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PROTON-PUMPING AND NITROGEN METABOLISM IN RICE UNDER HUMID TROPIC CONDITIONS

[BOMBEO DE PROTONES Y METABOLISMO DE NITRÓGENO EN ARROZ EN CONDICIONES DE TRÓPICO HUMEDO]

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SUMMARY

In rice plants, it is important to recognize N metabolism efficiency parameters in order to design strategies for improvements, increasing grain protein and enhancing people nutrition quality. Two rice varieties grown under humid tropic conditions were evaluated: Piaui, a landrace adapted to low N availability, and reduced light supply, and IAC-47, an improved variety. The assay was carried out in controlled conditions of 24°C and 12/12 hour light/dark periods (200 $\mu E.m^{-2}.s^{-1}$) using a growth chamber, simulating humid tropic environment. Rice plants were grown under 0.1 and 1.0 mM NH_4^+-N, in a Hoagland & Arnon nutrient solution, pH 5.5, up to 26 days after germination. The activity of PM H^+-ATPases in the plasma membrane, vacuole VH^+-ATPases, H^+-PPases as well as GS, GOGAT, and GDH were determined. PM H^+-ATPase presented higher activity in IAC-47 roots. On the other hand, Piaui roots showed an enhanced microsomal protein content and VH^+-ATPases activity, which apparently allows this variety to absorb N as well as the improved one. Increases in the cytosolic ammonium would result in high GDH deamination in Piaui variety at 1.0 mM NH_4^+-N. Meanwhile, GS increases were observed in IAC-47 shoots, together with GOGAT increases at same treatment.

Keywords: *Oryza sativa;* Ammonium; V-ATPase; P-ATPase; V-PPase; GS; GOGAT; GDH.

RESUMEN

En las plantas de arroz, es importante reconocer los los parámetros de la eficiencia del metabolismo N para desenvolver mejoras, optimizar la proteína del grano y calidad de la nutrición humana. Dos variedades de arroz cultivadas en condiciones del trópico húmedo fueron evaluados: Piauí, una variedad adaptada a la baja disponibilidad de N y a la reducción de luz, e IAC-47, una variedad mejorada. El experimento se llevó a cabo en condiciones controladas de 24°C y períodos de 12/12 horas de luz/obscuridad (200 µE.m-2.s-1), en cámaras de crecimiento, simulando el ambiente del trópico húmedo. Las plantas de arroz fueron cultivadas en 0,1 y 1,0 mM de NH_4^+-N en una solución nutritiva de Hoagland y Arnon, pH 5.5, durante 26 días después de la germinación. Se determinaron las actividades de las H^+-ATPasas de membrana plasmática; de las VH^+-ATPasas vacuolares, de las H^+-PPasas, así como de GS, de GOGAT y de GDH. Las H^+-ATPasa presentaron mayor actividad en las raíces de IAC-47, mientras que en las de Piauí se encontró um aumento en el contenido de proteína microsomal y de la actividad de las VH^+-ATPasas. Al parecer, esto le permite la variedad adaptada absorver N e La mismas cantidades que la mejorada. El aumento en el amonio citosólico resultaría en uma alta GDH desaminación en la variedad de Piaui en el 1,0 mM NH_4^+-N. Mientras tanto, aumenta la GS se observaron en IAC-47 follaje, junto con un aumento en GOGAT, em el mismo tratamiento.

Palabras clave: *Oryza sativa;* amonio; V-ATPasa; P-ATPasa; V PPasa; GS; GOGAT;GDH.

INTRODUCTION

About half planet population, more than three billion of people depend on rice for their nutrition (IRRI, 2012). Asia alone produces and consumes 90% of all world rice production. Out of the Asian continent, Brazil represents the greatest cereal producer (Pereira et al., 2005).

Particularly in Humid Tropic, rice growing starts when rainy summer begins, between December and July. It is characterized by low light and not so high temperatures (Agritempo, 2010). Tropical environmental conditions produce strongly acid soils with low natural fertility and frequently with high aluminum saturation. Under these conditions nitric-N is lixiviated from soil and, ammonium-N may raise its concentration by nitrification inhibition (Rodrigues and Garrido, 2005). Then, NH_4^+ can be the only mineral N source available for plants (Britto et al., 2001).

Ammonium assimilation is specially important since NH_4^+ use is faster and economic than NO_3^- assimilation, because ammonium by-pass two steps of nitrate reduction, however toxicity symptoms are common (Holzschuh et al., 2009). In flooded rice it was observed that up to three weeks old, NH_4^+ absorption and use is higher than NO_3^- (Kronzucker et al., 2000).

Actually, it is well known that ammonium uptake is carried out by two transporter systems: the hight affinity (HATS) system is active at low external NH_4^+ concentrations, and a low affinity (LATS) system is constitutive and is active above 1mM ammonium in the soil solution (Wang et al., 1994; Kuumar et al., 2003).

Ammonium influx is reduced due to PM H^+-ATPases inhibition. This influx is charge-dependent, but it is not directly affected by external pH variation (Howitt and Udvardi, 2000). In HATS, rice presents a light level N regulation: it decreases with external NH_4^+ increasing, and increases with prolonged light periods (Kumar et al., 2003).

The proton-motive force for this process is generated by PM H^+-ATPases. Beyond plasmalem, H^+-ATPases are widely distributed in cell membranes, as vacuole (VH^+-ATPases), endoplasmatic reticulum and vesicles (Palmgren, 2001). In vacuole, it is also known pyrophosphate dependent enzyme (H^+-PPases). Only in extreme metabolic stress, where PPi becomes the principal energy donor, H^+-PPases would be greater than VH^+-ATPases activity (Santos et al., 2009; Ramos et al., 2005).

The aim of the study is to prospect parameters of N absorption and assimilation efficiency, specially in rice landscape variety that enhances grain protein levels, despite environmental conditions. It allows to design strategies for improvements, contributing to enhance human nutrition quality. For both, two rice varieties were evaluated: Piaui, adapted to Humid Tropic conditions, and IAC-47, an improved variety. Piaui presents N assimilation efficiency, while the improved variety is efficient in N absorption (Ferraz Jr et al., 1997).
Two rice varieties were cultivated under Humid Tropic conditions, low NH_4^+-N levels and weak light, up to 26 DAG. Ammonium absorption and assimilation, PM H^+-ATPases, V H^+-ATPases and H^+-PPases as well as glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) kinetics, pH variation and NH_4^+ depletion in nutrition solution are elucidated.

MATERIAL AND METHODS

The study was carried out at the Universidade Federal Rural do Rio de Janeiro, Seropédica campus, Brazil, during 2007. Two rice (*Oryza sativa*, L.) varieties were used: Piaui and IAC-47. Rice plants were cultivated in a growth chamber, at 24°C of average temperature and with 12 hour light/dark periods (200 µE m^{-2} s^{-1}). The assay was designed with varieties and 2 treatments (0.1 and 1.0 mM NH_4^+-N, pH 5.5) and 3 replications. Replications are constituted of blocks with 5 repetitions and 5 plants each for extraction and enzyme assays. Means were compared using T test.

Rice plants were grown in nutrient solutions until 26 days after germination (DAG). A modified Hoagland & Arnon (1950) solution was used at ¼ of its ionic strength. At 4 DAG it was changed to ½ of its ionic strength and kept so up to 8 DAG. After that, plants were grown in a full strength solution up to the final harvest at 26 DAG.

Absorption NH_4^+ kinetic assay was carried out in plants which received nutrient solution without ammonium at 24 DAG. After 48 hours without N (26 DAG), it was reestablished 0.1 and 1.0 mM NH_4^+-N, pH 5.5 nutrient solutions. Variation in pH and NH_4^+ depletion in nutrient solution were followed during 72 hours. Ammonium was measured according Mitchell (1972).

For proton-pumping activities (PM H^+-ATPases, V H^+-ATPases and H^+-PPases) determination, tonoplast and plasma membrane vesicles were extracted by cell fractionation (Yoshida et al., 1983, Ramos et al., 2005, Garrido et al., 2008). At 26 DAG, plants were harvested, washed in distillated water, dried and 9 grams of roots was grounded in an ice cold mortar

and pestle, with 25 mL of a buffer solution (0.25 M sorbitol; glycerol 10%; 50 mM TRIS-acetate; 250 mM sucrose; 2 mM EDTA; 2 mM EGTA; 5 mM DTT; 2 mM 2-mercaptoethanol; 1 mM PMSF; 0.5% BSA; 1% PVP 40T; 150 mM KCl; 100 mM choline chlorate; pH 8.0). The grounded material was filtered through 4 layers of cheesecloth. The extract was centrifuged at 3.600 x g for 10 minutes, at 4º C. The supernatant was taken and centrifuged at 10.000xg for 10 minutes, at 4º C. The new supernatant was centrifuged at 105.000 x g for 45 minutes at 4ºC. Pellet was ressuspended in 1.5 mL of ressuspending buffer, pH 7.5 (30 mM TRIS-HCl; glicerol 15%; 1 mM EGTA, 1 mM EDTA, pH 7.5; 2 mM $MgCl_2$; 2 mM DTT; 1 mM PMSF). Total protein was determined by Bradford (1976) in purified fraction. It was stored in 0.5 ml samples and, after liquid N_2 frozen, kept at -20°C until used for the proton-pumps activity.

Hydrolytic proton-pump activities were measured by inorganic phosphate (Pi) releasing (Fiske, Subarrow, 1925). Proton pumping assays occurred as described by Yan et al. (2002), with minor modifications. The basic reaction mediums' contained KCl 1 M; 0.1 M $MgSO_4$; 40 mM ATP-BTP (0.1 M sodium pyrophosphate); 0.1 M Na_2MoO_4; 1% Brij 58; 0.1M NaN_3; 40 µg.mL^{-1} protein. After 45 min at 30°C, reaction was stopped by cool acid solution 1 mL (2% H_2SO_4; 3% SDS; 0.7% $(NH_4)MoO_4$). Buffers were modified according the proto-pump type: PM H^+-ATPase 0.5 M MOPS-BTP, pH 6.5; V H^+-ATPases 0.5 M HEPES-BTP, pH 7.5; H^+-PPase 0.5 M MOPS-Imidazole, pH 7.2. The controlled mediuns were assayed with/without 200 nM Bafilomicine; 1.0 M KNO_3; 10 mM Na_3VO_4; 100 mM KCl; 2.5mM NaF, respectively, according the inhibition profile; 40 mM ATP-BTP (0.1 M sodium pyrophosphate); and protein. Then, 50 µL ascorbic acid was added. Ten minutes afterward, 1.45 mL of citrate reagent containing 4% sodium citrate and 2% acetic acid was added to prevent ATP/PPi acid hydrolysis.

After absorption analysis of ammonium assimilation pattern for both varieties was determined at the same environmental conditions. For this, ammonium assimilatory enzymes: Glutamine Sinthetase (GS), Glutamate Sinthase (GOGAT) and Glutamate Dehydrogenase (GDH) (amination and deamination) were assayed

Ammonium assimilation enzymes GS, GOGAT and GDH were purified from plants harvested at 26 DAG. One gram of shoots and roots was grounded in liquid N_2 with a mortar and pestle, with 4 mL of extraction buffer (imidazole-HCl 0.5 M, pH 5.0). The grounded material was filtered and the cooled extract (1.5 mL) was centrifuged at 15.000 x g, for 15 min (0^{o}C). The supernatant was used in enzymatic assays. Enzymes activity was measured by spectrum absorption variation according Bergersen (1980).

RESULTS AND DISCUSSION

Under both NH_4^+-N (0.1 and 1.0 mM) levels and weak light (200 µE.m^{-2}.s^{-1}), Piaui plants presented higher protein concentration in microsomal fraction (Table 1). Garrido et al. (2008) showed similar result when the same variety was cultivated with NO_3^- pulses. These conditions would enable reduction in protein transcription, like that of ammonium transporters (Kumar et al. 2003). This apparent contradiction highlights the discussion of Piaui morphophysiologic adaptations to Humid Tropic conditions (Ferraz Jr. et al., 2001, Garrido et al., 2008).

Table 1. Protein Purified (µg. g^{-1} MF) in root microsomal fraction of Piaui and IAC-47 rice plants cultivated according to Material and Methods. Average of 5 pots with 5 plants each.

Rice Varieties	NH_4^+-N levels (mM)	Protein (µg. g^{-1} FW)
Piaui	0.1	1230,54± 38,08
	1.0	1308,67± 63,88
IAC-47	0.1	544,38±113,84
	1.0	901,61±159,72

IAC-47 plants showed higher PM H^+-ATPases hydrolytic activity in both treatments (Table 2). Similar profile is observed under nitrate nutrition (Santos et al., 2009, Garrido et al., 2009). Ammonium transport across a uniport is maintained due to proton-pumps activity that established a $\Delta\Psi$ (Howitt and Udvardi, 2000). Despite this, it was not reflected in solution acidification and ammonium depletion (Fig. 1).

Table 2. ATP and PPi (µmol Pi.mg^{-1} Ptn. h^{-1}) hydrolysis activity by PM H$^+$-ATPase, V H$^+$-ATPase, and H$^+$-PPase in root microsomal fraction of Piaui and IAC-47 rice plants cultivated according to Material and Methods. Average of 5 pots with 5 plants each.

Rice Varieties	NH_4^+-N levels (mM)	Enzyme Activity (µmol Pi.mg^{-1} Ptn. h^{-1})		
		PM H$^+$-ATPase	V H$^+$-ATPase	H$^+$-Ppase
Piaui	0.1	1.28 ± 1.13	8.05 ± 1.94	2.17 ± 0.57
	1.0	0.67 ± 0.33	8.72 ± 2.02	3.04 ± 0.45
IAC-47	0.1	4.93 ± 0.93	1.61 ± 0.86	2.72 ± 0.41
	1.0	4.96 ± 1.60	1.41 ± 0.47	3.30 ± 0.79

When V H$^+$-ATPases activity was analyzed, it showed a completely distinct pattern in Piaui plants (Table 2). Garrido et al. (2008) observed that V H$^+$-ATPases activity was higher in Piaui than in IAC-47, when plant received nitrate pulse. Santos et al. (2009) observed this pattern in Piaui plants under NO_3^--N starvation. So, it was noticed as a varietal characteristic, independently from treatments (Table 2).

Oppositely to what happens to nitrate, vacuolar ammonium stock would be more transitory and not allowed by acid pH (Howitt and Udivar, 2000). High VH$^+$-ATPases activities found in Piaui would diminish ammonium movement through tonoplast, maintaining its cytosolic levels for continuous assimilation even under low concentration.

Otherwise, IAC-47 plants, improved for higher N nutrition, would be able to maintain and stock ammoniun in the tonoplast when necessary. High Piaui tonoplast pumps activity would also compensate for low activity in plasmalem allowing for cytoplasm alkalization and probably keeping the $\Delta\Psi$ (not measured) in relation to apoplasm. This finding could explain the high N-ammonium and N-amino observed in Piaui plants by Garrido, et al. (2008)

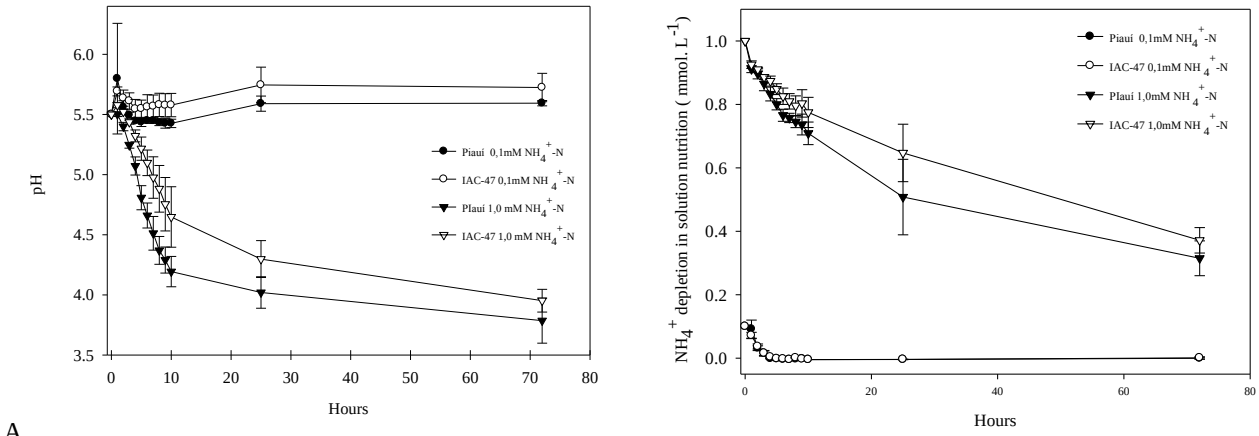
H$^+$-PPases activity was not different between varieties or between treatments (Table 2). In general, plants use H$^+$-PPases in a parallel to V H$^+$-ATPases. H$^+$-PPases would only be higher than V H$^+$-ATPases in case of extreme energetic stress, when PPi would act as the principal cell energy donor (Ramos et al., 2005).

Nutrient solution acidification by plants after 26th DAG (Figure 1A) was enhanced in higher NH_4^+-N concentration. Acidification happens due to positive charge equivalents exudation against excessive cation absorption, avoiding membrane depolarization and maintaining $\Delta\Psi$ and ΔpH necessaries for HATS (Howitt and Udivardi, 2000).

Ammonium depletion followed nutritive solution acidification (Figure 1A and B). The similar slopes observed for Piaui and IAC-47 would be explained by different adaptative mechanisms: higher protein membrane content in Piaui (Table 1), probably electrogenic pumps, and higher specific PM H$^+$-ATPase activity in IAC-47 (Table 2).

Figure 1B showed that NH_4^+ was exhausted in 0.1 mM solution during the earliest four hours. During this time, the curve slope was very similar for both nutritional levels. This fact would point out to the same ammonium transporter class, although, after 72h, both varieties in 1.0 mM treatment showed 0.4 mM ammonium in nutritive solution. Thus, under these nutritional levels there were transporters with different thresholds.

Piaui plants cultivated under 1.0 mM NH_4^+-N showed higher amination activity by glutamate dehydrogenase (GDH) in shoots (Table 3). Nevertheless, according to Labboun et al. (2009), studying tobacco in a real time analysis of NH_4^+ metabolism, GDH activity does not present considerable level *in vivo*, even when GDH genes are overexpressed.



A B

Figure 1. pH (A) and NH_4^+ ($mmol.L^{-1}$) depletion (B) in nutritive solution later than 26th DAG by Piaui and IAC-47 rice plants cultivated according to Material and Methods. Average of 5 pots with 5 plants each.

Table 3. Glutamate dehidrogenase amination (nmol NADH min^{-1} $g^{-1}FW$) and deamination (nmol NAD^+cons. min^{-1} $g^{-1}FW$); glutamate synthase (nmol NADH min^{-1} $g^{-1}FW$), and glutamine synthetase (nmol γ-glutamil min^{-1} $g^{-1}FW$) in shoots and roots of Piaui and IAC-47 rice plants cultivated according to Material and Methods. Average of 5 repetitions in purified material of 3 blocks with 3 pots and 5 plants each.

Rice Varieties	NH_4^+-N levels (mM)	ENZYMATIC ACTIVITY			
		Glutamate Dehidrogenase		Glutamate Synthase	Glutamine Synthetase
		Amination (nmol NADH min^{-1} $g^{-1}FW$)	Deamination (nmol NAD^+cons. min^{-1} $g^{-1}FW$)	(nmol NADH min^{-1} $g^{-1}FW$)	(nmol γ-glutamil min^{-1} $g^{-1}FW$)
		Shoots			
Piaui	0.1	342,33 ± 153.43	1.66 ± 0.27	0.00 ± 0.00	36.90 ± 4.38
	1.0	462,78 ± 185.02	3.05 ± 0.39	7.14 ± 4.76	57.81 ± 13.61
IAC-47	0.1	416,07 ± 165.03	2.76 ± 0.25	0.00 ± 0.00	93.45 ± 23.09
	1.0	136,88 ± 113.70	2.11 ± 0.04	29.13 ± 12.52	136.16 ± 30.40
		Roots			
Piaui	0.1	459.11 ± 206.16	19.61 ± 2.88	20.57 ± 4.67	29.40 ± 1.70
	1.0	499.24 ± 204.23	19.79 ± 1.71	25.37 ± 16.36	43.94 ± 14.87
IAC-47	0.1	537.85 ± 219.70	20.73 ± 2.73	15.94 ± 6.48	69.80 ± 29.19
	1.0	403.34 ± 161.90	15.01 ± 0.99	24.31 ± 17.96	129.42 ± 3.36

Thus, Piaui plants that also presented low GS/GOGAT activity in shoots (table 3), would compensate the assimilation with a NH_4^+ level maintenance in cytosol by a low PM H^+-ATPases and high V H^+-ATPases (table 2). This condition allows cytosolic ammonium concentration to supply the slow and gradual absorption, as an adaptative performance for low N conditions.

Despite this, high GDH amination *in vitro* was not found in roots of Piaui plants and under the lowest nutrition levels (Table 3). This reduction would explain the lowest soluble sugar concentration that resulted in similar NH_4^+ levels in Piaui roots cultivated in these conditions (Garrido et al., 2008).

GDH deamination was higher in roots of both varieties. In shoots, this activity was highest in Piaui under 1.0 mM N-NH_4^+ (Table 3). It seemed that GDH activity would be related essentially to the N recycling at high ammonium and amino-N (Lancien et al., 2000, Dubois et al., 2003), as observed for Piaui shoots under the same treatment conditions (Garrido et al., 2008). IAC-47 Plants showed higher GDH deamination in roots in the 0.1 mM NH_4^+-N treatment (Table 3). Its improvement conditions under high N concentration would produce a plant with an intense catabolism under low N conditions (Ferraz Jr et al., 2001)

Aminative activity of GDH was always higher than GS (GS1 + GS2) (Table 3). It is known that higher GS activity in roots and in the top part of rice plants during senescence is due to GSr and GS1 isoforms, while in green tissues, GS2 is the principal isoform. In shoots, the minor GDH amination (IAC-47 1.0 mM NH_4^+-N) was followed by high GS activity. In general, GS and GDH amination showed an inverse profile, principally when comparing developing and senescent parts (Souza et al., 2002, Stitt et al., 2002).

GS activity varied with N concentration in both treatments in shoots and roots (Table 3). It was not a surprise, because this enzyme is in ammonium primary assimilation pathway in higher plants (Souza et al., 2002, Souza, Fernandes, 2006; 2006a). However, in regular N assimilation, GS carries out the amination pathway with GOGAT, forming glutamine. Thus, when GOGAT NADH dependent were analyzed in shoots of IAC-47 plants under 1.0 mM NH_4^+-N treatment, it has showed highest levels of this enzyme activity than Piaui (Table 3).

The result was not repeated in roots (Table 3). However, in both Piaui and IAC-47 roots treatments, GOGAT and GDH activities were inverse to each other. This could point out to an enzyme competition for substrate.

The major ammonium assimilation pathway in IAC-47 shoots was GS/GOGAT, this was also observed for NO_3^- nutrition (Santos et al., 2009). In contrast with it, Piaui plants carried on slowly, but continuously, assimilation under 1.0 mM N-NH_4^+. Distinct levels of GS/GOGAT pathway between varieties can be explained by ammonium and amino-N accumulated in shoots (Garrido et al., 2008, Dubois et al., 2003) and high N-amino levels that inhibits GS in Piaui plants (Garrido et al., 2008, Souza, Fernandes, 2006a, Labboun et al., 2009). In roots, assimilatory ammonium, difference between varieties did not exist

CONCLUSION

Biochemical different mechanisms for ammonium absorption and assimilation were carried out by Piaui and IAC-47 under humid tropic conditions. Piaui plants were able to maintain nutritive solution acidification and ammonium depletion pattern similar to IAC-47.

Piaui plants presented higher V H^+-ATPases activity that supplied ammonium concentration in cytosol for slow and continuous assimilation by GS/GOGAT. However, GDH deamination in shoots, 1.0 mM N-NH_4^+, was also maintained high, acting as N recycle pathway when there was high ammonium and amino-N levels.

Improved variety carried out high GS/GOGAT assimilation, a standard pathway, and it have absorbed ammonium thanks to higher PM H^+-ATPases activity.

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