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Rice varieties exhibit different mechanisms for Nitrogen Use Efficiency (NUE)

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Abstract

Nitrogen use efficiency (NUE) is an important characteristic for increasing the yield and quality of rice grains and reducing the use of nitrogen fertilizers. This study evaluated parameters that contribute to NUE in two tropical japonica rice varieties contrasting in nitrogen remobilization efficiency (NRE) and nitrate-uptake kinetics: IAC-47 (bred for high-input farming), and Piauí (a landrace from the state of Maranhão, Brazil). The two varieties were grown with and without N supplementation at anthesis stage. Both varieties received urea equivalent to 60 kg N ha^{-1} in the first ten days after planting (DAP) and a subset of the two varieties received a supplementation with 40 kg N ha^{-1} at anthesis stage, composing a treatment with supplementation (Ts). The control treatment (Tc) was constituted of the plants that did not receive nitrogen supplementation at anthesis. We analyzed the nitrogen soluble fractions, soluble sugars, dry matter production, total N, crude protein in grains, NUE parameters, and expression and activity of glutamine synthetase (GS). The N uptake after anthesis affected the total N content in the shoots and grains of both rice varieties. In treatments with and without N supplementation, the nitrogen uptake efficiency (NUpE) was higher in IAC-47 than in Piauí plants. The NRE, a desirable characteristic for plants grown with low N input, was around two times higher in the Piauí plants that were not supplemented with N during anthesis. panicles of piauí plants grouwn without N supplementation presented higher expression of the glutamine synthetase gene *OsGS1.3* post-anthesis. The results indicate that NRE is the primary factor contributing to NUE in rice varieties adapted to a low N supply, whereas improved varieties exhibit a higher NUpE.

Keywords: Nitrogen remobilization efficiency, nitrogen uptake efficiency; glutamine synthetase; Nitrate Reductase.
Abbreviations: NUE_ Nitrogen Use Efficiency; NRE_ Nitrogen Remobilization Efficiency; NUpE_ Nitrogen Uptake Efficiency; NHI_ Nitrogen harvest index; NUtE_ Nitrogen utilization efficiency; Tc_ control treatment; Ts_ treatment with N supply at anthesis; GS_ glutamine synthetase; DBA_ days before anthesis; DAA_ days after anthesis; NRA_ nitrate reductase activity;

Introduction

Rice is an important cereal for human nutrition, especially for low-income populations, as it provides an affordable source for calories intake. However, rice production depends on intensive use of fertilizers, especially nitrogen fertilizers. The use of varieties with higher NUE can be an economically positive strategy for the production of cereal crops. In addition, the environmental benefits generated by reducing the use of nitrogen fertilizers for growing these plants should be taken into account. According to Chardon et al. (2012), with the stagnation of production after the green revolution, producers have as challenge circumvent the increased costs of production and, at the same time, increase crop yields to meet the increased demand of the world population for food. The development of rice varieties that use N efficiently is; therefore, important for increasing rice yield under systems with low N fertilizer use and for decreasing the use of fertilizers in systems that make intensive use of N fertilizers to lower production costs and environmental impact. It is necessary to have a comprehensive knowledge of the regulatory mechanisms controlling N use, particularly when N is limited in the environment, to permit the use of molecular breeding and genetic engineering approaches to improve crop plants for complex traits like NUE (Kant et al., 2011). However, the molecular basis underlying adaptation to low N environments is poorly understood owing to the complexity in plant responses to N-limitation (Guevara et al., 2014). Many farmers in developing countries cannot use much N fertilizer due to financial conditions and lack of crops that genetically improved for yielding well under limiting N (Kant et al., 2011). Specific techniques for N management, the improvement of traditional plants and biotechnology can be used to increase NUE in cereal crops to maintain or even increase yield (Beatty et al., 2010). Much of the EUN is linked to the efficiency of N remobilization during the reproductive period of crop plants. According to Kant et al. (2011), the use of N can be divided into two stages during the life cycle of plants, being the first vegetative stage, where biomass formation occurs, and the N is stored, and the second is the reproductive stage, where most of the remobilization occurs when N is transported to the seeds, especially like amino acids.

The overexpression of N transport enzyme do not lead to significant increase of NUE, which can be associated with the operation of the control system for "feedback" acting on these enzymes. So, perhaps the most challenging aspects to increase the nitrogen use efficiency in plants are the identification and management of interactions between the genes and coordinating the metabolism of carbon and nitrogen (CN) (Brito and Konzucher, 2004). Therefore, the study of enzymes associated with carbon and nitrogen metabolism, such as glutamine sintetase (GS) is important.

Bao et al. 2014 reported that GS/GOGAT cycle is the first step of incorporation of inorganic nitrogen into organic nitrogenous compounds in higher plants, which is a major checkpoint for controlling nitrogen assimilation. The recent studies have demonstrated that overexpression of *GS1;1*-, *GS1;2* genes in plants decreased growth and yield due to a imbalance in carbon and nitrogen metabolic status. Thus, it is important to study the expression parameters of this genes in plants to plants contrasting to EUN to verify if there are differences in expression patterns. The contribution of N remobilization from leaves to grains, which ranges from 50 to 90%, is a characteristic that is genetically controlled in rice, wheat and corn plants (Masclaux et al., 2001). Thus, nitrogen remobilization is a key factor for enhancing NUE (Mickelson et al., 2003; Masclaux-Daubresse et al., 2010). Among the enzymes involved in N metabolism, glutamine synthetase (GS) has been suggested as critical for plant NUE, particularly given its participation in N remobilization and recycling and its increased activity in the reproductive phase of the development cycle. In addition, cytosolic isoforms of GS participate in N remobilization during senescence (Swarbreck et al., 2011).

Piauí is a local rice variety from Maranhão State, Brazil, that is grown under rainfed conditions with low nitrogen fertilizer use. The climate of Maranhão State includes two main seasons: a rainy winter and a dry summer. According to Rodrigues et al. (2004), the onset of the rainy season in the region facilitates the rise of N-NO_3^- from the deeper soil layers, which, in addition to the accumulation of NO_3^- from the mineralization of organic matter in the superior soil layers, establishes a "seasonal flow of NO_3^-". Souza et al. (1998) reported that compared to improved rice varieties, the local Piauí variety was more efficient in remobilizing N from plant parts and, therefore, less dependent on external N supplementation during grain filling. According to Rodrigues et al. (2004), adaptations of the Piauí variety to the environmental conditions of Maranhão State might have enabled higher N use efficiency (NUE) in Piauí compared to improved varieties such as IAC-47. Therefore, it is important to study these plants to identify mechanisms associated with NUE under low nitrogen fertilization. We investigated the contributions of parameters affecting NUE in two contrasting rice varieties: IAC-47, improved for high input agriculture, and Piauí, a local variety of Maranhão State, Brazil.

Results

Nitrate reductase activity (NRA)

The NRA was low in all plant parts and treatments, but differences between the two varieties were observed (Fig 1). The N supplementation (Ts) increased NRA in the flag, young and old leaves of IAC-47 but did not affect or decreased NRA in Piauí (Fig 1A, B and D). The NRA in flag leaves at 15 DBA and 15 DAA in the treatment with N supplementation (Ts) was lower in Piauí than in IAC-47 (Fig 1A). In IAC-47 plants, the NRA in the flag leaves and old leaves decreased at anthesis and increased at 15 DAA (Fig 1A and D). Small or no difference between varieties was found in the second and older leaves, sheaths and panicles (Fig C-F).

Varieties show marked differences in nitrogen soluble fractions

In all plant parts analyzed, N-NO_3^- levels were approximately 10 µmoles g^{-1} of fresh weight in the Piauí variety at 15 DBA, whereas the improved IAC-47 variety did not exhibit detectable N-NO_3^- levels in any parts analyzed in this period (Fig 2A). At anthesis, N-NO_3^- levels in the different plant parts were similar between the two varieties (Fig 2B). At 15 DAA, N-NO_3^- level was higher in the Piauí variety than in all plant parts of IAC-47 (Fig 2C, D, E and F) independent of N supplementation. Low NH_4^+ levels were detected in all plant parts of both treatments (Fig 3A - G). Amino-N levels at 15 DBA were higher in the sheath of the Piauí variety (Fig 4A). At anthesis, amino-N levels were higher in the second leaves, young leaves, old leaves and sheaths of IAC-47 than of the Piauí variety (Fig 4B), similar to the NH_4^+ levels (Fig 3B). The highest amino-N values in both varieties were detected in the panicles during anthesis, reaching up to 60 µmoles g^{-1} of fresh matter in IAC-47 (Fig 4B). After anthesis, amino-N levels were higher in Piauí leaves and sheaths (Fig 4C, D, E and F). At 15 DAA, the highest amino-N levels in panicles were observed in IAC-47 plants, but no differences were observed between treatments in either variety (Fig 4G).

At 15 DBA, soluble sugar levels were higher in the sheaths of IAC-47 than that of Piauí plants (Fig 5A). At anthesis, sugar levels in IAC-47 were higher than in the Piauí variety only in the flag leaves (Fig 5B). Sugar levels were higher at 15 DAA in the flag leaves of IAC-47 than of Piauí plants (Fig 5C), and plants of both varieties exhibited higher soluble sugar levels in the old leaves and sheaths when submitted to T_S than to T_C (Fig 5E and F). The soluble sugar increased from 15 DBA to anthesis and decreased from anthesis to 15 DAA in all parts of the plants in both varieties (Fig 5A- G).

Nitrogen uptake efficiency and nitrogen remobilization efficiency differ between varieties, but not NUE

In both treatments, the filled grain weight did not vary significantly between varieties (Table 1). In both T_C and T_S, the IAC-47 variety exhibited a greater shoot N content than the Piauí variety (Table 1). Plants of both varieties receiving N supplementation (T_S) exhibited higher shoot N content than non-supplemented plants (Table 1), but the N content in the grains was similar between the varieties in T_S and T_C (Table 1). In both varieties, the grain N content was higher in plants submitted to T_S (Table 1). NHI and NUtE were not affected by the N supply; however, the Piauí variety exhibited a higher NHI and NUtE than IAC-47 in both treatments (Fig 6A and B). In both treatments, NUpE was higher in IAC-47 than in Piauí plants (Fig 6C). Plants from T_C exhibited higher NUpE and NUE than those from T_S for both varieties (Fig 6C and D), but NUE did not differ between varieties (Fig 6D).

The grain protein content was also similar between the rice varieties (Fig 6E). Both IAC-47 and Piauí plants submitted to T_S exhibited higher grain protein content than those submitted to T_C (Fig 6 E). A decrease in the shoot N content from anthesis to the maturity stage was more severe in plants that were not supplemented with N and in the Piauí variety than in IAC-47 (Fig 7A).

Table 1. Filled grains weight (g pot^{-1}) and shoot and grain N content (mg pot^{-1}) at the maturity stage of IAC-47 and Piauí grown under T_C and Ts treatments. Uppercase letters indicate differences between the varieties, and lowercase letters indicate differences between the treatments in the same column (Tukey's test, $P \leq 0.05$).

Varieties	Treatments (kg N ha^{-1})	Filled grain weight (g pot^{-1})	±SE	Shoot N content (mg pot^{-1})	±SE	Shoot dry weight (g per pot^{-1})	±SE	Grain N content (mg pot^{-1})	±SE
Piauí	Tc (40+20)	24.38Aa	±1.72	484.17Bb	±8.68	45.85Ab	1.70	310.73Ab	±10.96
	Ts (40+20+40)	26.30Aa	±0.75	626.29Ba	±16.95	56.51Aa	2.89	408.84Aa	±2.04
IAC	Tc (40+20)	24.54Aa	±1.57	600.93Ab	±37.86	46.03Ab	2.46	341.35Ab	±23.43
	Ts (40+20+40)	27.78Aa	±0.60	722.62Aa	±10.57	49.04Aa	1.80	433.45Aa	±2.96

Tc (40 kg N ha^{-1} at planting + 20 kg ha^{-1} at 10 DAP); Ts (40 kg N ha^{-1} at planting + 20 kg ha^{-1} at 10 DAP + 40 kg ha^{-1} at anthesis). DAP=days after planting.

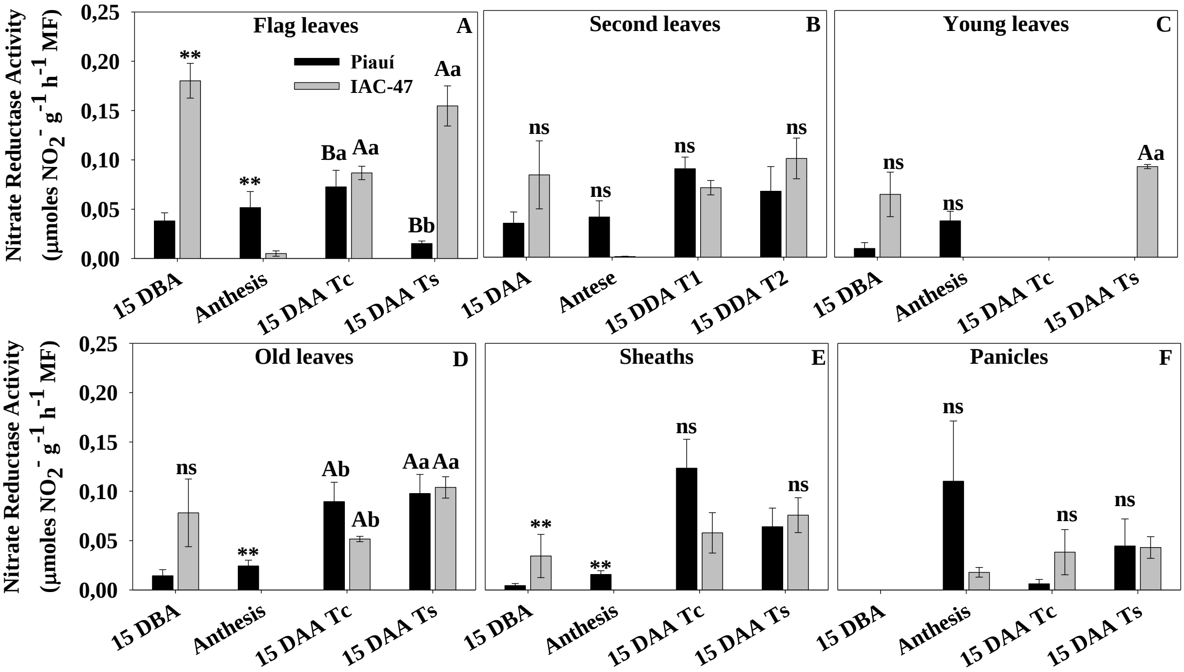

Fig 1. Nitrate reductase activity (NRA). NRA (mean + SE; N=3) 15 days before anthesis (15 DBA), at anthesis and 15 days after anthesis (15 DAA) in flag leaves (A), second leaves (B), old leaves (C) sheaths (D) and panicles (F) of the IAC-47 and Piauí rice varieties grown under treatments T_C (40 kg N ha^{-1} at planting and 20 kg ha^{-1} at 10 DAP) or T_S (40 kg N ha^{-1} at planting + 20 kg ha^{-1} at 10 DAP + 40 kg ha^{-1} at anthesis). (*) and (**) significant differences ($P \leq 0.05$ and $P \leq 0.01$, respectively) and (ns) Non-significant differences between IAC-47 and Piauí at 15 DBA and anthesis harvests according to the F test. Uppercase letters indicate differences between the varieties, and lowercase letters indicate differences between the treatments (Tukey's test, $P \leq 0.05$).

Piauí and IAC-47 plants from T_S exhibited more N uptake after anthesis than those from T_C (Fig 7B), which resulted in an increase of 134 mg N for the IAC variety and 154.5 mg N for the Piauí variety in Ts plants (data not shown). T_C Piauí plants exhibited the highest N remobilization to the grains and the lowest values when submitted to T_S (Fig 7C). In IAC-47, N remobilization to the grains was not affected by N supplementation (Fig 7C). The efficiency of N remobilization in Piauí plants without N supplementation was higher than in Piauí plants with N supplementation and IAC-47 plants under both treatments (Fig 7D). The NRE in IAC-47 plants was not affected by N supplementation (Fig 7D).

GS activity and expression differ between varieties

Small differences in the expression of *GS1.1* and *GS2* were observed between varieties (Fig 8A-F). *OsGS1.3* expression in panicles at 15 DAA was five-fold higher in the Piauí variety without supplemental N than with supplemental N and higher than in the IAC-47 variety under both treatments (Fig 8H).

The GS activity was lower at 15 DBA and 15 DAA than at anthesis for the Piauí variety (Fig 9). At anthesis, the GS activity in the flag leaf, second leaves and panicles was higher in Piauí than IAC-47 (Fig 9B).

Discussion

The piauí variety exhibits metabolic characteristics that may contribute to nitrogen remobilization and adaptation to low N supply environments

We observed differences in NRA between both varieties once Piauí plants presented lower NRA in flag leaves and sheaths at 15DBA. Associated with this NRA, Piauí Plants presented higher NO_3^- levels than IAC-47 before anthesis (Fig 2). This low NRA in Piauí plants may have contributed to its nitrate accumulation in tissues before anthesis. This behavior of ANR and nitrate accumultion in Piauí plants are in accordance with the findings of Rodrigues et al. (2004), Souza and Fernandes (2006) and Santos et al. (2009a, 2009b), who also observed an accumulation of nitrate in

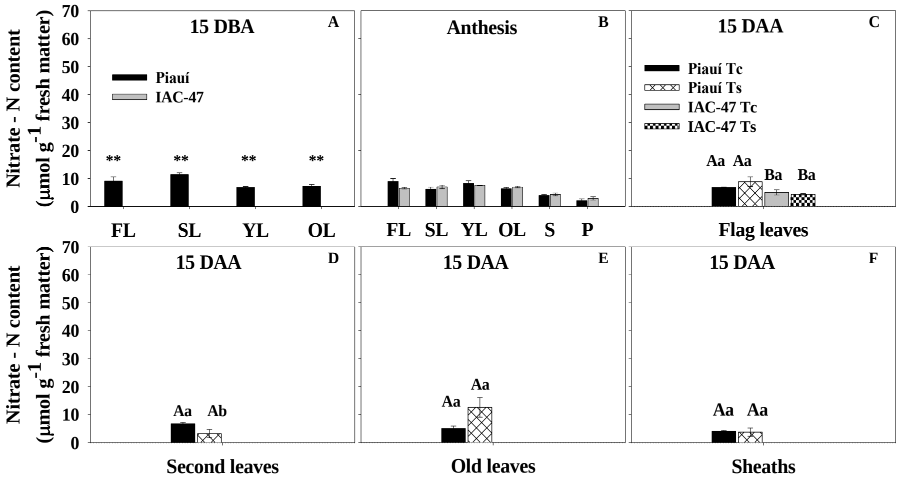

Fig 2. Nitrate levels in the flag leaves (FL), second leaves (SL), young leaves (YL), old leaves (OL), sheaths (S) and panicles (P) in IAC-47 and Piauí. Plants were grown under treatments T_C (40 kg N ha^{-1} at planting and 20 kg ha^{-1} at 10 DAP) or T_S (40 kg N ha^{-1} at planting + 20 kg ha^{-1} at 10 DAP + 40 kg ha^{-1} at anthesis) and sampled at (A) 15 DBA , (B) anthesis and (C, D, E, F)15 DAA. (**) Statistically significant differences between IAC-47 and Piauí at 15 DBA and anthesis (Tukey's test, $P \leq 0.01$). Uppercase letters indicate differences between the varieties, and lowercase letters indicate differences between the treatments (Tukey's test, $P \leq 0.05$).

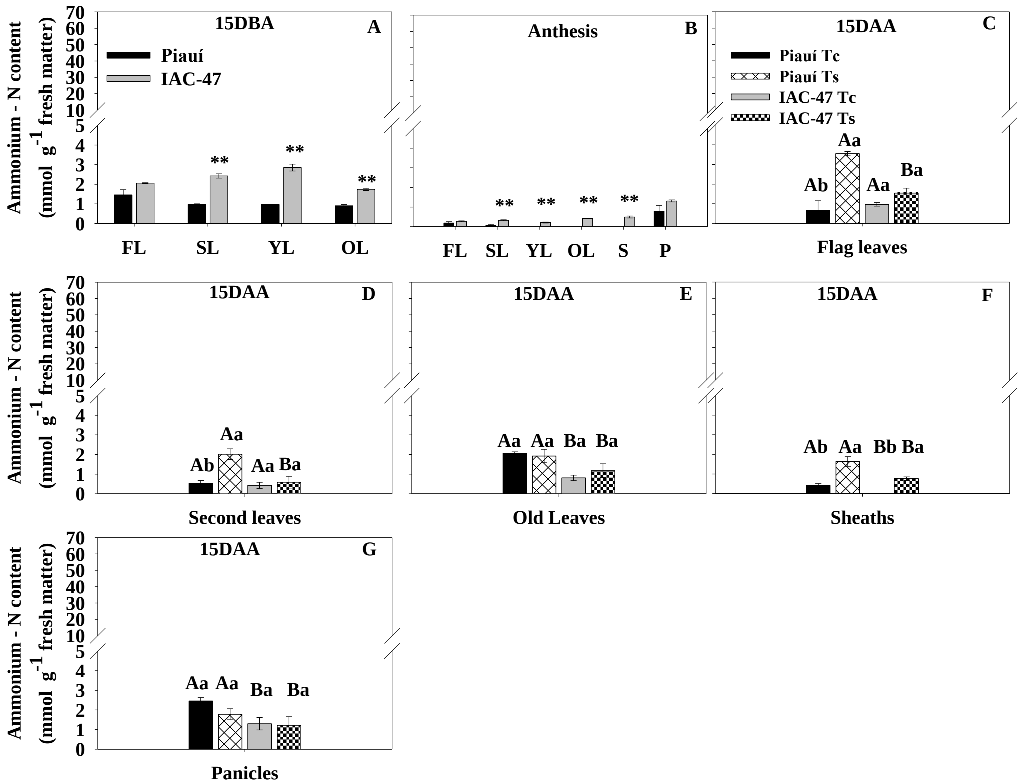

Fig 3. Ammonium levels in the flag leaf (FL), second leaves (SL), young leaves (YL), old leaves (OL), sheaths (S) and panicles (P) in IAC-47 and Piauí. Plants were grown under treatments T_C (40 kg N ha^{-1} at planting and 20 kg ha^{-1} at 10 DAP) or T_S (40 kg N ha^{-1} at planting + 20 kg ha^{-1} at 10 DAP + 40 kg ha^{-1} at anthesis). The plants were sampled at (A)15 DBA, (B) anthesis and (C, D, E, F)15 DAA. (**) Statistically significant differences between IAC-47 and Piauí at 15 DBA and anthesis (Tukey's test, $P \leq 0.01$). Uppercase letters indicate differences between the varieties, and lowercase letters indicate differences between the treatments (Tukey's test, $P \leq 0.05$).

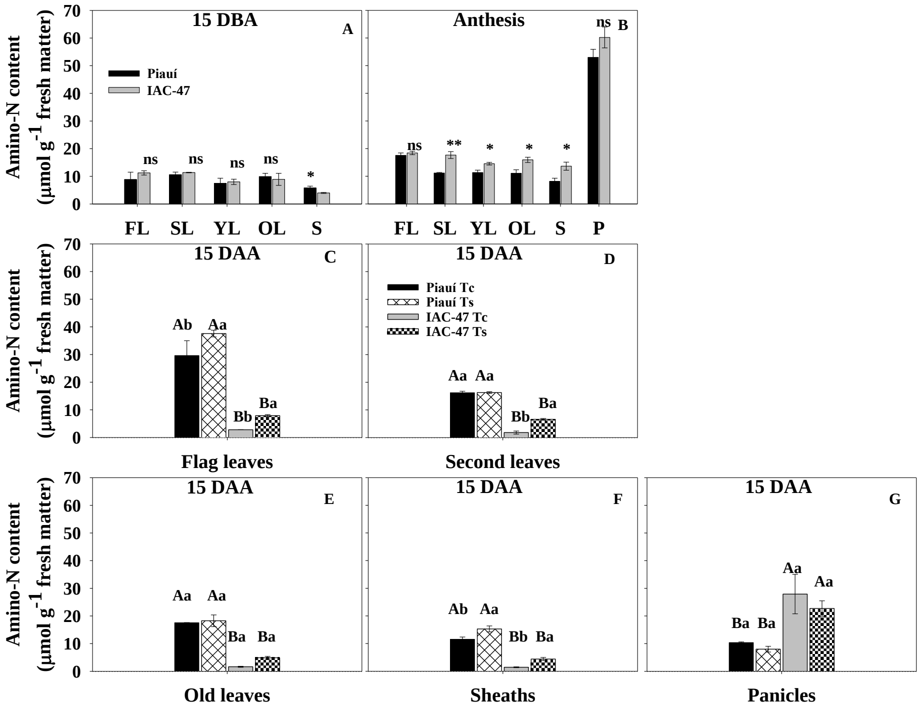

Fig 4. Amino-N levels in the flag leaf (FL), second leaves (SL), young leaves (YL), old leaves (OL), sheaths (S) and panicles (P) in IAC-47 and Piauí. Plants were grown under treatments T_C (40 kg N ha^{-1} at planting and 20 kg ha^{-1} at 10 DAP) or T_S (40 kg N ha^{-1} at planting + 20 kg ha^{-1} at 10 DAP + 40 kg ha^{-1} at anthesis). The plants were sampled at (A)15 DBA, (B) anthesis and (C, D, E, F)15 DAA. (*) and (**) statistically significant differences (Tuckey´s test $P \leq 0.05$ and $P \leq 0.01$, respectively) and (ns) non-significant differences between IAC-47 and Piauí at 15 DBA and anthesis harvests according to the F test. Uppercase letters indicate differences between the varieties, and lowercase letters indicate differences between the treatments (Tukey's test, $P \leq 0.05$).

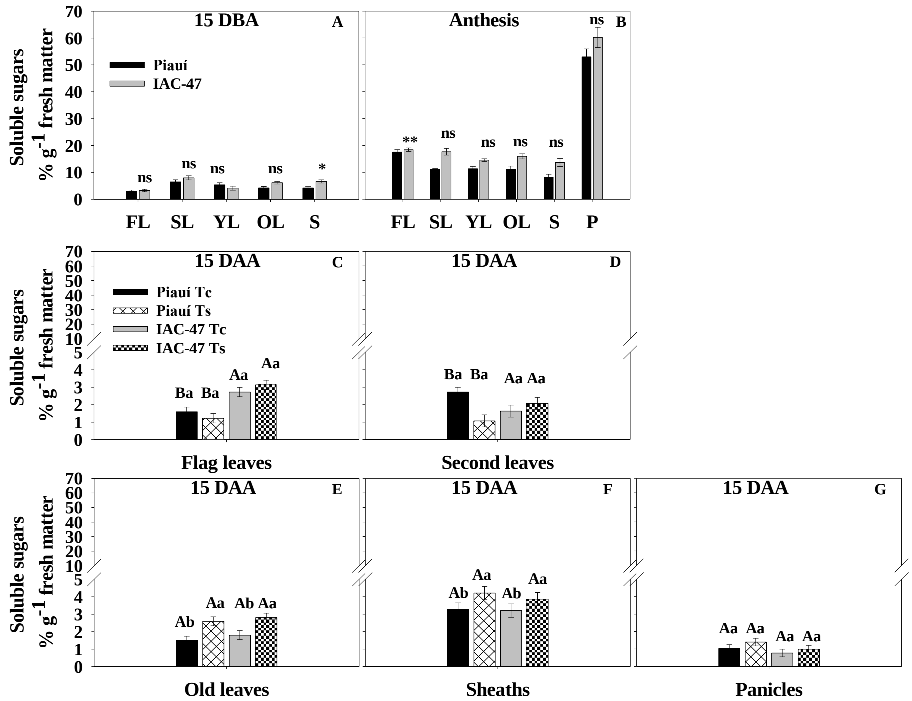

Fig 5. Sugar levels in IAC-47 and Piauí grown under treatments T_C (40 kg N ha^{-1} at planting and 20 kg ha^{-1} at 10 DAP) or T_S (40 kg N ha^{-1} at planting + 20 kg ha^{-1} at 10 DAP + 40 kg ha^{-1} at anthesis). The plants were sampled at (A) 15 DBA, (B) anthesis and (C, D, E, F)15 DAA. (*) and (**) statistically significant differences (Tuckey´s test $P \leq 0.05$ and $P \leq 0.01$, respectively) and (ns) non-significant differences between IAC-47 and Piauí at 15 DBA and anthesis harvests according to the F test. Uppercase letters indicate differences between the varieties, and lowercase letters indicate differences between the treatments (Tukey test, $P \leq 0.05$).

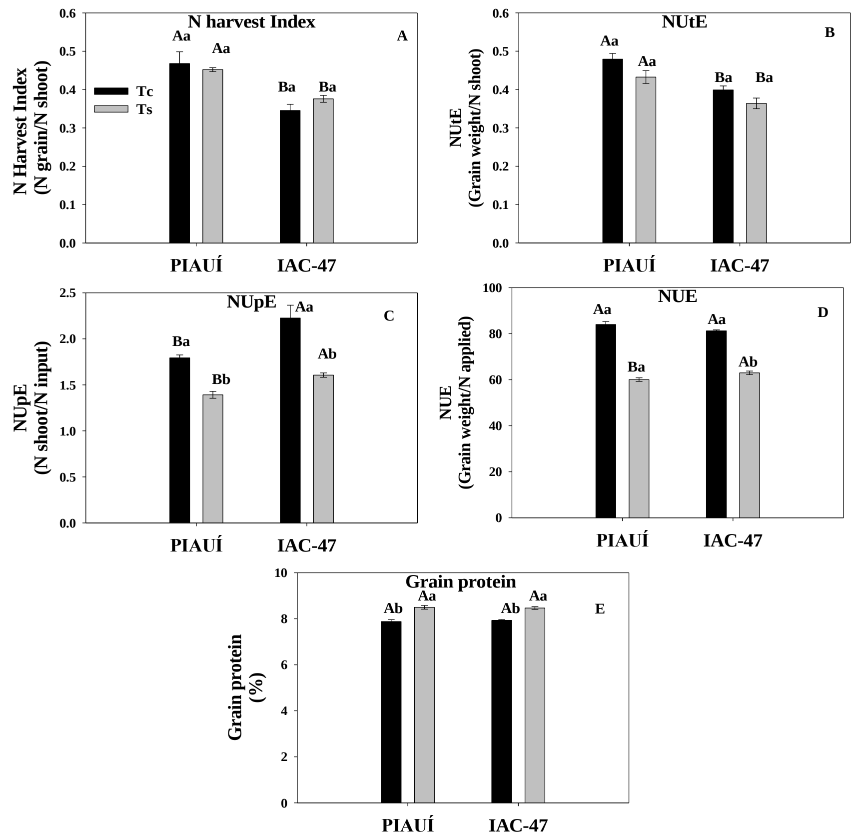

Fig 6. Nitrogen Use Efficiency parameters. (A) Nitrogen harvest index (NHI), (B) N utilization efficiency (NUtE), (C) N uptake efficiency (NUpE), (D) N use efficiency (NUE) and (E) grain protein content in IAC-47 and Piauí varieties grown under treatments T_C (40 kg N ha^{-1} at planting and 20 kg ha^{-}1 at 10 DAP) or T_S (40 kg N ha^{-1} at planting + 20 kg ha^{-1} at 10 DAP + 40 kg ha^{-1} at anthesis). Uppercase letters indicate an interaction effect between variety *vs*. treatment, and lowercase letters indicate an interaction effect between treatment *vs*. variety (Tukey's test, $P \leq 0.05$).

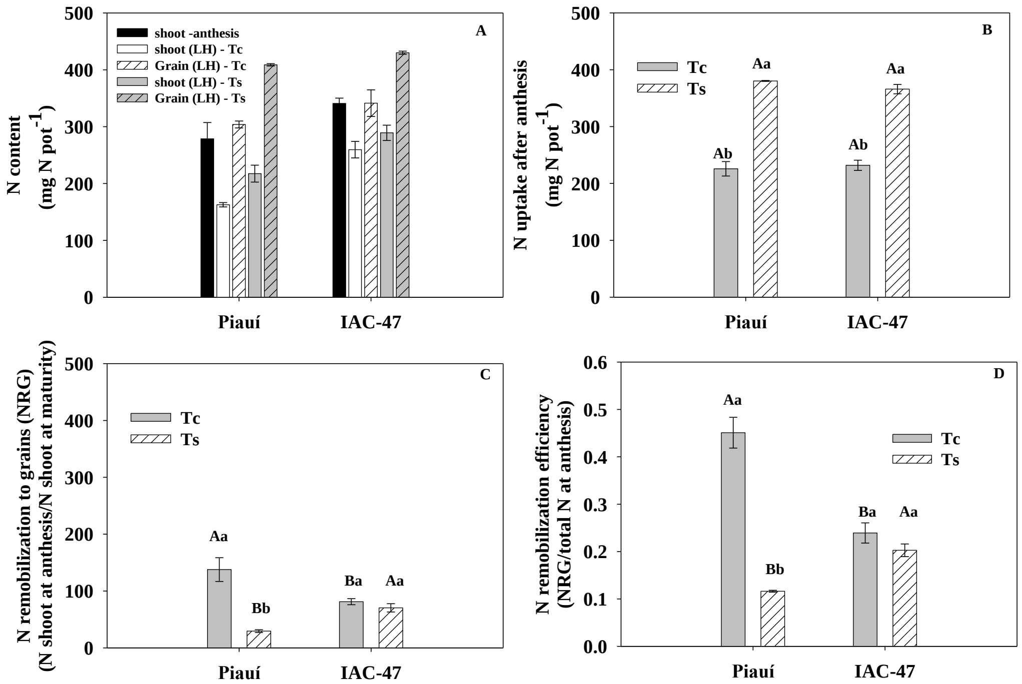

Fig 7. Nitrogen Remobilization Efficiency parameters. (A) Total shoot (without panicles) and grain N content of plants collected at anthesis and at the end of the development cycle (last harvests, LH); (B) N uptake after anthesis; (C) N remobilization to grains at the end of the development cycle; (D) N remobilization efficiency at the end of the development cycle of Piauí and IAC-47 grown under treatments Tc (40 kg N ha^{-1} at planting and 20 kg ha^{-1} at 10 DAP) or Ts (40 kg N ha^{-1} at planting + 20 kg ha^{-1} at 10 DAP + 40 kg ha^{-1} at anthesis). Uppercase letters indicate differences between the varieties, and lowercase letters indicate differences between the treatments (Tukey's test, $P \leq 0.05$).

Piauí tissues in the early stages of the development cycle of plants. According to Souza et al. (1998, 1999) and Hirel et al. (2001), the capacity for rapid NO_3^- accumulation in the initial growth stage may promote a higher N stock for plant metabolism in the later stages, especially at grain filling. Therefore, the apparent capacity of the Piauí variety to take up and accumulate NO_3^- with lower NRA, from the initial growth stages until anthesis, is likely a mechanism that enables the accumulation of substantial N content in the grains. Therefore, this mechanism may contribute to efficiency of remobilization of N in Piauí plants. The higher NRA in IAC-47 plants may explain the ammonium accumulation in the tissues. The higher NH_4^+ levels in IAC-47 before anthesis (Fig 3A) may indicate that this variety takes up and readily metabolizes the available N. This behavior is expected in improved varieties as a response to fertilizer use.

Previous studies have demonstrated that amino acid synthesis and transport are important for seed development (Seebauer et al., 2004; Cañas et al., 2009). At anthesis, the free amino-N content was higher in the leaves and sheaths of IAC-47, but at 15 DAA, it was higher in the Piauí variety. Furthermore, higher amino-N levels were observed in the panicles than in the other plant parts at anthesis for both varieties (Fig 4 B), indicating the onset of N remobilization at grain filling. These results indicate a temporal variation between those varieties in its remobilization or maintenance of remobilization activity, which was longer in the Piauí variety (Fig 4). Therefore, compared to IAC-47, the higher free amino-N levels in the leaves and sheaths of the Piauí variety at 15 DAA (Fig 4) suggests its greater capacity for N remobilization or utilization after anthesis.

The increased sugar levels in all parts of plants in both varieties from 15 DBA to anthesis (Fig 5A and B) may be associated with the senescence progress after the onset of the reproductive period, when N is remobilized for grain filling (Van Doorn, 2008). Greater nitrate accumulation before and higher amino-N content after anthesis may be a indicator of higher remobilization capacity and as consequence of superior adaptation to low N conditions The higher N-amino content after anthesis in Piauí tissues (Fig 4C-F) is an indicator of better N remobilization in the reproductive stage. These characteristics indicate a superior adaptation of the local and low-input variety (Piauí) to growth under low N conditions.

Nitrogen supplementation during anthesis increased grain protein levels in both varieties studied and reduced drastically NRE in Piauí plants

The varieties studied did not significantly differ in their grain weight with or without N supplementation (Table 1). However, there was a higher shoot and grain N levels at the maturity stage in plants receiving N supplementation (Table 1). This indicates that N uptake after anthesis affected positively these two parameters simultaneously as also shown by Souza et al. (1998, 1999). Bogard et al. (2010) observed that N uptake after anthesis predominantly affected grain protein levels, and this was not correlated with N-uptake in the period of time until anthesis, and was negatively correlated with N remobilization. This negative association was also observed in the present study, where plants supplemented with N at anthesis exhibited higher grain protein levels and lower NRE in both varieties (Fig 6E and Fig 7D). This finding was most evident for the Piauí variety due to the greater influence of the N supply at anthesis on the NRE of these plants. The higher shoot N in IAC-47 plants under N supplementation demonstrates that this variety is more responsive to N supply than the Piauí variety (Table 1). This may be because IAC-47 was bred to respond to high N. Despite the lower values of NUpE in the Piauí variety (Fig 6C), it exhibited a higher N harvest index (NHI) (Fig 6A), suggesting that Piauí is more efficient than the improved variety in directing the absorbed N to the grains. However, the higher NHI of the Piauí variety did not increase its grain protein content.

Nitrogen use efficiency by the Piauí variety may be associated with its greater N remobilization efficiency

Nitrogen content between anthesis and maturity decreased faster in the Piauí plant shoots, suggesting a greater N remobilization in the absence of N supplementation than IAC-47 (Fig 7A). This observation is supported by the higher N remobilization to grains and higher NRE observed in Piauí plants (Fig 7D). In both varieties, N supplementation caused a reduction in N remobilization to grains and NRE, indicating a negative effect of supplementation on N remobilization (Fig 7C). Chardon et al. (2010; 2012) observed that the NUE and NRE were significantly higher in *Arabidopsis thaliana* plants treated with a low nitrate supply than in those with a high supply.

The higher NRE exhibited by the Piauí variety from T_C (Fig 7D) is a favorable characteristic for cropping under low N supply. In addition, NRE can be considered as the main factor affecting NUE in this variety. In contrast, NUpE (Fig 6C) seems to be the main factor affecting NUE in the IAC-47 variety (Fig 6D).

Glutamine synthetase (GS) may be correlated with the high NRE of the Piauí variety

The higher expression of the *OsGS1.1* gene in the second leaves of the Piauí variety at 15 DBA (Fig 8D) may be associated with the effects of this GS isoform on N remobilization. Expression analyses were performed in the second leaves because they enter senescence before the flag leaves.

The higher expression of the *OsGS1.3* gene at 15 DAA in plants of the Piauí variety that did not receive N supplementation (Fig 8H) may be correlated with their greater N remobilizing ability, as GS1 isoforms might be associated with remobilization activity during senescence. According to Tabuchi et al. (2007), *GS1.3* is expressed specifically in the panicles of rice plants. In a study performed to verify highly co-expressed genes with the rice GS gene family, Swarbreck et al. (2011) noted that, *OsGS1.3* was co-expressed with genes coding for proteins in the E2F-DP, bZIP, C3H, NAC and CCAAT-HAP2 transcription factor families and an F-box domain-containing protein critical for the controlled degradation of cellular proteins. This observation highlights the involvement of the *OsGS1.3* gene in glutamine metabolism during senescence.

Nitrogen uptake after anthesis (Fig 7B) contributed to greater protein content in the grains of the IAC-47 and Piauí varieties subjected to Ts (Fig 6E). However, the supplemental N reduced NRE (Fig 7D) and thus affected NUE in Piauí plants (Fig 6D). The greatest expression of the *OsGS1.3* gene, specific for the panicles and associated with processes of N remobilization to grains may be correlated with a higher capacity for N remobilization by the Piauí variety.

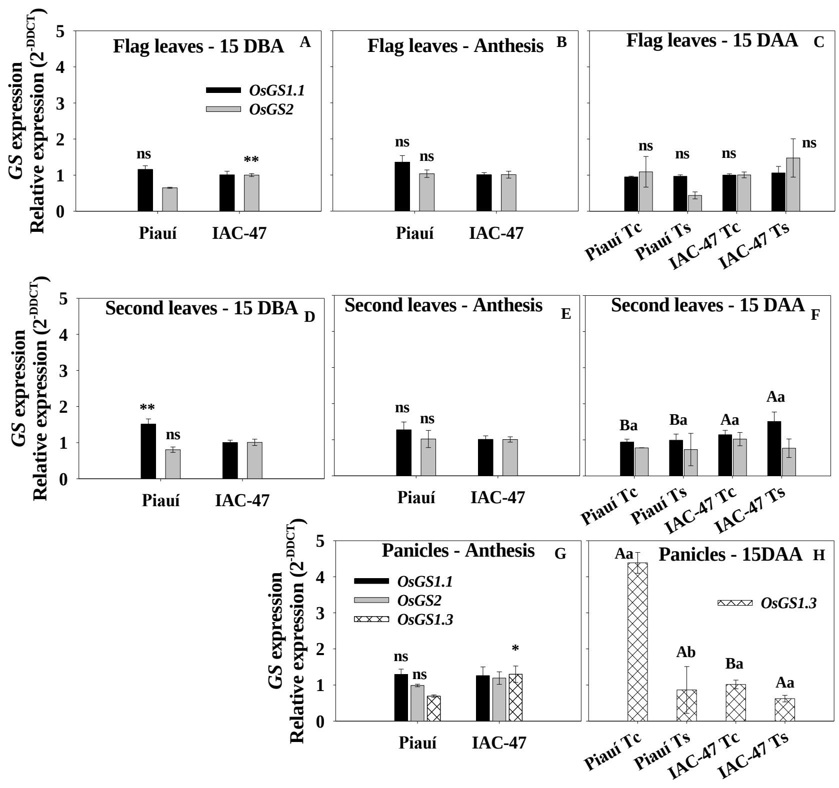

Fig 8. Expression of the *OsGS1.1*, *OsGS2* and *OsGS1.3* genes at (A, D) 15 DBA, (B, E, G) anthesis and (C, E, H) 15 DAA in different parts of Piauí and IAC-47 grown under treatments T_C (40 kg N ha^{-1} at planting and 20 kg ha^{-1} at 10 DAP) or T_S (40 kg N ha^{-1} at planting + 20 kg ha^{-1} at 10 DAP + 40 kg ha^{-1} at anthesis). Significant differences between the varieties are represented by (*) (F test, $P \leq 0.05$) and (**) (F test, $P \leq 0.01$) for samples collected at 15 DBA and anthesis. Uppercase letters indicate differences between the varieties, and lowercase letters indicate differences between the treatments (Tukey's test, $P \leq 0.05$).

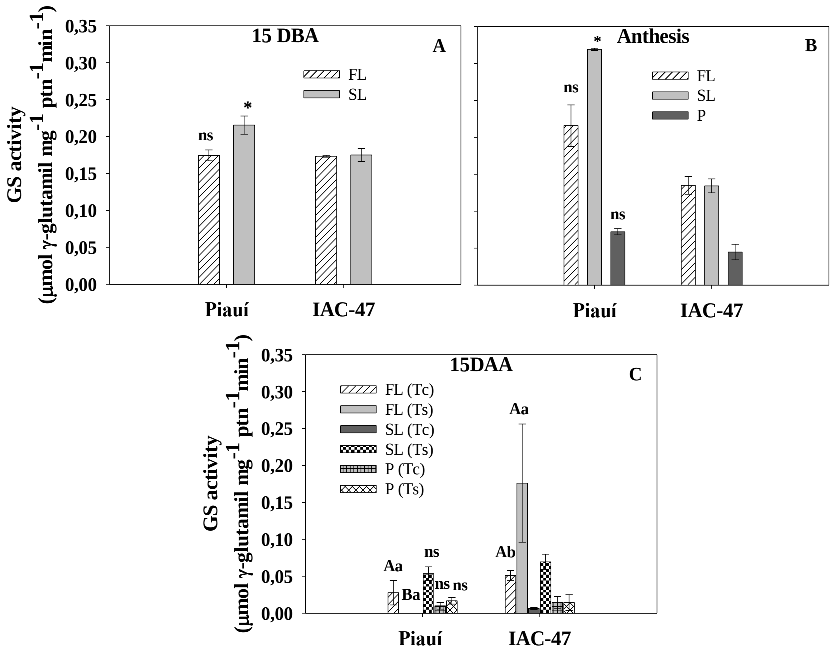

Fig 9. Glutamine synthetase (GS) activity at (A) 15 DBA, (B) anthesis and (C) 15 DAA in flag leaves (FL), second leaves (SL) and panicles (P) in IAC47 and Piauí grown under treatments Tc (40 kg N ha^{-1} at planting and 20 kg ha^{-1} at 10 DAP) or Ts (40 kg N ha^{-1} at planting + 20 kg ha^{-1} at 10 DAP + 40 kg ha^{-1} at anthesis). Significant differences between the varieties are represented by (*) (F test, $P \leq 0.05$) for samples collected at 15 DBA and anthesis. At 15 DAA, uppercase letters indicate differences between the varieties, and lowercase letters indicate differences between the treatments (Tukey's test, $P \leq 0.05$).

Materials and Methods

Rice varieties

Two rice varieties contrasting in NRE (Souza et al., 1998) and nitrate-uptake kinetics (Santos et al., 2011) were used: IAC-47, an improved variety for cropping systems that use high N inputs, and Piauí, a local variety from the state of Maranhão-Brazil that is used in cropping systems with a low N input. Piauí variety has a higher NRE (Souza et al., 1998) and lower Km to nitrate (Santos et al., 2011).

Experimental design and nitrogen treatments

The experiment was performed in a greenhouse at the Soil Department of the Rural Federal University of Rio de Janeiro (UFRRJ), Seropédica, RJ (22º45' S; 43º41' W). A completely randomized 2 x 2 x 4 factorial design (varieties x N levels x harvest time) with four replicates was used. The soil was collected from the A horizon (0-20 cm) of a Typic Argiudoll and stored in pots filled with 8 kg of ADFS (air-dried fine soil). The soil analysis before fertilization showed 2.95 $g.kg^{-1}$ of total nitrogen levels and 1.78% of organic carbon. Nitrogen and organic carbon levels was determined using the method proposed by Embrapa (2009).

The soil moisture was kept at 80 to 90% of field capacity, and each pot held two plants. All plants received urea at doses equivalent to 40 kg N ha^{-1} during planting and 20 Kg N ha^{-1} ten days after planting (DAP). A subset of these plants received N supplementation of 40 kg N ha^{-1} at anthesis. We used two treatments: without N supply, which was the control treatment (Tc), and with N supply at anthesis (Ts).

The N doses and other nutrients were determined according to soil analysis and crop recommendations. Phosphorus and potassium were provided as K_2HPO_4 to all pots at 17.4 kg ha^{-1} of P and 43.8 kg ha^{-1} of K before sowing.

Four harvests were performed in the reproductive phase: at the booting stage, 15 days before anthesis (15 DBA), at anthesis and 15 days after anthesis (15 DAA). The last harvest was performed at the maturity stage. The plants were considered to be at anthesis when 50% of them had emitted panicles. The plants were harvested at 9:00 am and separated into the flag leaf, second leaf, young leaves, old leaves, sheaths and panicles for analysis. The middle third of the leaves and panicles was cut into small fragments to compose the samples. The sheath samples (culm and sheath) were collected from the middle part of the plant and cut into small fragments.

Nitrate reductase activity (NRA)

Nitrate reductase (NR, EC 1.7.1.1) activity (NRA) was determined in 0.2 g of fresh plant tissue samples from each plant using the method proposed by Jaworski (1971). The samples were placed in test tubes containing 5 ml of phosphate buffer solution (0.1 M KH_2PO_4, pH 7.5, 3% N-propanol and KNO_3 0.2 M) and maintained in the bath at 30ºC for 60 minutes. Aliquots of the samples (0.4 ml) were combined with 0.3 ml of 1% sulfanilamide in 3 M HCl and 0.3 ml n-naphthyl-ethylenediamine (0.02%). After 20 minutes, 4 ml of water was added, and the absorbance (540 nm) was measured against $NaNO_2$ standard using a Multiskan GO UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Vantaa, Finland).

In some harvests, the NRA was not determined due to a lack of plant material. This situation occurred for leaves in harvests performed at anthesis and 15 DAA, when green leaves were not observed in most biological replicates. In harvests performed at 15 DBA and 15 DAA, the NRA was not measured in the panicles because they had not yet been emitted at 15 DBA and were dry at 15 DAA.

Soluble fraction analysis

One gram of the middle third of the leaves, sheaths and panicles sampled at 15 DBA, anthesis and 15 DAA was homogenized in 80% ethanol. After chloroform partitioning (Fernandes, 1984), the soluble fraction was used to determine N-NH_4^+ (Felker, 1977), N-NO_3^- (Cataldo et al., 1975), free amino-N (Yemm et al., 1955) and free sugar levels (Yemm and Willis, 1957).

Dry matter production and total N

The samples were weighed and dried in a forced-air oven at 65°C for 72 h to determine dry matter in the flag leaf, second leaf, young leaves, old leaves, sheaths, panicles and roots. The samples were ground and weighed to evaluate the total N according to the Kjeldahl method. The total grain N was multiplied by 5.95 to yield the percent crude protein (Juliano, 1985).

Determination of nitrogen use efficiency parameters

We determined Nitrogen harvest index (NHI): the ratio of grain N content (%) to total shoot N (%); N uptake efficiency (NUpE): the ratio of total shoot N, including the panicles (g pot^{-1}), to N input (g pot^{-1}); N utilization efficiency (NUtE): the ratio of filled grain weight (g pot^{-1}) to shoot N content (%); N use efficiency (NUE): the ratio of filled grain weight (g pot^{-1}) to N fertilizer applied (g pot^{-1}); post-anthesis N uptake: the total N (mg) at the end of the reproductive cycle as a function of the total N (mg) at anthesis; N remobilization for the grains: the total shoot N (mg) as a function of the straw N content at maturity (mg); and N remobilization efficiency (NRE): the ratio between N remobilization to the grains (mg) and total N at anthesis (mg).

Expression and activity of glutamine synthetase (GS)

RNA was extracted according to Gao et al. (2001) with NTES buffer (0.2 M Tris-Cl, pH 8.0; 25 mM EDTA; 0.3 M NaCl; 2% SDS). Total RNA was treated with DNase I (Life Technologies) according to the manufacturer's instructions, and 1 µg was used for cDNA synthesis using the High-Capacity RNA to cDNA Master Kit (Life Technologies) along with the oligo-dT primer according to the manufacturer's instructions.

Real-time PCR reactions were performed with the Power SYBR Green PCR Master Mix kit (Applied Biosystems) according to the manufacturer's recommendations. The actin gene (NM_001057621.1) was used as a reference to normalize gene expression. We evaluated the expression of genes encoding glutamine synthetase, including *OsGS1.1* (NM_001054580.1), *OsGS1.3* (NM_001057602.1) and *OsGS2* (NM-001060668.1), in flag leaves, second leaves and panicles. The samples were collected at 15 DBA, anthesis and 15 DAA. Specific primers were designed for the actin gene, *OsGS1.1* and *OsGS2* using the Primer Express program (Applied Biosystems) (F, forward, R, reverse):
OsGS1.1 (F:5´-CCACGACATCCTCGTCATC-3´;
R:5´-CCAGCACAAATGCAATTCAC-3´);
OsGS2 (F:5´-GGCAAATAAATCCCAGCAAA-3´;

R:5´-TTAACTGGCGAATGGAAGGT-3´) and actin (F:5´-CTTCATAGGAATGGAAGCTGCGGGTA-3´; R:5´-CGACCACCTTGATCTTCATGCTGCTA-3´). The following primers were used for *OsGS1.3*: F: 5´-AGCCGATTCCGACGAACAAC-3´ and R: 5´-GTAGCGTGCCACCCAGACAT-3´ according to Zhao and Shi (2006). Expression was calculated according to Livak and Schmittgen (2001) using variety IAC-47 without N supplementation as a reference.

Glutamine synthetase (GS, EC 6.3.1.2) activity was determined from 100 mg of frozen plants ground in liquid nitrogen according to Farnden and Robertsen (1980). Total soluble protein was extracted with Gamma-glutamyl hydroxamate (GHD) used as a standard, and readings were taken at λ=540 nm. Protein quantification was conducted as described by *Bradford* (1976).

Statistical analysis

The data were evaluated with analysis of variance (ANOVA). The effects of N-supplementation and rice variety were determined with the F-test (at 5% and 1%). When significant differences were indicated by ANOVA, the treatment means were separated using the minimum significant differences calculated by Tukey's test at $P \leq 0.05$.

Conclusion

Piauí plants have adaptive characteristics that are compatible with higher NUE under N-limiting cropping. Among those characteristics are: (1) Low ANR and high nitrate accumulation at initial stages of crop development; (2) higher expression of *OsGS1.1* and *OsGS1.3* genes (3) Higher NHI (4) Higher NRE without supplementary N fertilization. Plants of IAC-47 are more responsive to N supplementation and show a higher NUpE. These results suggest that due these different mechanisms Piauí is more efficient than the improved variety IAC-47 in directing absorbed N to grains.

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