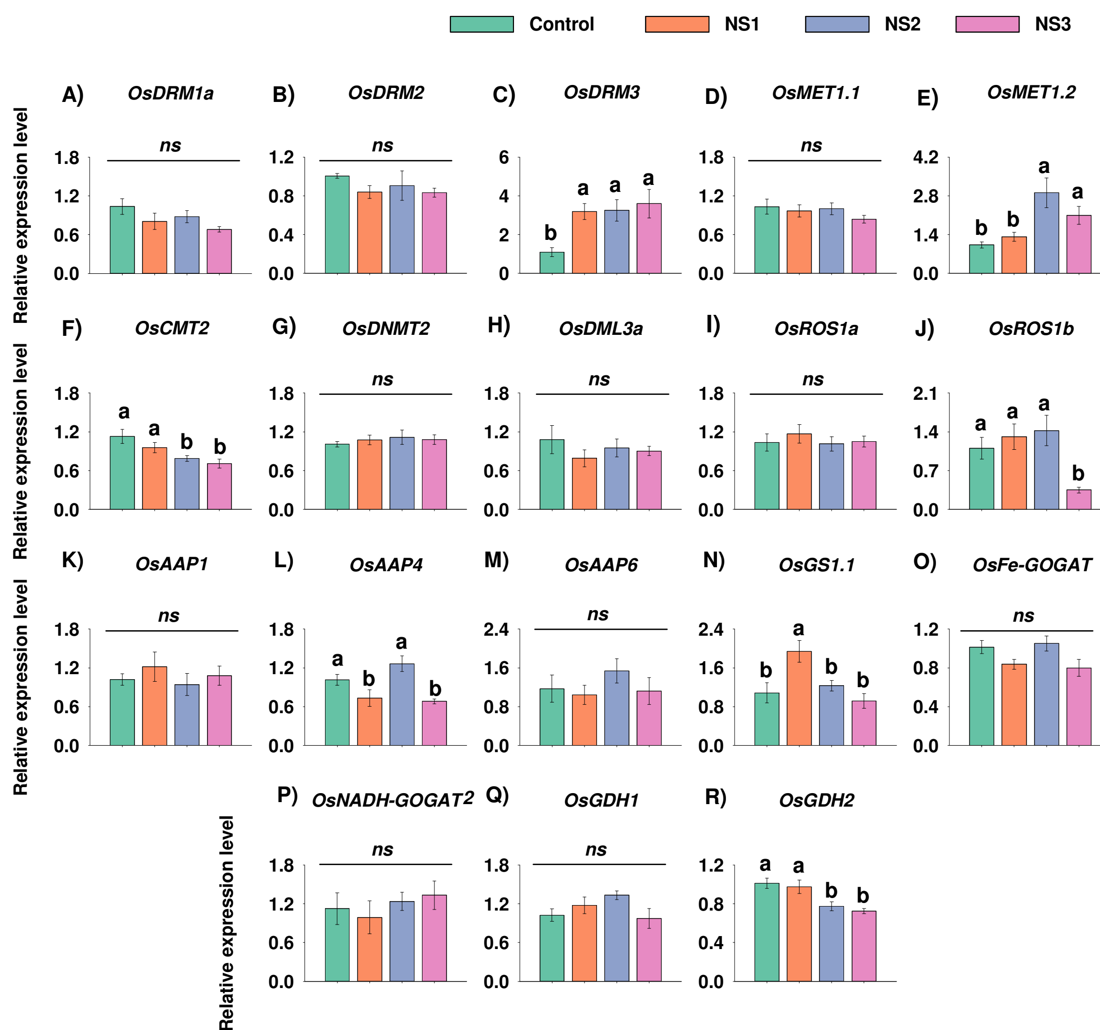

Figure 8. Relative expression of genes related to methylation (*OsDRMs*, *OsMETs*, *OsCMT2*, *OsDNMT2*), demethylation (*OsDML3a* and *OsROSs*) and N metabolism (*OsAAPs*, *OsGS1.1*, *OsFe-GOGAT*, *OsNADH-GOGAT 2*, *OsGDHs*) in rice plants (variety Manteiga) subjected to N-stress regimes. NS1 (1st and 2nd cycles: sufficient N; 3rd cycle: N stress); NS2 (1st cycle: N stress; 2nd cycle: sufficient N; and 3rd cycle: N stress); NS3 (1st, 2nd and 3rd cycle: N stress). C - control: (1st, 2nd and 3rd cycle: sufficient N). The results refer to the third cultivation cycle; NS1, NS2, and NS3 received fertilizer equivalent to 10 kg N ha^{-1}, while control plants received fertilizer equivalent to 60 kg N ha^{-1}. The data represent the averages of four biological replicates. The error bars represent the standard errors (SEs). Different lowercase letters indicate significant differences according to the Scott–Knott test ($p < 0.05$) between treatments. ns: absence of significant difference.

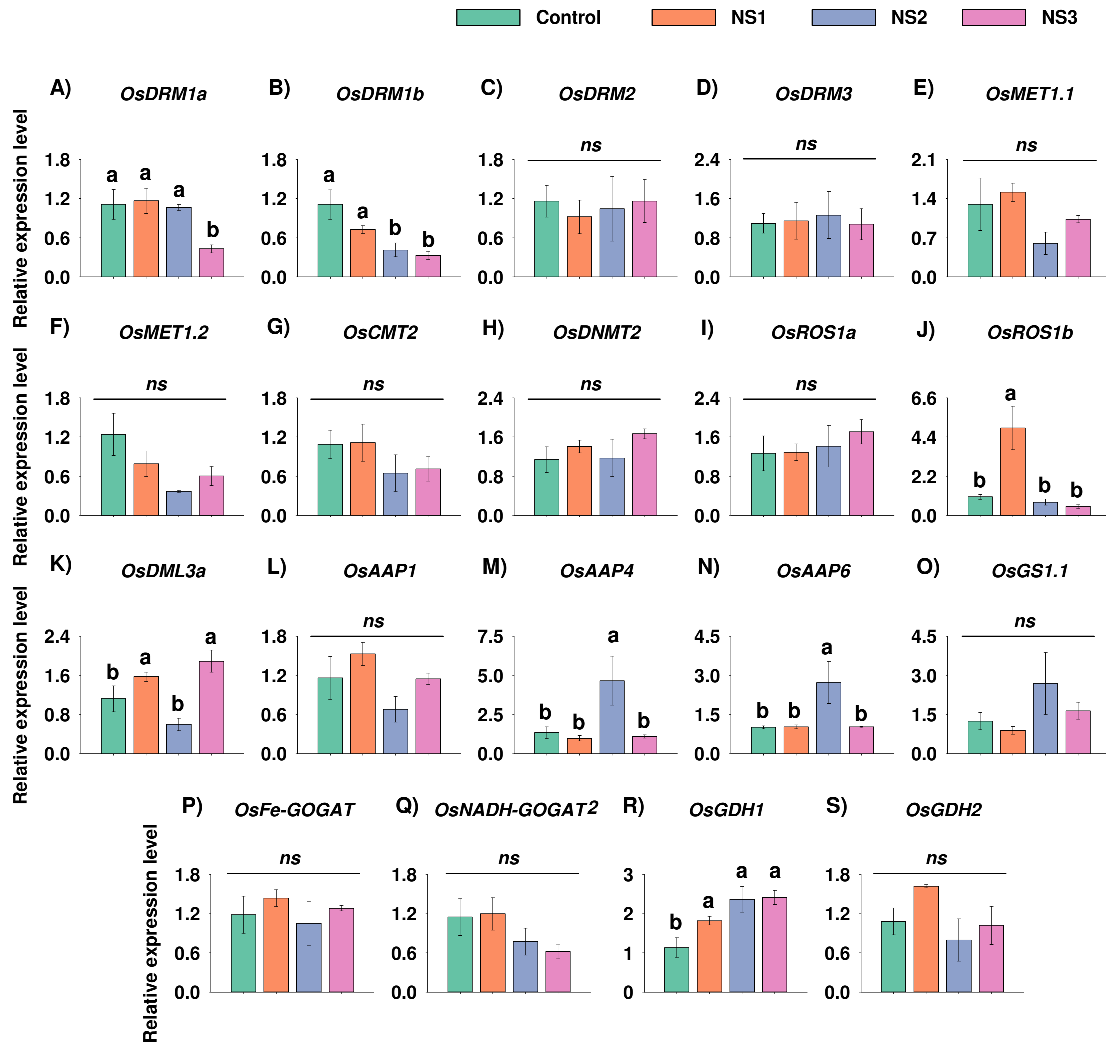

Figure 9. Relative expression of genes related to methylation (*OsDRMs*, *OsMETs*, *OsCMT2*, *OsDNMT2*), demethylation (*OsROSs*, *OsDML3a*) and N metabolism (*OsAAPs*, *OsGS1.1*, *OsFe-GOGAT*, *OsNADH-GOGAT 2*, *OsGDHs*) in rice plants (variety Piauí) subjected to N-stress regimes. NS1 (1st and 2nd cycles: sufficient N; 3rd cycle: N stress); NS2 (1st cycle: N stress; 2nd cycle: sufficient N; and 3rd cycle: N stress); NS3 (1st, 2nd and 3rd cycle: N stress). C - control: (1st, 2nd and 3rd cycle: sufficient N). The results refer to the third cultivation cycle; NS1, NS2, and NS3 received fertilizer equivalent to 10 kg N ha^{-1}, while control plants received fertilizer equivalent to 60 kg N ha^{-1}. The data represent the averages of four biological replicates. The error bars represent the standard errors (SEs). Different lowercase letters indicate significant differences according to the Scott–Knott test (p <0.05) between treatments. ns: absence of significant difference.

3.5.6 N use parameters

In both varieties, the lowest N use efficiency was observed in the NS1 treatment, in which the plants underwent N stress for the first time (Fig. 10C). On the other hand, treatments NS2 and NS3 (intermittent and recurrent N stress, respectively) showed NUEs similar to that in the control, indicating some adaptive process to the N stress in previous cycles. In both varieties, all the N stress treatments reduced NUtE (Fig. 10B).

The Piauí and Manteiga varieties showed different values and behaviors regarding NUpE and NRE (Fig. 10A and D). In the N stress treatments, only the Piaui variety showed a reduction in NUpE (Fig. 10A), while only the Manteiga variety showed a reduction in NRE (Fig. D). In addition, the NUpE values were higher for the Piauí variety, and the NRE values were higher for the Manteiga variety. Regarding the N harvest index (NHI), despite presenting lower values than the Manteiga variety, the Piauí variety had higher NHI values in the N-stress treatments than in the control (Fig. 10E). These results suggest different mechanisms of adaptation to N stress between varieties.

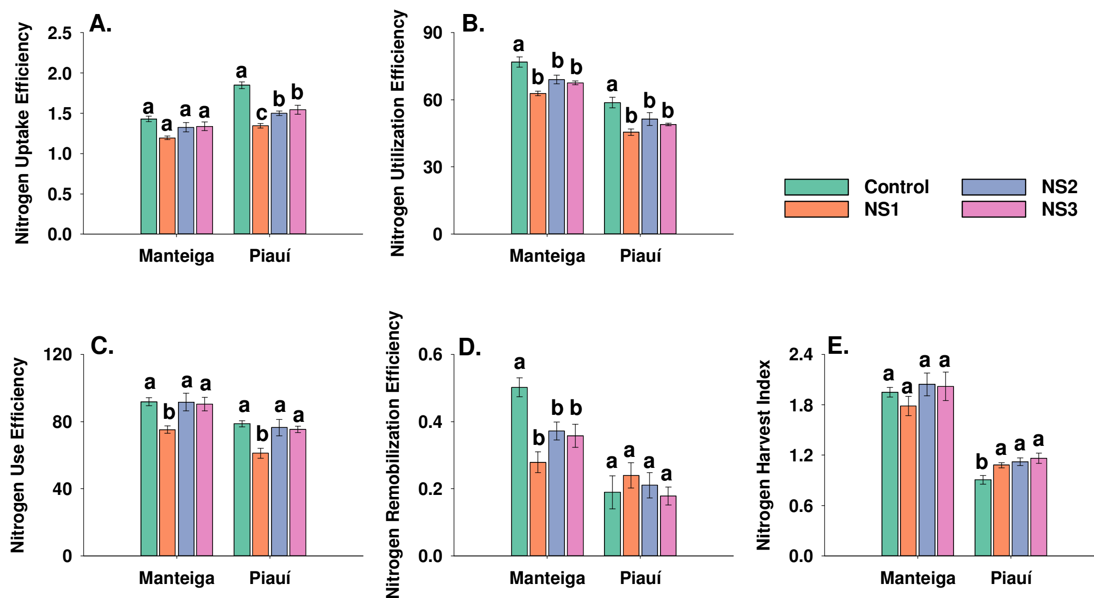

Figure 10. Parameters related to N use in rice plants (varieties Manteiga and Piauí) subjected to N-stress regimes. NS1 (1st and 2nd cycles: sufficient N; 3rd cycle: N stress); NS2 (1st cycle: N stress; 2nd cycle: sufficient N; and 3rd cycle: N stress); NS3 (1st, 2nd and 3rd cycle: N stress). C - control: (1st, 2nd and 3rd cycle: sufficient N). The results refer to the third cultivation cycle; NS1, NS2, and NS3 received fertilizer equivalent to 10 kg N ha^{-1}, while control plants received fertilizer equivalent to 60 kg N ha^{-1}. Nitrogen uptake efficiency (NUpE): total nitrogen of the aerial part (including panicles)/nitrogen supplied; Nitrogen utilization efficiency (NUtE): weight of full grains/nitrogen supplied; Nitrogen use efficiency (NUE): full grain weight/aerial part nitrogen content; Nitrogen remobilization efficiency (NRE): nitrogen remobilization to grains/total nitrogen in anthesis; N harvest index (NHI): nitrogen in grains/total nitrogen in the straw at maturity. The data represent the averages of four biological replicates. The error bars represent the standard errors (SEs). Different lowercase letters indicate significant differences according to the Scott–Knott test ($p < 0.05$) between treatments for each variety separately.

3.5.7 Photosynthetic efficiency

The photosynthetic efficiency was analyzed based on parameters related to the energy flow per reaction center (ABS/RC, DIo/RC, TRo/RC,), quantum yield (φ (Po), φ (Eo) and φ (Ro)) and performance of the photosynthetic apparatus (Pi abs and Pi total) (appendix – Tables 12, 13). The N-stress treatments altered the photosynthetic efficiency of rice plants (Fig. 11). During the vegetative phase (29 DAE), plants of the Manteiga variety that were subjected to the N-stress treatments showed lower photosynthetic efficiency than the control plants (Fig. 11A). This lower efficiency was evidenced by the greater energy flow trapped by the reaction center (TRo/RC) and greater energy dissipation in the form of heat (DIo/RC), which resulted in reductions in the partial (Pi abs) and total (Pi total) photosynthetic performances. On the other hand, it was observed that at 44 and 56 DAE, the plants subjected to the NS2 and NS3 treatments showed photosynthetic efficiency similar to that of the control treatment and higher than that of the plants in the NS1 treatment (Figs. 11B, C). In this phase, the NS1 treatment resulted in higher energy absorption flow per reaction center (ABS/RC), followed by greater energy trapping (TRo/RC). This greater energy flow was dissipated in the form of heat, which resulted in decreases in the partial (Pi abs) and total (Pi total) photosynthetic performance. At 79 DAE, plants subjected to the NS2 and NS3 treatments maintained a total photosynthetic performance (Pi total) higher than that in plants subjected to the NS1 treatment (Fig. 11D). Similar behavior was observed at 90 DAE. It is interesting to note that the photosynthetic performance of the plants in the NS2 and NS3 treatments was also superior to that of the control plants at 90 DAE (Fig. 11F).

At the beginning of the vegetative phase (29 DAE), plants of the Piauí variety that were subjected to the N-stress treatments showed lower photosynthetic efficiency than the control plants (Fig. 11G), as observed in the Manteiga variety (Fig. 11A). At the end of the vegetative (44 and 56 DAE) and reproductive (79 DAE) phases, no significant differences were observed for the photosynthetic parameters (Figs. 11H, I, J), except in the NS3 treatment, which resulted in a higher total photosynthetic performance (Pi total) than in the other treatments, including the control, at 44 DAE (Fig. 11H). On the other hand, in the measurements performed during the ripening phase (83 and 90 DAE), it was observed that plants subjected to the NS2 and NS3 treatments had a better photosynthetic performance than plants subjected to the NS1 treatment and a similar photosynthetic performance to that of the control plants (Figs. 11K, L).

In general, the data show specific differences between the varieties but better adaptation among plants that underwent N stress in previous cycles (i.e., plants in the NS2 and NS3 treatments) than among plants subjected to N stress for the first time (i.e., plants in the NS1 treatment).

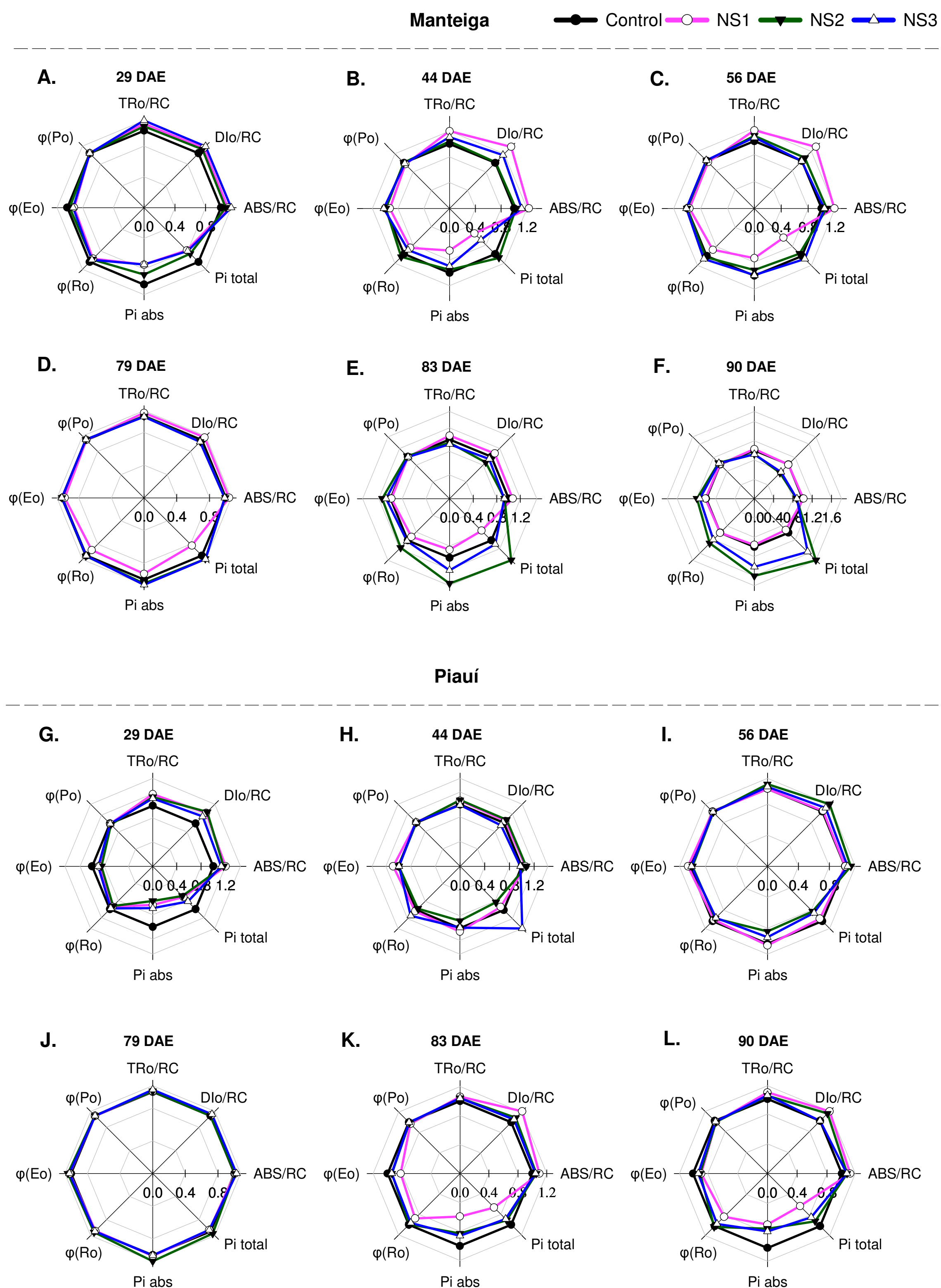

Figure 11. Photosynthetic parameters determined by the analysis of the fluorescence of chlorophyll a in rice plants (varieties Manteiga and Piauí) subjected to N-stress regimes. NS1 (1st and 2nd cycles: sufficient N; 3rd cycle: N stress); NS2 (1st cycle: N stress; 2nd cycle: sufficient N; and 3rd cycle: N stress); NS3 (1st, 2nd and 3rd cycle: N stress). C - control: (1st, 2nd and 3rd cycle: sufficient N). The results refer to the third cultivation

cycle; NS1, NS2, and NS3 received fertilizer equivalent to 10 kg N ha^{-1}, while control plants received fertilizer equivalent to 60 kg N ha^{-1}. The JIP test was used to calculate the parameters related to the following processes: i) energy fluxes: TRo/RC (maximum capture rate per RC); DIo/RC (energy flux dissipated as heat per reaction center (RC)); ABS/RC (RC absorption flux); ii) Productivity: φ(Po) (photochemical maximum quantum yield); φ(Eo) (Quantum yield of electron transport from Quinone A (Q_A-) to the electron acceptor intersystem); φ(R_o) (Quantum yield of electron transport from Q_A- to the final PSI electron acceptor); iii) Performance: Pi abs (Partial Photosynthetic Performance Index); Pi total (Total Photosynthetic Performance Index).

3.5.8 Metabolic profile and pathway analysis

The metabolic profiles of flag leaves of the Manteiga and Piauí varieties were analyzed to identify the metabolic changes promoted by the N-stress treatments. A total of 42 and 44 metabolites were identified and annotated in the Manteiga and Piauí varieties, respectively (appendix – Tables 14, 15), and these metabolites were divided into six groups: 7 organic acids (17%), 11 sugars (26%), 10 amino acids and derivatives (24%), 4 alkanes (10%), 5 lipids (12%) and 5 other substances (12%) for the Manteiga variety (Fig. 12A); and 4 organic acids (9%), 14 sugars (33%), 15 amino acids and derivatives (33%), 2 alkanes (4%), 3 lipids (7%) and 6 other substances (15%) for the Piauí variety (Fig. 12B). Based on the ANOVA and the Scott–Knott test ($p < 0.05$), the metabolites with significant changes in concentration between treatments were identified. In total, 23 and 27 differentially regulated metabolites were identified in the Manteiga and Piauí varieties, respectively (appendix – Tables 14, 15). Two heatmap plots were constructed to show metabolites with high relative abundances and to group the treatments based on the changes promoted in the metabolite profiles (Fig. 12C, D). The formation of two distinct groups was verified for the Manteiga variety, with one group consisting of the N-stress treatments (NS1, NS2 and NS3) and the other including the control treatment (Fig. 12C). Although the N-stress treatments comprised the same group, it was observed that the NS2 and NS3 treatments promoted greater changes in the relative abundances of the metabolites than the NS1 treatment, which indicates more similar behaviors between the NS2 and NS3 treatments. When compared to the control and NS1 treatment, the NS2 and NS3 treatments significantly increased ($p < 0.05$) the concentrations of levulinic acid, oxoglutaric acid, salicylic acid, succinic acid, raffinose, saccharic acid, glutamic acid, and pyroglutamic acid (appendix – Tables 14, 15). In addition, the NS3 treatment induced higher levels of glucopyranose, melezitose, GABA, ethanolamine and thiazole than the other treatments. For the Piauí variety, the formation of two groups was verified, with the first consisting of the control and NS1 treatment and the second consisting of the NS2 and NS3 treatments (Fig. 12D). In the Piauí variety, there was a clear contrast in the levels of differentially abundant metabolites between the group composed of the control and NS1 treatment and the group composed of the NS2 and NS3 treatments.

Six metabolic pathways with impact values> 0.1 and P value < 0.05 were screened for the Manteiga variety and four were screened for the Piauí variety (Fig. 12E, F). In both varieties, amino acid metabolism was the predominant metabolic pathway, including alanine, aspartate and glutamate metabolism, glyoxylate and dicarboxylate metabolism, glutathione metabolism, and arginine and proline metabolism, followed by energy metabolism (citric acid cycle). When analyzing the metabolites with significant differences between the treatments (Fig. 12C, D, appendix – Tables 14, 15), it is evident that the differentially abundant metabolites are mostly different among the varieties. However, all the metabolic pathways affected in the Piauí variety were also affected in the Manteiga variety, which additionally showed changes in two other pathways (Fig. 12E, F). It was also observed that the NS2 and

NS3 treatments resulted in deeper changes in the metabolic profile than the control (Fig. 12C, D).

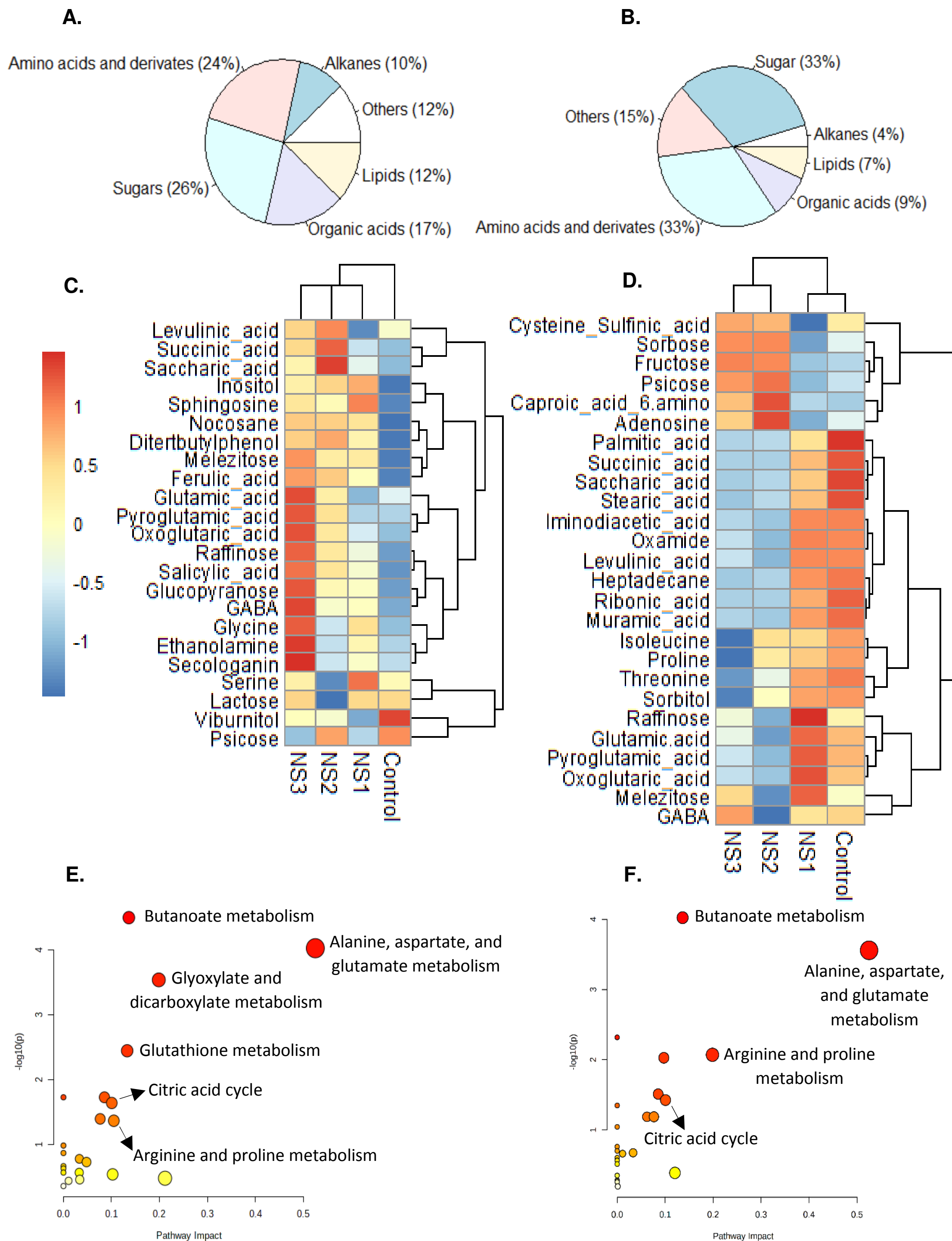

Figure 12. (A, B) Relative distribution of metabolites detected via GC/MS, (C, D) cluster analysis via heatmap of differentially abundant metabolites ($p < 0.05$) according to the Skott-Knot test ($n = 3$), and (E, F) metabolic pathway analysis using the MetaboAnalyst platform, where each circle represents a metabolic pathway, with red circles indicating high impact and yellow circles low impact. Panels A, C and E refer to the Manteiga variety, while panels B, D and F refer to the Piauí variety.

3.6 DISCUSSION

N stress is a condition to which rice plants may be subjected during the same cultivation cycle or in different cycles. Increasing the tolerance of a crop to stress conditions is a critical factor for agriculture (CHANG et al., 2020). In this study, we initially investigated whether rice plants grown under different N-stress conditions differed in terms of phenotypic parameters, grain yield and grain quality. Subsequently, changes were investigated at the molecular, physiological and metabolic levels, as well as in the efficiency of N use.

It was found in this study that rice plants of the varieties Manteiga and Piauí that were subjected to intermittent (NS2) and recurrent (NS3) N stress had higher grain yields than plants exposed for the first time to N stress (NS1) (Fig. 1E). In addition, plants subjected to the NS2 treatment produced better quality grains, which was evidenced by the increased levels of glutelin, a protein of high nutritional value for the human diet (JULIANO, 1992) (Fig. 7). The higher grain yield and quality observed in NS2 and NS3 compared to those observed in NS1 suggests a better adaptation/acclimation to N stress among plants exposed to intermittent or recurrent N stress compared to plants exposed for the first time to N stress. Plants that are subjected to a specific abiotic stress, such as N stress, may exhibit different molecular, physiological and biochemical responses than plants not previously subjected to the same stress (SRIKANT and DROST, 2021). These responses characterize a type of memory to stress and may improve the performance of a plant under stressful conditions (KOU et al., 2011; NEVES et al., 2017).

DNA methylation is a molecular response that can be acquired momentarily in response to a specific stress but can also be hereditary, depending on the nature, intensity and persistence of the stress condition (MAGER and LUDEWIG, 2018). Using the MSAP technique, a lower hemimethylated band ratio was observed in both varieties, especially in Piauí, in the NS2 and NS3 treatments (Table 5). Interestingly, the decrease in the ratio of hemimethylated bands was accompanied by a proportional and more intense increase in the ratio of fully methylated bands, especially for the NS2 and NS3 treatments. With the separation of the MSAP fragments into classes A (A1-A4, no change), B (B1-B6, demethylation) and C (C1-C6, methylation), it was possible to verify that the plants subjected to the NS2 and NS3 treatments had a higher frequency of methylation than the plants in the control and NS1 treatment (Table 6), which goes against the most significant increase observed in the ratio of fully methylated bands (Table 5).

Based on the overall methylation profile obtained using the MSAP technique, it was possible to highlight some points: I) N stress induced changes in the DNA methylation profile; II) the type and intensity of the changes were dependent on the genotype and the frequency of N stress; and III) DNA hypermethylation was more frequent than hypomethylation in rice plants subjected mainly to intermittent (NS2) and recurrent (NS3) N stress. Changes in the DNA methylation profile have been reported in plants subjected to stress due to low nitrogen supply. MAGER and LUDEWIG (2018) reported an extensive loss of DNA methylation in maize plants subjected to N deficiency. On the other hand, KOU et al. (2011) reported that N deficiency did not significantly alter the total DNA methylation level in rice leaves, in contrast to what was observed in our study. As shown by LI et al. (2021b), this discrepancy may be due to different methylation responses to N supply in different species and/or experimental designs.

DNA methylation maintenance is performed by DNA methyltransferases (WOO et al., 2008), while active demethylation is coordinated by DNA glycosylase/lyase (NIEHRS, 2009). Some studies have shown that there is a strong relationship between changes in the DNA methylation profile and gene induction or repression (DO AMARAL et al., 2020; SECCO et

al., 2015; WU et al., 2020). In our study, the expression of genes related to the methylation and demethylation processes differed among the N-stress treatments (Figs. 8 and 9). The *OsMET1.2* gene, which codes for a methyltransferase, was more highly expressed in plants of the Manteiga variety that were subjected to the NS2 and NS3 treatments (Fig. 8E), which may be related to the higher frequency of methylation and the increase in fully methylated bands (Tables 5 and 6). On the other hand, the lower expression of the *OsROS1b* gene (Fig. 9J), a DNA glycosylase/lyase, may explain the lower frequency of demethylation observed in plants of the Piauí variety that were subjected to the NS2 and NS3 treatments than in plants subjected to the NS1 treatment (Table 6).

Previous studies have reported changes in the expression of DNA methyltransferases and DNA glycosylase/lyase under abiotic stress conditions. YONG-VILLALOBOS et al. (2015) found an induction of *AtMET1* gene expression in treatments with low phosphate (Pi) availability in Arabidopsis plants. MAGER and LUDEWIG (2018) reported a downregulation in the expression of the *ZmROS1* gene in maize plants under nitrogen deprivation. In our study, we also analyzed the expression of genes related to N metabolism to determine possible changes in N assimilation and amino acid transport pathways. There was a reduction in the expression of *OsGS1.1* and *OsGDH1* in plants of the Manteiga variety that were subjected to the NS2 and NS3 treatments compared to those that were subjected to the NS1 treatment (Figs. 8N, R), suggesting a lower N assimilation at the flowering stage (79 DAE). Interestingly, upregulation of the *OsAAP4* gene was observed in the Manteiga and Piauí varieties subjected to the NS2 treatment compared to plants subjected to the NS1 and NS3 treatments (Figs. 8L and 9M) and of the *OsAAP6* gene in the Piauí variety (Fig. 9N) suggests greater amino acid transport activity in the flowering stage. FANG et al. (2021) showed that *OsAAP4* is an important regulator of the allocation of neutral amino acids and thus contributes to better efficiency of N utilization (NUtE), grain production and tillering. PENG et al. (2014) reported that *OsAAP6*, in addition to improving the absorption and distribution of amino acids, is an important regulator of the nutritional quality of grains, regulating the synthesis and accumulation of glutelin, globulin, prolamine, albumin and starch.

The upregulation of the *OsAAP4* and *OsAAP6* genes suggests a contribution of these transporters to better N use efficiency (NUE) (Fig. 10C), which may have contributed to the higher grain yield (Fig. 6) and quality (Fig. 7) observed in plants subjected to the NS2 treatment than in those subjected to the NS1 treatment. On the other hand, the increase in production and better grain quality observed in plants subjected to the NS3 treatment compared to those subjected to the NS1 treatment may be more associated with the modification of photosynthetic efficiency and regulation of specific metabolic pathways. To some extent, plants can retain information on previous stress responses to ensure faster adaptation to the same adversity (CHANG et al., 2020). In this study, analysis of the chlorophyll a content of plants in the transient flowering stage showed that the NS2 and NS3 treatments promoted a significant improvement in photosynthetic efficiency compared to that in the NS1 treatment (appendix – Tables 12, 13). This improvement was observed in the Manteiga variety in the vegetative (44 and 56 DAE), reproductive (79 DAE) and ripening (90 DAE) phases and in the Piauí variety in the ripening (83 and 90 DAE) phase (Fig. 11), showing that previous exposure to N stress, either intermittent (NS2) or recurrent (NS3), modifies the photosynthetic efficiency in rice plants and that the intensity and phases of the development cycle in which these modifications occur depend on two factors: genotype and the type of N-stress treatment.

Although differences in photosynthetic capacity among rice varieties are commonly reported (HIRASAWA et al., 2009), photosynthetic changes resulting from N-stress conditioning, as shown in this study, are not frequently reported. The photosynthetic process

involves a large number of proteins that together represent most of the leaf nitrogen (EVANS and CLARKE, 2019). As a result, low levels of N in the soil can affect the synthesis of these proteins, reducing the photosynthetic capacity of plants and directly affecting the synthesis of carbohydrates, amino acids, and organic acids, among other factors (ZHAO et al., 2018a). Tolerance to abiotic stresses, such as low-N stress, is related, among other factors, to metabolic adjustments (SADDHE et al., 2021). In this sense, there is an increase in the production and concentration of specific metabolites, depending on the nature and intensity of the abiotic stress (SHI et al., 2015). In this study, changes in the metabolic profile were observed as a function of the N-stress treatments (Figs. 12C, D).

In the Manteiga variety, compared to the control and NS1 treatment, the NS2 and NS3 treatments promoted an increase in the concentrations of glutamic acid, pyroglutamic acid, oxoglutaric acid and raffinose, among other metabolites (Fig. 12C). On the other hand, in the Piauí variety, the concentration of these metabolites was reduced (Fig. 12D), suggesting that the genetic background of the varieties results in different metabolic regulations for adaptation to similar N-stress treatments. Under conditions of low N, ZHAO et al. (2018b) found that the G9 rice genotype, which is resistant to low N when compared from the susceptible genotype (SH527), showed relatively high levels of sugars, amino acids and derivatives, as observed in this study for the Manteiga variety, and suggested a positive correlation between increased tolerance to low-N stress and increased levels of specific metabolites. The study of metabolic pathways, based on metabolites significantly altered as a function of N-stress treatments, showed regulation of pathways related to amino acid and energy metabolism (Figs. 12E, F). The stimulation of the citric acid cycle and glyoxylate and dicarboxylate metabolism observed in the Manteiga variety (Fig. 12E) indicates an improvement in the energy production processes (XIONG et al., 2018), which corroborates the increase in photosynthetic efficiency in different phases of the culture cycle (Fig. 11). In addition, the stimulation of glyoxylate and dicarboxylate metabolism has been reported as an important factor in adaptation to abiotic stresses (XU et al., 2018). The increase in metabolic levels related to glutathione metabolism indicates a stimulus of the antioxidant system that may have contributed to the better performance of plants of the Manteiga variety when subjected to the NS2 and NS3 treatments. The same metabolic pathways were affected by N stress in the Manteiga and Piauí varieties, except for two pathways affected exclusively in Manteiga. Similarly, the intermittent (NS2) and recurrent (NS3) N-stress treatments were responsible for the more extensive changes in the metabolic profiles of the two varieties compared to the control. The differences in the profiles and contents of differentially abundant metabolites between the varieties suggest that adaptation to N stress has intrinsic peculiarities specific to each genotype.

3.7 CONCLUSIONS

Based on the data obtained in this study, it was possible to conclude that exposure to N stress induces molecular, physiological, metabolic and phenotypic changes in rice plants. The type and intensity of the modifications will depend on the number of N stress cycles to which the plants are subjected and differ among varieties. When compared with first experience to low-N stress, exposure to intermittent and recurrent N stress improves photosynthetic efficiency and N use efficiency, which together with changes in the DNA methylation profile and changes in the metabolic profile and gene expression, increase tolerance to N stress and improve grain yield and quality.

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4 GENERAL CONCLUSIONS

Rice plants grown with a low dose of N show modifications in the overall DNA methylation profile, followed by morpho-physiological, metabolic, N use efficiency, and gene expression modifications related to N metabolism and the DNA methylation, and demethylation process.

The low N conditioning time has a direct influence on the methylation profile and the other parameters analyzed in this study. The intensity of these modifications differs between varieties.

Exposure of Manteiga and Piaui varieties to intermittent (NS2) and recurrent (NS3) N-stress induces a DNA methylation pattern distinct from plants of the same varieties first exposed to N-stress (NS1). This divergent pattern of NS2 and NS3 relative to NS1, was also observed for the other parameters analyzed. These changes seen in NS2 and NS3 were accompanied by better development and higher grain yield, indicating a beneficial effect of prior exposure of these varieties to N-stress.

In the Esmeralda variety the greatest differences were seen between NS2 in relation to NS1 and NS3. The modifications observed in NS2 was harmful, since it significantly decreased the grain yield in this variety.

Overall, it is concluded from this study that N-stress induces modifications in the DNA methylation pattern, followed by morpho-physiological, metabolic, N use, gene expression and grain quality changes. The time of N-stress conditioning and the variety used strongly influence the type and intensity of the modifications.

5 APPENDIX

Table 7. Chemical analysis of the superficial layer of Planosol used for rice cultivation during three cycles.

Na	Ca	Mg	K	H+Al	Al	SB	CEC	V	m	n	SOC	pH	P	N
$cmol_c/dm^3$								%				1:2.5	mg/dm^3	$kg\ ha^{-1}$
0.091	2.00	0.70	0.10	5.23	0.0	2.92	8.15	36.15	0	1.13	1.19	5.40	53.82	30.47

In which: ***Na*** sodium; ***Ca*** calcium; ***Mg*** magnesium; ***K*** potassium; ***H+Al*** hydrogen + aluminum (potential acidity); ***Al*** aluminum; ***SB*** sum of bases; ***CEC*** cation exchange capacity; ***V*** base saturation; ***m*** aluminum saturation; ***n*** sodium saturation; ***SOC*** soil organic carbon; ***pH*** hydrogen potential; ***P*** phosphor; ***N*** total nitrogen. Units: $cmol_c/dm^3$ centimol of charge per cubic decimeter; ***%*** percentage; ***1:2.5*** proportion of soil and water used in pH analysis; ***mg/L*** milligrams per cubic decimeter; $kg\ ha^{-1}$ kilograms per hectare.

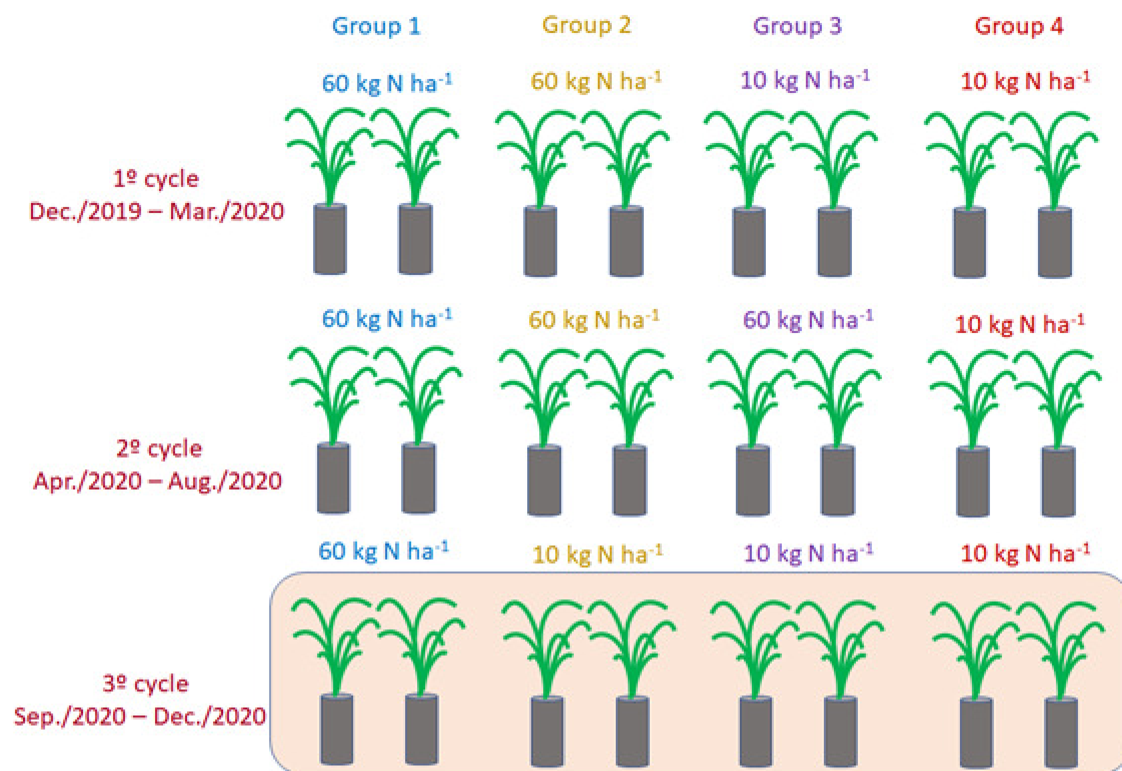

Figure 13. Experimental design of N-stress conditioning. ***Dec*** December; ***Mar*** March; ***Apr*** April; ***Aug*** August; ***Sep*** September

Table 8. Sequence of adapters and primers used to analyze the methylation profile employing the MSAP technique.

Primers/adapters	Oligonucleotide sequences (5' – 3')
*Eco*RI adapter	CTCGTAGACTGCGTACC AATTGGTACGCAGTCTAC
*Hpa*II/*Msp*I adapter	GACGATGAGTCCTGAG CGCTCAGGACTCAT
Preamplification primer	
E00	GACTGCGTACCAATTC
HM00	GATGAGTCCTGAGCGG
*Eco*RI selective amplification primers	
E00 + TCT	GACTGCGTACCAATTCTCT
E00 + GTT	GACTGCGTACCAATTCGTT
E00 + GTC	GACTGCGTACCAATTCGTC
E00 + TCG	GACTGCGTACCAATTCTCG
*Hpa*II/*Msp*I selective amplification primers	
HM00 + AGC	GATGAGTCCTGAGCGGAGC
HM00 + AGG	GATGAGTCCTGAGCGGAGG
HM00 + AGT	GATGAGTCCTGAGCGGAGT

Table 9. Photosynthetic parameters of rice plants (cv. Esmeralda) using the JIP test. NS1 (two successive N-sufficient cycles + one N-stress cycle); NS2 (one N-stress cycle + one N-sufficient cycle + one N-stress cycle); NS3 (three successive N-stress cycles). Control (three successive N sufficient cycles).

Treatments	Stages	ABS/RC	Dio/RC	TRo/RC	REo/RC	φ(Po)	φ(Eo)	φ(Ro)	Pi_{abs}	Pi_{total}
Control	14 DAE	1.90±0.07ns	0.37±0.02ns	1.53±0.06ns	0.46±0.02ns	0.80±0.00a	0.47±0.01a	0.24±0.01ns	3.09±0.27ns	3.24±0.35ns
NS1		1.92±0.10	0.39±0.03	1.54±0.07	0.46±0.02	0.80±0.00a	0.46±0.02a	0.24±0.00	2.85±0.40	3.05±0.34
NS2		2.00±0.19	0.43±0.07	1.57±0.12	0.49±0.06	0.79±0.01b	0.47±0.01a	0.24±0.01	2.76±0.43	3.02±0.44
NS3		1.92±0.04	0.41±0.02	1.51±0.02	0.46±0.02	0.79±0.01b	0.45±0.01b	0.24±0.01	2.54±0.22	2.90±0.25
Control	29 DAE	2.39±0.24ns	0.56±0.08ns	1.83±0.16ns	0.52±0.06ns	0.77±0.01ns	0.41±0.03a	0.22±0.02ns	1.43±0.25a	1.68±0.22a
NS1		2.61±0.20	0.64±0.09	1.97±0.10	0.55±0.08	0.76±0.02	0.36±0.03b	0.21±0.02	1.15±0.28a	1.62±0.30a
NS2		2.68±0.24	0.67±0.10	2.00±0.15	0.52±0.06	0.75±0.01	0.35±0.06b	0.19±0.02	0.86±0.14b	1.19±0.25b
NS3		2.60±0.10	0.67±0.04	1.94±0.08	0.54±0.01	0.74±0.01	0.32±0.02b	0.21±0.01	0.86±0.13b	1.59±0.23a
Control	41 DAE	2.54±0.10ns	0.60±0.04ns	1.94±0.06ns	0.51±0.02b	0.76±0.01ns	0.38±0.02ns	0.20±0.01ns	1.26±0.21ns	1.43±0.19ns
NS1		2.62±0.17	0.64±0.08	1.98±0.10	0.54±0.03a	0.76±0.02	0.39±0.03	0.21±0.01	1.30±0.36	1.47±0.26
NS2		2.80±0.21	0.69±0.07	2.11±0.14	0.56±0.03a	0.75±0.01	0.38±0.02	0.20±0.01	1.11±0.18	1.27±0.23
NS3		2.73±0.24	0.67±0.10	2.07±0.14	0.52±0.02b	0.76±0.02	0.36±0.04	0.19±0.01	1.07±0.32	1.23±0.31
Control	64 DAE	2.82±0.13b	0.59±0.04b	1.20±0.03b	0.54±0.03b	0.79±0.01a	0.43±0.01ns	0.19±0.01b	1.58±0.20a	1.31±0.22ns
NS1		2.88±0.11a	0.61±0.03b	1.21±0.04b	0.56±0.02b	0.79±0.00a	0.42±0.01	0.19±0.00b	1.46±0.12b	1.27±0.10
NS2		2.98±0.15a	0.65±0.03a	1.26±0.04a	0.60±0.03a	0.78±0.01b	0.42±0.01	0.20±0.00a	1.43±0.17b	1.31±0.13
NS3		2.75±0.12b	0.57±0.05b	1.18±0.03b	0.55±0.02b	0.79±0.00a	0.43±0.02	0.20±0.01a	1.65±0.22a	1.46±0.16

Be continued...

Table 9 - Continuation

Control		2.62±0.19b	0.53±0.07b	2.09±0.13b	0.41±0.03c	0.80±0.01a	0.41±0.02a	0.15±0.01b	1.62±0.41a	0.98±0.20ns
NS1	68	2.70±0.11b	0.55±0.03b	2.15±0.08b	0.43±0.02b	0.80±0.00a	0.41±0.01a	0.16±0.00a	1.52±0.14a	0.98±0.07
NS2	DAE	2.88±0.06a	0.63±0.05a	2.25±0.06a	0.47±0.01a	0.78±0.01b	0.38±0.01b	0.16±0.00a	1.18±0.08b	0.88±0.07
NS3		2.61±0.06b	0.55±0.04b	2.07±0.04b	0.42±0.01b	0.79±0.01a	0.40±0.01a	0.16±0.01a	1.51±0.17a	1.03±0.11
Control		3.29±0.07ns	0.84±0.05ns	2.44±0.05ns	0.41±0.02ns	0.74±0.01ns	0.33±0.01a	0.13±0.01ns	0.71±0.07ns	0.43±0.04a
NS1	75	3.25±0.17	0.82±0.09	2.44±0.09	0.40±0.04	0.75±0.01	0.33±0.01a	0.12±0.00	0.73±0.12	0.43±0.02a
NS2	DAE	3.32±0.09	0.81±0.04	2.51±0.06	0.44±0.02	0.76±0.01	0.33±0.01a	0.13±0.01	0.71±0.06	0.49±0.07a
NS3		3.47±0.23	0.94±0.13	2.53±0.11	0.40±0.03	0.73±0.02	0.30±0.02b	0.12±0.00	0.55±0.12	0.35±0.04b
Control		3.69±0.33b	0.99±0.16c	2.69±0.18ns	0.47±0.06ns	0.73±0.02a	0.30±0.02a	0.130.01a	0.55±0.14a	0.38±0.04a
NS1	83	4.10±0.10a	1.21±0.06b	2.89±0.05	0.46±0.03	0.70±0.01b	0.27±0.01b	0.11±0.01b	0.35±0.02b	0.26±0.02c
NS2	DAE	4.01±0.10a	1.15±0.04b	2.85±0.07	0.49±0.02	0.71±0.01b	0.27±0.01b	0.12±0.01a	0.39±0.03b	0.31±0.03b
NS3		4.37±0.14a	1.37±0.09a	3.00±0.09	0.52±0.03	0.69±0.01b	0.26±0.02b	0.12±0.00a	0.31±0.07b	0.27±0.01b

In which: ***Dio/RC*** energy flux dissipated as heat per reaction center (RC); ***ABS/RC*** absorption flux per RC; ***TRo/RC*** maximum capture rate per RC; ***REo/RC*** reduction flux of electrons in the final electron acceptor in photosystem I (PSI); ***φ(Po)*** photochemical maximum quantum yield; ***φ(Eo)*** quantum yield of electron transport from Quinone A (Q_A-) to the electron acceptor intersystem; ***φ(R_o)*** quantum yield of electron transport from Q_A- to the final PSI electron acceptor; ***Pi_{abs}*** partial photosynthetic performance index; ***Pi_{total}*** total photosynthetic performance index. ***DAE*** days after emergency. Data represent the average ± SD of four biological replicates. Different lowercase letters between treatments within a column indicate statistical differences by the Scott-Knott test ($p < 0.05$) in each stage of rice growing. ***ns*** absence of significant difference.

Table 10. Metabolic profiles in flag leaves in rice plants (cv. Esmeralda) subjected to distinct N-stress regimes: NS1 (two successive N-sufficient cycles + one N-stress cycle); NS2 (one N-stress cycle + one N-sufficient cycle + one N-stress cycle); NS3 (three successive N-stress cycles). Control: (three successive N sufficient cycles).

Metabolites	Treatments				
Organic acids (µg/g fresh matter)	Control	NS1	NS2	NS3	P value
2-Oxoglutaric acid	15.43±2.00	13.12±1.21	11.49±1.07	15.93±0.94	0.0513
Citric acid	**37.62±13.21**	**72.35±11.53**	**75.76±7.27**	**87.29±5.38**	**0.0053**
Dehydroascorbic acid	38.90±3.42	28.68±8.67	33.06±3.05	38.20±3.65	0.2486
Erythronic acid	**8.99±0.30**	**7.78±0.39**	**8.60±0.31**	**8.18±0.17**	**0.0122**
Glyceric acid	55.38±3.96	34.93±2.09	37.66±3.25	43.58±2.66	0.2532
Isocitric acid	**24.01±12.63**	**62.39±5.00**	**56.95±10.78**	**49.71±1.53**	**0.0100**
Lactic acid	**4.38±0.91**	**5.51±0.63**	**9.59±0.73**	**9.46±0.71**	**0.0002**
Malic acid	92.00±5.63	70.26±7.38	79.03±2.80	101.42±6.55	0.0651
Oxalic acid	**11.42±1.11**	**13.30±0.15**	**15.21±0.49**	**17.98±1.34**	**0.0006**
Propanoic acid	16.40±2.38	13.06±0.59	15.17±1.56	16.80±0.53	0.1246
Succinic acid	22.18±6.66	26.10±3.08	24.16±2.11	25.60±1.88	0.7571
Threonic acid	7.92±2.74	8.00±1.69	7.35±0.54	7.44±0.32	0.9669

Sugars (µg/g fresh matter)	Control	NS1	NS2	NS3	P value
D-Mannose	8.41±5.95	8.66±2.97	10.86±1.26	5.11±0.10	0.4470
D-turanose	3.30±4.67	2.75±3.89	2.59±3.67	5.13±3.66	0.9132
Fructose	**1178.52±104.74**	**595.49±49.57**	**765.20±73.62**	**836.58±55.53**	**0.0003**
Galactose	62.24±4.66	38.78±7.86	47.14±4.21	47.34±2.06	0.0614
Glucose	**937.27±103.96**	**428.42±37.56**	**565.81±49.36**	**639.84±42.73**	**0.0003**
Maltose	24.34±0.92	86.31±91.71	38.37±23.12	21.81±2.15	0.5266
Sedoheptulose	18.98±0.94	16.05±3.07	16.92±1.13	15.06±1.11	0.2369
Sucrose	**1027.35±55.78**	**878.83±34.15**	**960.69±34.26**	**976.08±31.93**	**0.0360**
Xylose	18.39±3.46	23.26±17.59	18.19±6.23	11.91±1.34	0.7061

Be continued…

Table 10 – Continuation

Amino acids and derivates (µg/g fresh matter)	Control	NS1	NS2	NS3	P value
Alanine	**113.87±1.78**	**100.37±2.74**	**104.78±0.43**	**106.41±5.34**	**0.0160**
Aminobutyric acid	6.02±1.14	6.87±1.89	5.54±0.16	5.12±0.30	0.4793
Gamma aminobutyric acid	6.35±0.62	6.41±0.24	7.21±0.54	6.88±0.46	0.3135
Glutamic acid	23.56±4.70	29.00±3.58	23.31±2.89	28.58±3.45	0.3134
Oxoproline	8.82±1.92	8.40±0.44	6.63±0.64	8.79±0.92	0.2473
Palmitic acid	**57.61±5.07**	**54.95±2.49**	**68.00±6.86**	**67.00±1.26**	**0.0470**
Serine	4.26±0.35	5.11±1.17	1.33±1.88	3.15±2.24	0.1766

Polyalcohol (µg/g fresh matter)	Control	NS1	NS2	NS3	P value
D-glucitol	12.14±2.42	13.99±5.32	12.12±2.16	11.53±0.44	0.8708
Erythritol	27.10±5.25	40.15±22.91	39.37±8.91	28.79±2.78	0.6390
Glycerol	13.23±8.03	17.65±0.76	13.13±2.24	15.07±3.11	0.7243
Hexyl alcohol	**7.81±0.78**	**8.39±0.31**	**10.12±0.82**	**10.22±0.26**	**0.0080**
Myo-Inositol	107.57±7.66	94.58±4.78	101.67±12.70	95.29±8.11	0.4559
Xylitol	14.81±3.04	14.79±6.14	14.85±3.45	11.53±0.44	0.7799

Data represent the average of metabolite content (µg/g fresh matter) ± SD in three biological replicates. The compounds that significantly differed ($p < 0.05$) through analysis of variance are in bold.

Table 11. List of primers used in the real-time PCR.

Genes	Locus (MSU)	Forward	Reverse	Length (pb)
OsDRM1a	LOC_Os11g01810	GCTGCTAGGAAGAGGGGTTA	TCCTGCTAACTCCCCTGGTAT	73
OsDRM1b	LOC_Os12g01800	CTGTTCTTCCCATTCCCCCA	CGTTCTGTATGCTTTGCGCT	133
OsDRM2	LOC_Os03g02010	ACTCTGATGGTTCTGGTGACG	GGCGCATCGAGACATCTAT	133
OsDRM3	LOC_Os05g04330	AGCTGTGTGAGAGACTGGGA	CTGGTGCATGATGTGCGTTT	83
OsMET1-1	LOC_Os03g58400	CCAGAGTGGCCTGAACCAAT	TTGACAGCGGCGTAGAACTT	86
OsMET1-2	LOC_Os07g08500	CCACCCTGACCAGGATAGGA	AATCTGCCTGTGCTTGCTCT	109
OsCMT2	LOC_Os05g13790	TCACGAGTGGTCAGCAAGAC	GCCCTCTGTGTCTTTGGAGG	71
OsDNMT2	LOC_Os01g42630	AGGGCAGGTCACAACAGATG	AGACCCATTAACTGAAGAATCCTGA	126
OsROS1a	LOC_Os01g11900	ACAGTTCGGGCTCATGACAG	TTGGCAACTTCCAGTTTGCG	97
OsROS1b	LOC_Os02g29230	TAGCTGCAACAGTACACGGG	TGGGACTCTCAAGAGGGAACT	79
OsDML3a	LOC_Os02g29380	CGGACAGCAACCAGAGGAAA	GGATGGTAATCGGGGAACGG	100
OsAAP1	LOC_Os07g04180	GGTGGCGTTCTCGGTCATAA	GCTTGGCCGTAGGTGTAGTT	105
OsAAP4	LOC_Os12g09300	ACATCTACCCTCCTCGCCAA	GCACACCCACACAACATCAC	125
OsAAP6	LOC_Os01g65670	GTCAGAACAGGAACGGTATGG	TAGTAGGTGATGTAGGCGCAGA	149
OsGS1.1	LOC_Os02g50240	ACTGTGGTATCGGTGCTGAC	CTCGCCGTTGATTCCACTGA	104
OsNADH-GOGAT2	LOC_Os05g48200.1	AACCCAGGTGCCAGAATCAG	TTCACCACACCACTAGCGAC	74
OsFeGOGAT	LOC_Os07g46460.1	TGTGGAGCGGACAATGACTC	TTTGTTCTGTCCAGGGGAGC	113
OsGDH1	LOC_Os03g58040.1	CACAGACCCTGAGGCAGATG	CGTCCCACATGAAACCCTGA	134
OsGDH2	LOC_Os04g45970.1	CGCTTTAGGTGGAGTGCTCA	ATGGTCACTCCCTTCTTGGC	126
OsACT1	LOC_Os03g50885.1	CTTCATAGGAATGGAAGCTGCGGGTA	CGACCACCTTGATCTTCATGCTGCTA	197
OseEf	LOC_Os03g08020.1	TTTCACTCTTGGTGTGAAGCAGAT	GACTTCCTTCACGATTTCATCGTAA	103

[a]Primers sequences used were obtained from Plant Mineral Nutrition Laboratory/UFRRJ.

Table 12. Photosynthetic parameters of rice plants (variety Manteiga) according to the JIP test. NS1 (1st and 2nd cycles: sufficient N; 3rd cycle: N stress); NS2 (1st cycle: N stress; 2nd cycle: sufficient N; and 3rd cycle: N stress); NS3 (1st, 2nd and 3rd cycle: N stress). C - control: (1st, 2nd and 3rd cycle: sufficient N)

Treatments	Stages	ABS/RC	DIo/RC	TRo/RC	φ(Po)	φ(Eo)	φ(Ro)	Pi abs	Pi total
Control	29 DAE	2.14±0.020c	0.45±0.008b	1.69±0.013d	0.79±0.002ns	0.45±0.006a	0.21±0.002a	2.30±0.085a	2.01±0.057a
NS1		2.34±0.019b	0.50±0.006a	1.84±0.015b	0.79±0.002	0.41±0.004b	0.20±0.003b	1.70±0.049c	1.58±0.048b
NS2		2.27±0.032b	0.49±0.008a	1.79±0.026c	0.79±0.002	0.43±0.010a	0.20±0.002b	2.00±0.119b	1.70±0.064b
NS3		2.42±0.028a	0.51±0.007a	1.91±0.023a	0.79±0.002	0.41±0.002b	0.20±0.002b	1.70±0.030c	1.61±0.043b
Control	44 DAE	2.99±0.092c	0.73±0.034c	2.25±0.055c	0.75±0.004a	0.34±0.006a	0.18±0.006a	0.86±0.049a	1.03±0.119a
NS1		3.69±0.025a	0.99±0.010a	2.69±0.014a	0.73±0.002b	0.32±0.005b	0.16±0.002b	0.56±0.016b	0.56±0.024b
NS2		3.08±0.039c	0.74±0.012c	2.34±0.028c	0.76±0.002a	0.34±0.006a	0.19±0.006a	0.82±0.034a	1.11±0.080a
NS3		3.35±0.099b	0.86±0.037b	2.48±0.062b	0.74±0.004b	0.35±0.003a	0.17±0.004b	0.77±0.042a	0.71±0.044b
Control	56 DAE	2.87±0.097b	0.67±0.033b	2.21±0.066ns	0.77±0.005a	0.40±0.007ns	0.16±0.006a	1.26±0.093ns	0.84±0.094a
NS1		3.42±0.138a	0.86±0.046a	2.56±0.095	0.75±0.004b	0.38±0.014	0.14±0.001b	0.94±0.106	0.51±0.027b
NS2		3.09±0.052b	0.71±0.020b	2.39±0.034	0.77±0.003a	0.40±0.003	0.16±0.005a	1.16±0.044	0.80±0.056a
NS3		2.99±0.067b	0.66±0.018b	2.33±0.050	0.78±0.002a	0.40±0.007	0.17±0.004a	1.26±0.069	0.89±0.043a
Control	79 DAE	2.53±0.050ns	0.53±0.015ns	2.00±0.037ns	0.79±0.002ns	0.43±0.006ns	0.17±0.003a	1.79±0.080ns	1.22±0.039a
NS1		2.63±0.063	0.55±0.019	2.07±0.045	0.79±0.002	0.42±0.008	0.16±0.003b	1.66±0.122	1.01±0.068b
NS2		2.51±0.057	0.52±0.016	1.99±0.041	0.79±0.002	0.43±0.005	0.18±0.004a	1.87±0.094	1.29±0.067a
NS3		2.49±0.061	0.51±0.018	1.98±0.044	0.79±0.003	0.43±0.007	0.17±0.004a	1.90±0.112	1.29±0.078a

Be continued…

Table 12 - Continuation

Treatments	Stages	ABS/RC	DIo/RC	TRo/RC	φ(Po)	φ(Eo)	φ(Ro)	Pi abs	Pi total
Control	83 DAE	2.91±0.169ns	0.65±0.065ns	2.26±0.106ns	0.78±0.009ns	0.37±0.016ns	0.14±0.009b	1.14±0.168ns	0.68±0.123ns
NS1		3.12±0.141	0.71±0.041	2.41±0.103	0.77±0.005	0.36±0.009	0.12±0.006b	0.98±0.096	0.52±0.074
NS2		2.70±0.097	0.57±0.040	2.14±0.059	0.79±0.007	0.42±0.013	0.16±0.005a	1.63±0.194	1.00±0.112
NS3		2.70±0.102	0.62±0.075	2.08±0.040	0.77±0.018	0.39±0.011	0.14±0.004b	1.38±0.171	0.74±0.094
Control	90 DAE	3.35±0.140a	0.88±0.078a	2.47±0.064a	0.74±0.012b	0.31±0.020b	0.10±0.009b	0.67±0.115b	0.32±0.066b
NS1		3.41±0.132a	0.87±0.060a	2.54±0.073a	0.75±0.008b	0.31±0.012b	0.10±0.003b	0.64±0.078b	0.30±0.029b
NS2		2.94±0.076b	0.66±0.030b	2.28±0.047b	0.78±0.005a	0.37±0.011a	0.13±0.005a	1.07±0.099a	0.58±0.052a
NS3		2.96±0.079b	0.69±0.029b	2.27±0.051b	0.77±0.004a	0.35±0.009a	0.12±0.009a	0.94±0.076a	0.50±0.077a

ABS/RC: absorption flux per reaction center (RC); DIo/RC: energy flux dissipated as heat per RC; TRo/RC: maximum capture rate per RC; φ(Po): photochemical maximum quantum yield; φ(Eo): quantum yield of electron transport from Quinone A (Q_A-) to the electron acceptor intersystem; φ(Ro): quantum yield of electron transport from Q_A- to the final PSI electron acceptor; Pi abs: partial photosynthetic performance index; Pi total: total photosynthetic performance index. DAE: days after emergency. Data represent the average ± SE of four biological replicates. Different lowercase letters between treatments within a column indicate statistical differences by the Scott-Knott test ($p < 0.05$) in each stage of rice growing. ns: absence of significant difference.

Table 13. Photosynthetic parameters of rice plants (variety Piauí) according to the JIP test. NS1 (1st and 2nd cycles: sufficient N; 3rd cycle: N stress); NS2 (1st cycle: N stress; 2nd cycle: sufficient N; and 3rd cycle: N stress); NS3 (1st, 2nd and 3rd cycle: N stress). C - control: (1st, 2nd and 3rd cycle: sufficient N).

Treatments	Stages	ABS/RC	DIo/RC	TRo/RC	φ(Po)	φ(Eo)	φ(Ro)	Pi abs	Pi total
Control	29 DAE	2.16±0.042b	0.46±0.016b	1.70±0.027b	0.79±0.004a	0.42±0.007a	0.20±0.003a	2.00±0.135a	1.79±0.103a
NS1		2.60±0.078a	0.58±0.023a	2.02±0.055a	0.78±0.002a	0.37±0.009b	0.19±0.005b	1.27±0.106b	1.28±0.111b
NS2		2.53±0.019a	0.59±0.012a	1.94±0.008a	0.77±0.003b	0.36±0.007b	0.18±0.005b	1.14±0.064b	1.23±0.049b
NS3		2.44±0.038a	0.54±0.007a	1.91±0.031a	0.78±0.001a	0.38±0.005b	0.20±0.001a	1.38±0.048b	1.47±0.030b
Control	44 DAE	3.38±0.117ns	0.91±0.055ns	2.47±0.067ns	0.73±0.008ns	0.29±0.014ns	0.17±0.006b	0.55±0.061ns	0.79±0.073b
NS1		3.57±0.082	0.94±0.035	2.63±0.048	0.74±0.004	0.31±0.007	0.17±0.004b	0.59±0.034	0.73±0.062b
NS2		3.61±0.101	0.97±0.033	2.65±0.069	0.73±0.002	0.29±0.009	0.16±0.006b	0.49±0.017	0.65±0.030b
NS3		3.30±0.059	0.85±0.024	2.45±0.037	0.74±0.003	0.29±0.010	0.19±0.004a	0.55±0.030	1.12±0.081a
Control	56 DAE	3.00±0.089ns	0.67±0.030ns	2.32±0.060ns	0.78±0.004ns	0.38±0.007ns	0.17±0.003ns	1.12±0.091ns	0.95±0.074ns
NS1		3.01±0.048	0.68±0.019	2.32±0.030	0.77±0.003	0.39±0.006	0.17±0.007	1.15±0.052	0.90±0.080
NS2		3.22±0.100	0.76±0.038	2.46±0.062	0.76±0.005	0.37±0.008	0.16±0.005	0.94±0.083	0.78±0.080
NS3		3.10±0.110	0.72±0.041	2.38±0.069	0.77±0.005	0.37±0.007	0.16±0.005	1.03±0.086	0.80±0.066
Control	79 DAE	2.58±0.043ns	0.53±0.012ns	2.06±0.032ns	0.80±0.002ns	0.41±0.005ns	0.16±0.002ns	1.62±0.075ns	1.07±0.045ns
NS1		2.60±0.031	0.53±0.008	2.07±0.023	0.80±0.001	0.42±0.002	0.16±0.002	1.64±0.041	1.04±0.029
NS2		2.60±0.048	0.53±0.013	2.07±0.036	0.80±0.002	0.42±0.004	0.17±0.001	1.74±0.069	1.11±0.037
NS3		2.65±0.039	0.54±0.009	2.11±0.031	0.80±0.001	0.42±0.002	0.16±0.001	1.63±0.035	1.04±0.025
Control	83 DAE	2.84±0.044b	0.64±0.021b	2.21±0.025b	0.78±0.004a	0.37±0.006a	0.14±0.002a	1.12±0.062a	0.70±0.028a
NS1		3.12±0.032a	0.77±0.016a	2.34±0.026a	0.75±0.004b	0.30±0.012b	0.12±0.003b	0.66±0.054b	0.47±0.020b
NS2		2.99±0.046b	0.69±0.021b	2.30±0.026a	0.77±0.004a	0.35±0.006a	0.14±0.004a	0.93±0.053a	0.64±0.047a
NS3		2.96±0.027b	0.67±0.018b	2.29±0.012a	0.77±0.004a	0.35±0.011a	0.14±0.003a	0.96±0.073a	0.62±0.037a

Be continued…

Table 13 - Continuation

Control	90 DAE	3.15±0.092ns	0.80±0.050ns	2.34±0.043ns	0.75±0.008ns	0.33±0.011ns	0.13±0.002a	0.76±0.087ns	0.48±0.022a
NS1		3.50±0.047	0.95±0.035	2.55±0.018	0.73±0.007	0.29±0.004	0.11±0.005b	0.52±0.029	0.30±0.028b
NS2		3.36±0.155	0.91±0.070	2.45±0.086	0.73±0.009	0.30±0.003	0.13±0.006a	0.56±0.052	0.44±0.015a
NS3		3.35±0.066	0.89±0.033	2.46±0.030	0.73±0.005	0.31±0.006	0.12±0.003a	0.59±0.024	0.40±0.024a

ABS/RC: absorption flux per reaction center (RC); DIo/RC: energy flux dissipated as heat per RC; TRo/RC: maximum capture rate per RC; φ(Po): photochemical maximum quantum yield; φ(Eo): quantum yield of electron transport from Quinone A (Q_A-) to the electron acceptor intersystem; φ(Ro): quantum yield of electron transport from Q_A- to the final PSI electron acceptor; Pi abs: partial photosynthetic performance index; Pi total: total photosynthetic performance index. DAE: days after emergency. Data represent the average ± SE of four biological replicates. Different lowercase letters between treatments within a column indicate statistical differences by the Scott-Knott test ($p < 0.05$) in each stage of rice growing. ns: absence of significant difference.

Table 14. Metabolic profile of samples from flag leaves of rice plants (variety Manteiga) subjected to N-stress regimes. NS1 (1st and 2nd cycles: sufficient N; 3rd cycle: N stress); NS2 (1st cycle: N stress; 2nd cycle: sufficient N; and 3rd cycle: N stress); NS3 (1st, 2nd and 3rd cycle: N stress). C - control: (1st, 2nd and 3rd cycle: sufficient N).

Metabolites	Treatments				
Organic acids	Control	NS1	NS2	NS3	P value
	peak intensity				
Dehydroascorbic acid dimer	10565±2139ns	17928±3121	11350±705	14175±774	0.304
Ferulic acid	**4104±795b**	**8043±469a**	**9740±593a**	**10557±242a**	**<0.001**
Gluconic acid-1,4-lactone	143279±4398ns	127009±6270	132652±4994	133371±4761	0.3101
Levulinic acid	**2484±20b**	**1840±182c**	**3074±94a**	**2843±48a**	**<0.001**
Oxoglutaric acid	**1966±1605b**	**3677±1550b**	**7393±150a**	**11464±605a**	**0.006**
Salicylic acid	**1551±105d**	**2248±41c**	**2571±110b**	**3012±32a**	**<0.001**
Succinic acid	**2002±399b**	**2288±198b**	**3870±53a**	**3268±133a**	**0.006**

Sugars	Control	NS1	NS2	NS3	P value
	peak intensity				
Galactose	87591±10401ns	50828±9048	62509±5197	62820±8788	0.165
Glucopyranose	**5365±493c**	**6968±84b**	**7214±238b**	**8806±130a**	**<0.001**
Inositol	**118776±5413b**	**166457±2687a**	**161627±5115a**	**154331±2248a**	**<0.001**
Lactose	**37880±3251a**	**37934±1838a**	**27819±863b**	**38126±377a**	**0.037**
Melezitose	**946±405c**	**3778±179b**	**3757±117b**	**4843±221a**	**<0.001**
Palatinose	2252±216ns	1951±133	2052±39	2185±89	0.760
Psicose	**6744±424a**	**5140±283b**	**6638±177a**	**4986±97b**	**0.009**
Raffinose	**3854±173b**	**5236±333b**	**6078±406a**	**7350±575a**	**0.006**
Saccharic acid	**2933±52b**	**3351±211b**	**4606±35a**	**3746±339a**	**0.008**
Sorbitol	9165±325ns	9132±219	9431±227	9279±122	0.371
Viburnitol	**4208±24a**	**3192±40c**	**3595±25b**	**3653±137b**	**<0.001**

Be continued…

Table 14 - Continuation

Amino acids and derivates	Control	NS1	NS2	NS3	P value
	peak intensity				
Agmatine	15247±2846ns	15215±1340	12114±1053	14711±1037	0.727
Alanine	5713±701ns	4936±895	5566±174	4095±1672	0.765
GABA	**4802±253b**	**5635±80b**	**5149±110b**	**6682±269a**	**0.003**
Glutamic acid	**37456±8043b**	**29499±2481b**	**47238±3040a**	**62365±2613a**	**0.018**
Glycine	**5118±792b**	**7835±520a**	**5802±72b**	**9349±1081a**	**0.034**
Proline	1796±272ns	2400±711	1920±145	2625±211	0.179
Pyroglutamic acid	**4621±3773b**	**4657±3802b**	**14270±870a**	**21087±1389a**	**0.024**
Serine	**10526±552a**	**12273±307a**	**8169±654b**	**10787±323a**	**0.008**
Threonine	2730±496ns	2925±133	2730±128	3744±346	0.281
Tyramine	2416±205ns	2407±140	2068±28	2733±176	0.127

Alkanes	Control	NS1	NS2	NS3	P value
	peak intensity				
Eicosane	1399±181ns	1318±173	826±344	1616±67	0.232
Heptadecane	2404±198ns	2852±9	2938±66	2760±143	0.183
Nocosane	**411±336b**	**1255±4a**	**1328±52a**	**1346±51a**	**0.038**
Octacosane	2908±125ns	3132±14	3044±72	3220±82	0.262

Lipids	Control	NS1	NS2	NS3	P value
	peak intensity				
Nonoic acid methyl ester	2419±62ns	2665±56	2582±94	2581±97	0.416
Octadecanoic acid-2,3-dihydroxypropyl ester	5930±833ns	7576±251	6691±362	6754±636	0.509
Palmitic acid	25583±2128ns	27083±483	29558±1026	29255±405	0.269
Sphingosine	**466±380b**	**2216±192a**	**1513±74a**	**1747±143a**	**0.012**
Stearic acid	36952±4080ns	41696±681	43152±1579	41827±1724	0.509

Be continued...

Table 14 - Continuation

Others	Control	NS1	NS2	NS3	P value
	peak intensity				
Ditertbutylphenol	**1929±190b**	**2438±44a**	**2645±34a**	**2565±63a**	**0.016**
Ethanolamine	**47673±5238b**	**56138±2511b**	**48831±762b**	**67997±1273a**	**0.016**
Quinic acid	5326±827ns	9336±1284	5974±570	14473±4435	0.180
Secologanin	**5734±286b**	**6068±138b**	**5774±163b**	**6916±53a**	**0.018**
Thiazole, 4-methyl-5-hydroxyethyl-	820±335ns	907±371	1248±21	1311±61	0.618

Data represent the average of three biological replicates ± SE. The compounds that significantly differed ($p < 0.05$) through analysis of variance are in bold. ns: absence of significant difference.

Table 15. Metabolic profile of samples from flag leaves of rice plants (variety Piauí) subjected to N-stress regimes. NS1 (1st and 2nd cycles: sufficient N; 3rd cycle: N stress); NS2 (1st cycle: N stress; 2nd cycle: sufficient N; and 3rd cycle: N stress); NS3 (1st, 2nd and 3rd cycle: N stress). C - control: (1st, 2nd and 3rd cycle: sufficient N).

Metabolites	Treatments				
Organic acids	Control	NS1	NS2	NS3	P value
	peak intensity				
Gluconic acid-1,4-lactone	**121051±1586a**	**103926±6493b**	**134377±2649a**	**130691±1242a**	**0.001**
Levulinic acid	**10862±230a**	**10859±237a**	**7705±241b**	**8287±228b**	**<0.001**
Oxoglutaric acid	**21764±2155a**	**25759±2288a**	**12270±1280b**	**13709±747b**	**0.001**
Succinic acid	**4818±110a**	**4231±227a**	**2668±171b**	**2672±279b**	**<0.001**

Sugars	Control	NS1	NS2	NS3	P value
	peak intensity				
Fructose	**178911±4751b**	**173102±24306b**	**238153±10560a**	**239247±5222a**	**0.015**
Fucose	90569±944ns	88557±995	88013±1114	86773±2018	0.409
Galactose	55356±4317ns	48449±11432	77282±8275	74344±9625	0.178
Lamiribiose	13809±1748ns	12473±633	10185±453	14415±1434	0.193
Melezitose	**6670±619b**	**9352±539a**	**4165±490c**	**7944±336b**	**<0.001**
Palatinose	4567±426ns	4591±208	4522±176	4617±271	0.997
Psicose	**7333±514b**	**6834±304b**	**9736±1016a**	**9453±637a**	**0.046**
Raffinose	**5950±372b**	**8702±375a**	**3405±180c**	**5193±184b**	**<0.001**
Ribonic acid	**7916±472a**	**7080±845a**	**3714±256b**	**3728±106b**	**<0.001**
Ribose	1627±128ns	1659±149	1705±128	1596±65	0.953
Ribulose	8353±808ns	8303±449	7908±470	7213±222	0.549
Saccharic acid	**10607±534a**	**8776±663a**	**5448±776b**	**5264±350b**	**<0.001**
Sorbitol	**6101±174a**	**6068±189a**	**5480±116a**	**4516±188b**	**<0.001**
Sorbose	**225951±9750b**	**203369±23069b**	**269410±6330a**	**268291±10655a**	**0.033**

Be continued…

Table 15 - Continuation

Amino acids and derivates	Control	NS1	NS2	NS3	P value
	peak intensity				
Caproic acid, 6-amino-	**15969±1115b**	**18188±606b**	**64796±13432a**	**50702±10265a**	**0.009**
Cysteine Sulfinic acid	**3750±63a**	**3347±116b**	**3852±77a**	**3879±37a**	**0.005**
GABA	**7968±984a**	**7763±336a**	**5271±592b**	**8366±458a**	**0.046**
Glutamic acid	**117290±9763a**	**133240±6916a**	**54183±3798c**	**82676±4281b**	**<0.001**
Glycine	13423±1261ns	12272±1346	7784±438	10881±1105	0.053
Homoserine lactone, N-octanoyl-	**4812±587a**	**4580±178a**	**3230±341b**	**3137±153b**	**0.025**
Iminodiacetic acid	**12481±1234a**	**12428±1070a**	**3296±317b**	**4132±399b**	**<0.001**
Isoleucine	**7821±625a**	**7103±312a**	**6977±1442a**	**3140±203b**	**0.017**
N-acetyl-Muramic acid	**4947±207a**	**4723±232a**	**3211±122b**	**3271±39b**	**<0.001**
Oxamide	**45151±1102a**	**45454±649a**	**21586±1683b**	**25240±1905b**	**<0.001**
Proline	**3604±96a**	**3435±252a**	**3246±186a**	**2082±123b**	**<0.001**
Pyroglutamic acid	**48020±6509a**	**56473±5194a**	**19601±1032b**	**25899±1218b**	**<0.001**
Serine	31918±3468ns	26705±3891	23942±3766	34697±4461	0.363
Threonine	**5894±690a**	**5664±221a**	**4425±420b**	**3479±483b**	**0.033**
Valine	6424±735ns	6844±217	6386±933	4534±227	0.153

Alkanes	Control	NS1	NS2	NS3	P value
	peak intensity				
Heptadecane	**2943±81a**	**2883±108a**	**2110±211b**	**2066±97b**	**0.002**
Octacosane	3263±70ns	3250±34	3331±213	3065±60	0.565

Lipids	Control	NS1	NS2	NS3	P value
	peak intensity				
Nonoic acid methyl ester	2921±48a	2663±67b	2483±70b	2486±64b	0.003
Palmitic acid	**34646±448a**	**31059±364a**	**26876±2265b**	**26729±1246b**	**0.009**
Stearic acid	**48081±1745a**	**45286±987a**	**38809±3249b**	**37945±1289b**	**0.024**

Be continued…

Table 15 - Continuation

Others	Control	NS1	NS2	NS3	P value
	peak intensity				
Adenosine	**14247±217b**	**13551±216b**	**16142±807a**	**15349±230a**	**0.018**
beta-D-Fructofuranosyl-(2,1)-beta-D-Fructofuranose	38390±3630ns	37449±1527	42548±1309	40082±1078	0.509
Ethanolamine	80636±10251ns	72446±1059	55624±1610	69840±6572	0.148
Octadecanoic acid-2,3-dihydroxypropylester	7563±405ns	7699±380	6177±487	6857±526	0.201
Propane-1,2-diol	2062±108ns	2104±157	1844±107	1685±137	0.215
Quinic acid	31918±3468ns	26705±3891	23942±3766	34697±4461	0.363

Data represent the average of three biological replicates ± SE. The compounds that significantly differed ($p < 0.05$) through analysis of variance are in bold. ns: absence of significant difference.