Humic acid as a biotechnological alternative to increase N-NO$_3^-$ or N-NH$_4^+$ uptake in rice plants

Orlando Carlos Huertas Tavares[a,b,*], Leandro Azevedo Santos[a], Osmário José Lima de Araújo[a], Cassia Pereira Coelho Bucher[a], Andrés Calderín García[b], Leilson Novaes Arruda[a], Sonia Regina de Souza[c,1], Manlio Silvestre Fernandes[a]

[a] Plant Nutrition Laboratory, Department of Soils, Federal Rural University of Rio de Janeiro, Seropédica, RJ, Brazil
[b] Soil Biological Chemistry Laboratory, Department of Soils, Federal Rural University of Rio de Janeiro, Seropédica, RJ, Brazil
[c] Plant Biochemistry Laboratory, Department of Chemistry, Federal Rural University of Rio de Janeiro, Seropédica, RJ, Brazil

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The aim of this work was to evaluate the vermicompost humic acid (HA) stimulatory effects on the N-uptake kinetics and the N metabolism of rice plants under high and low N-NO$_3^-$ or N-NH$_4^+$ supply in nutrient solution. Plants were grown in a growth chamber, and at 21 days after germination, they were submitted to N deprivation for 96 h, followed by HA treatment or no treatment. After 96 h of N deprivation, the plants received 0.2 or 2.0 mmol L^{-1} N-NO$_3^-$ or N-NH$_4^+$. Changes in pH, NO$_3^-$ or NH$_4^+$ net influx, fresh weight, soluble nitrogen fractions and sugars in both the roots and shoots were evaluated. Pretreatment of rice plants with HA stimulated NO$_3^-$ uptake, preserved plant metabolic status and increased fresh weight. On the other hand, plants submitted to N-NH$_4^+$ facilitated the accumulation of this N form, promoting symptoms of toxicity and leading to a reduction in fresh weight. The results suggest that HA pretreatment modifies the net influx of NO$_3^-$ or NH$_4^+$, which cause differences in plant physiology. Vermicompost humic acids could be part of biotechnology packages for the purpose of increasing the nitrogen nutrition of rice plants.

1. Introduction

Most of the total nitrogen (N) in the soil (> 98%) is in organic matter and is unavailable to plants (Dechorgnat et al., 2011). However, a small fraction of this N is available in soils under different forms, such as ammonium (NH$_4^+$), nitrate (NO$_3^-$), urea, amino acids, and soluble peptides, as well as in complex insoluble forms (Williams and Miller, 2001; Bloom, 2015; Liu and Von Wirén, 2017; Tegeder and Masclaux-Daubresse, 2018; Wang et al., 2018). Generally, in aerated soils, the NO$_3^-$ form is predominant. However, NH$_4^+$ may be the dominant form in acidic or anaerobic environments (Xuan et al., 2017; Wang et al., 2018).

Nitrogen (N) is an essential macronutrient for plant growth and development. The low availability of N in the soil constitutes a limitation for crop yields (Robertson and Vitousek, 2009; Andrews and Lea, 2013). Thus, the application of N fertilizers has become an important and economical strategy to increase crop yields in intensive farming systems worldwide (Andrews et al., 2013). However, excessive

applications of N fertilizer may not result in yield improvements but can lead to severe environmental problems (Vitousek et l., 2009; Good e Beatty, 2011). High N fertilizer input leads to low nitrogen-use efficiency (NUE) due to the fast N losses caused by ammonia volatilization to the atmosphere, denitrification, leaching to the groundwater table, and erosion into lakes and rivers. Consequently, there are significant environmental problems and an increase in the cost of production (Smil, 1999; Diaz and Rosenberg, 2008; Guo et al., 2010). To maximize productivity and increase NUE under well-fertilized conditions, new solutions are needed to increase yields by maintaining or reducing the amounts of nitrogen fertilizer applied (Han et al., 2015). One of the possibilities is to promote increases in nitrogen-use efficiency by crops.

Plants show differences in preference for N sources. In general, plants mainly take up inorganic forms—NO$_3^-$ and/or NH$_4^+$—and can absorb N in the organic form (Tegeder and Masclaux-Daubresse, 2018). The uptake of the different nitrogen forms causes different effects on plant growth, vigor and development (Britto and Kronzucker, 2013; Rocha et al., 2014).

* Corresponding author. Plant Nutrition Laboratory, Department of Soils, Federal Rural University of Rio de Janeiro, BR-465, km 7 Seropédica, RJ, 23890-000, Brazil.
E-mail address: ochtavares@gmail.com (O.C. Huertas Tavares).
[1] In memory.

NO_3^- uptake by plants occurs with its translocation through the plasma membrane (PM) as part of a secondary active process that involves $2H^+$ symporters. The uptake of NH_4^+ also occurs through the PM by transporters and other systems (Xuan et al., 2017; Santos et al., 2011). The plants take up NO_3^- or NH_4^+ via energy-dependent processes; this energy is generated by H^+-ATPase proton pumps present in the plasma membrane (PM). Classic kinetics and functional genomics studies in plants have shown that the influx of NO_3^- into root cells is mediated by members of the peptide/nitrate transporter family or the family of nitrate transporters 2 (NPF/NRT1 or NRT2). Ammonium influx is mediated by AMTs (Léran et al., 2014; Xuan et al., 2017; Tegeder and Masclaux-Daubresse, 2018). These transporter proteins act on two transport systems, a high-affinity transport system (HATS) and a low-affinity transport system (LATS), which occurs at external concentrations below or above $1\,mmol\,L^{-1}$ NO_3^- or NH_4^+, respectively (Williams and Miller, 2001; Souza and Fernandes, 2006; Rashid et al., 2018).

Among humic substances, the humic acid (HA) fraction exerts a direct effect on the growth and metabolism of different plant species (Nardi et al., 2017; Canellas et al., 2015). The effects are mainly exerted on the root system architecture and morphology, biochemical pathways and plant signaling in the same way as some plant phytohormones act (IAA, cytokinins and gibberellins) (Nardi et al., 2017). Among the effects of HA directly linked to nutrition in different plant species is its ability to stimulate root PM H^+-ATPase activity (Zandonadi et al., 2007; Canellas et al., 2015) and the uptake of some nutrients such as N, P, S and Fe (Zanin et al., 2018; Jannin et al., 2012; Jindo et al., 2016; Aguirre et al., 2009; Nardi et al., 2017).

Although there are studies on the positive effects of humic acids on nutrient uptake, there are few reports involving the action of HA on NO_3^- or NH_4^+ nutrition and its effects on the uptake kinetics of these nitrogen forms in conjunction with low- and high-affinity transport systems of N uptake. The aims of this study were to evaluate the changes in the net uptake of NO_3^- or NH_4^+ and the changes in nitrogen metabolism in the traditional Piaui rice variety after being submitted to a pretreatment period with humic acids applied in the nutrient solution and then subjected to conditions of low or high N-NO_3^- or N-NH_4^+ supply.

2. Material and methods

2.1. Extraction, purification and characterization of humic acid

Humic acid from vermicompost was isolated and purified according to the methodology recommended by the International Humic Substances Society (IHSS, 2016) and previously characterized by ^{13}C CP-MAS NMR (^{13}C nuclear magnetic resonance with cross polarization and rotation around the magic angle). The relative distribution of carbon in the regions of chemical displacement (ppm) of the ^{13}C-CPMAS-NMR spectra showed C-alkyl-H,R (29.29%), methoxyl and C-alkyl-O,N (19.19%), C-alkyl-O (13.13%), C-alkyl-di-O (anomeric) (5.05%), C-aromatic-H,R (13.13%), C-aromatic-O,N (7.07%), C-carboxyl-H,R (9.09%), and C-carbonyl (4.04%) forms, as well as 20.2% aromaticity, 79.79% aliphaticity, and a hydrophobicity index of 0.73. These characteristics were similar to those reported in García et al. (2018).

2.2. Conditions of plant growth

Rice plants were cultivated under a 12/12 h (light/dark) photoperiod with approximately $480\,\mu mol\,m^{-2}\,s^{-1}$ photosynthetic photon flux density, a relative humidity of 70% and temperatures of $28\,°C/24\,°C$ (light/dark). Rice (variety Piaui) seed samples were initially disinfested with 2% hypochlorite for 15 min on an orbital shaker and then washed 10 times with distilled water. At six days after germination (DAG) in distilled water, the seedlings were transferred to 0.7 L pots (four plants per pot) containing Hoagland solution (Hoagland and

Arnon, 1950) adjusted to ¼ of the ionic strength (IS) with $2\,mmol\,L^{-1}$ N ($1,5\,mmol\,L^{-1}$ N-NO_3^- and $0,5\,mmol\,L^{-1}$ N-NH_4^+). After three days, the ½-strength IS solutions with the same amount and sources of N were exchanged every three days. At 21 DAG, the plants were submitted to a nutrient solution without N for 96 h. After the first 48 h of N deprivation, the plants were separated into two groups for the remaining 48 h: with or without the supply of $80\,mg\,L^{-1}$ humic acid (HA). After the period of N deprivation and pretreatment with HA, the two groups of plants were submitted to nutrient solutions at full IS without HA but containing doses of 0.2 or $2.0\,mmol\,L^{-1}$ N-NO_3^- or N-NH_4^+. Thus, the treatments consisted of the combination of pretreatment with humic acid and two concentrations of NO_3^- or NH_4^+: 0.2 and $2.0\,mmol\,L^{-1}$ (complementing the high- and low-affinity systems of N uptake (HATS and LATS), respectively). The pH of all starting solutions was adjusted to 5.8. The experimental design was a completely randomized design with four replicates (four independent pots each with four plants).

2.3. Net NO_3^- or NH_4^+ uptake measurements

To determine the kinetic parameters in the treatments with $0.2\,mmol\,L^{-1}$ NO_3^- or NH_4^+, aliquots of 1.0 mL of the nutrient solution were collected every half hour for 28 hours. In the treatment with $2.0\,mmol\,L^{-1}$, aliquots of 1.0 mL of the nutrient solution were collected every hour for 12 h, every two hours for 24 h, and finally every 4 h until 56 h. The nutrient solution samples were stored in microtubes at $4\,°C$ for further determination of the concentrations of NO_3^- and NH_4^+ (Santos et al., 2011). During collection, the pH of the solutions was measured every 4 hours. At the end of the experiments, the roots were collected to obtain the mass and to calculate the kinetic parameters of the absorption of NO_3^- and NH_4^+. These parameters were determined in each treatment according to the decrease in concentration of these ions in the nutrient solution as a function of time, in accordance with the method described by Claassen and Barber (1974). The adjustment of the depletion curves of these nutrients and the calculations of kinetic parameters were performed using the mathematical graphical process proposed by Ruiz (1985) with Cineticawin 1.0 software (Universidade Federal de Viçosa - Brasil). The relationship between the N (C) concentration in the solution and the net influx was plotted via the modified Michaelis-Menten model (Eq. (1)) (Marschner, 2012):

$$\text{Net Influx} = \frac{Vmax(C - Cmin)}{Km + C - Cmin} \tag{1}$$

where the net influx is the net uptake rate of the ion ($\mu mol\,g^{-1}\,h^{-1}$) in a solution of concentration C ($\mu mol\,L^{-1}$), the constant Vmax ($\mu mol\,g^{-1}\,h^{-1}$) represents the maximum rate of absorption, Km ($\mu mol\,L^{-1}$) represents the concentration at which the absorption rate corresponds to half of Vmax, and Cmin ($\mu mol\,L^{-1}$) represents the concentration of the nutrient for which there is no net uptake (Marschner, 2012).

2.4. Extraction and determination of nitrogen fractions and soluble sugars

At the end of the nutrient solution collections, the plants were collected, and 1 g of root and shoot tissue was homogenized in ethanol (80%). After partitioning with chloroform (Fernandes, 1983), the soluble fraction obtained was used for the determination of NO_3^- or NH_4^+, free N-amino compounds and soluble sugars, according to the methods of Fernandes (1983) and Santos et al. (2011). All measurements were quantified in a 96-well plate using a standard spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Vantaa, Finlândia), and the readings for each replicate in each treatment were collected in duplicate.

2.5. Statistical analysis

The experimental design was completely randomized with 4
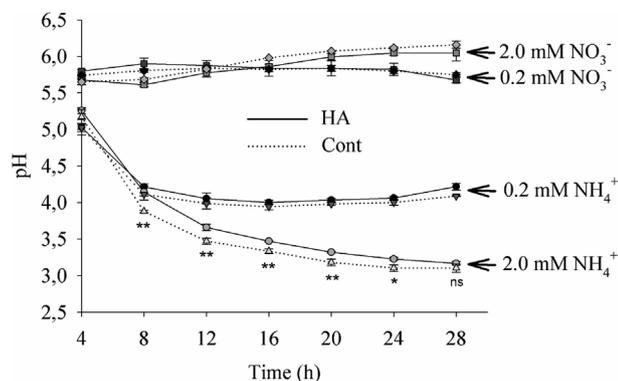
Fig. 1. pH-time course of the nutrient solution of rice plants pretreated with or without addition of humic acid (HA), followed by replenishment with only N-NO_3^- or N-NH_4^+ at an initial concentration of 0.2 and 2.0 mmol N L^{-1}. F Test between HA and Control (Cont) under 2.0 mmol N-NH_4^+ L^{-1}, $**p < 0.01$, $*p < 0.05$. Bars = standard error.

replicates. The data were submitted to analysis of variance (ANOVA) via the F test ($p < 0.05$), and the results were presented as the mean values and standard errors (± SEs). The Lilliefors and Cochran tests were performed to test the normality and homoscedasticity, respectively. Pearson correlations were performed with a *t*-test ($p < 0.05$). The ordering of the data was performed by principal component analysis and for clustering (via a heatmap). The data analyses were performed using software R (R Core Team, 2017).

3. Results

3.1. pH variation in the nutrient solution

The pH value variations in the nutrient solution were monitored over time (Fig. 1). There were differences depending on the nitric or ammoniacal N sources. NO_3^- uptake likely promoted increases in the pH value, especially in plants treated with 2.0 mmol N-NO_3^- L^{-1}, and the HA pretreatment did not influence these variations. On the other hand, the NH_4^+ uptake most likely reduced the pH, mainly at high concentrations. For treatments with 0.2 and 2.0 mmol L^{-1}, there was a greater decrease in pH values in the first hours in which the plants were submitted to these treatments, followed by a pH stabilization of approximately 4 in response to 0.2 mmol N-NH_4^+ L^{-1} and below 3.5 in response to 2.0 mmol N-NH_4^+ L^{-1}. Interestingly, HA promoted a pH value reduction under ammoniacal nutrition ($p < 0.05$), especially at high concentrations (Fig. 1).

3.2. Net influx of NO_3^- or NH_4^+

Plants pretreated with HA presented higher net influx of NO_3^- compared to the control plants (Fig. 2). The largest stimuli of absorption occurred in the HA pretreatment with both 0.2 and 2.0 mmol N-NO_3^- L^{-1}, which encompassed the systems of both the high and low transport affinity, respectively. The HA pretreatment led to a significant ($p < 0.05$) reduction in Km under 0.2 mmol N-NO_3^- L^{-1} (Fig. 2A) and a significant ($p < 0.05$) increase in Vmax under 2.0 mmol N-NO_3^- L^{-1} (Fig. 2B), providing an increase in the net uptake rate of nitrate (Fig. 2A and B), which promoted an increase in the nitrate-uptake efficiency.

The uptake rate in plants treated with 2.0 mmol N-NH_4^+ L^{-1} was stimulated by HA pretreatment, leading to a significant ($p < 0.05$) reduction in Km (Fig. 3B), providing an increase in the net uptake rate of NH_4^+. However, there was no difference in the uptake rate in plants treated with 0.2 mmol N-NH_4^+ L^{-1} or the uptake kinetic parameters and uptake rate between the HA and control treatments (Fig. 3A). Therefore, under high-ammonium supply conditions, pretreating plants with HA promoted an increase in ammonium-uptake efficiency.
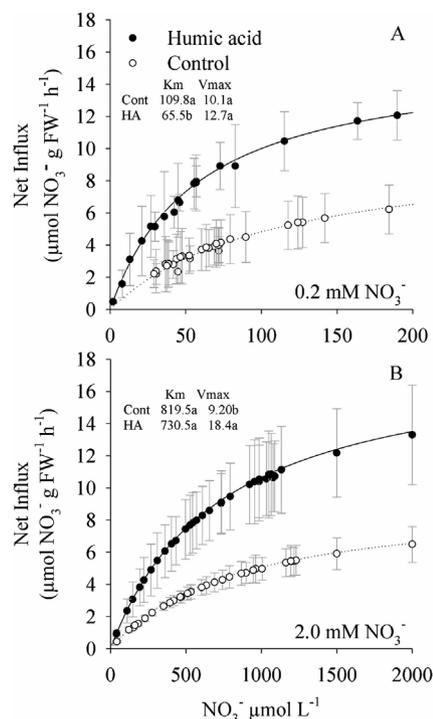
Fig. 2. NO_3^- net uptake rate in rice plants, with or without pretreatment with humic acid (HA) in the nutrient solution, followed by N-NO_3^- replenishment. (A) 0.2 mmol N-NO_3^- L^{-1}; (B) 2.0 mmol N-NO_3^- L^{-1}. Means followed by different letters are statistically different by the F Test ($p < 0.05$) between HA and control. Bars = standard error.
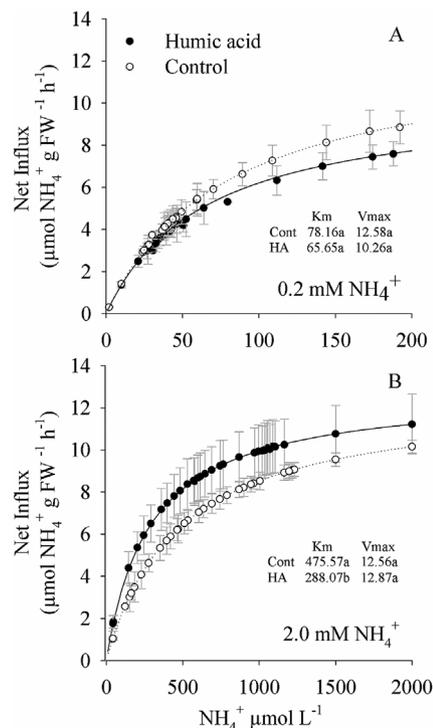
Fig. 3. Net uptake rate of NH_4^+ in rice plants, with or without pretreatment with humic acid (HA) in the nutrient solution, followed by N-NH_4^+ replenishment. (A) 0.2 mmol N-NH_4^+ L^{-1}; (B) 2.0 mmol N-NH_4^+ L^{-1}. Means followed by different letters are statistically different by the F Test ($p < 0.05$) between HA and control. Bar = standard error.
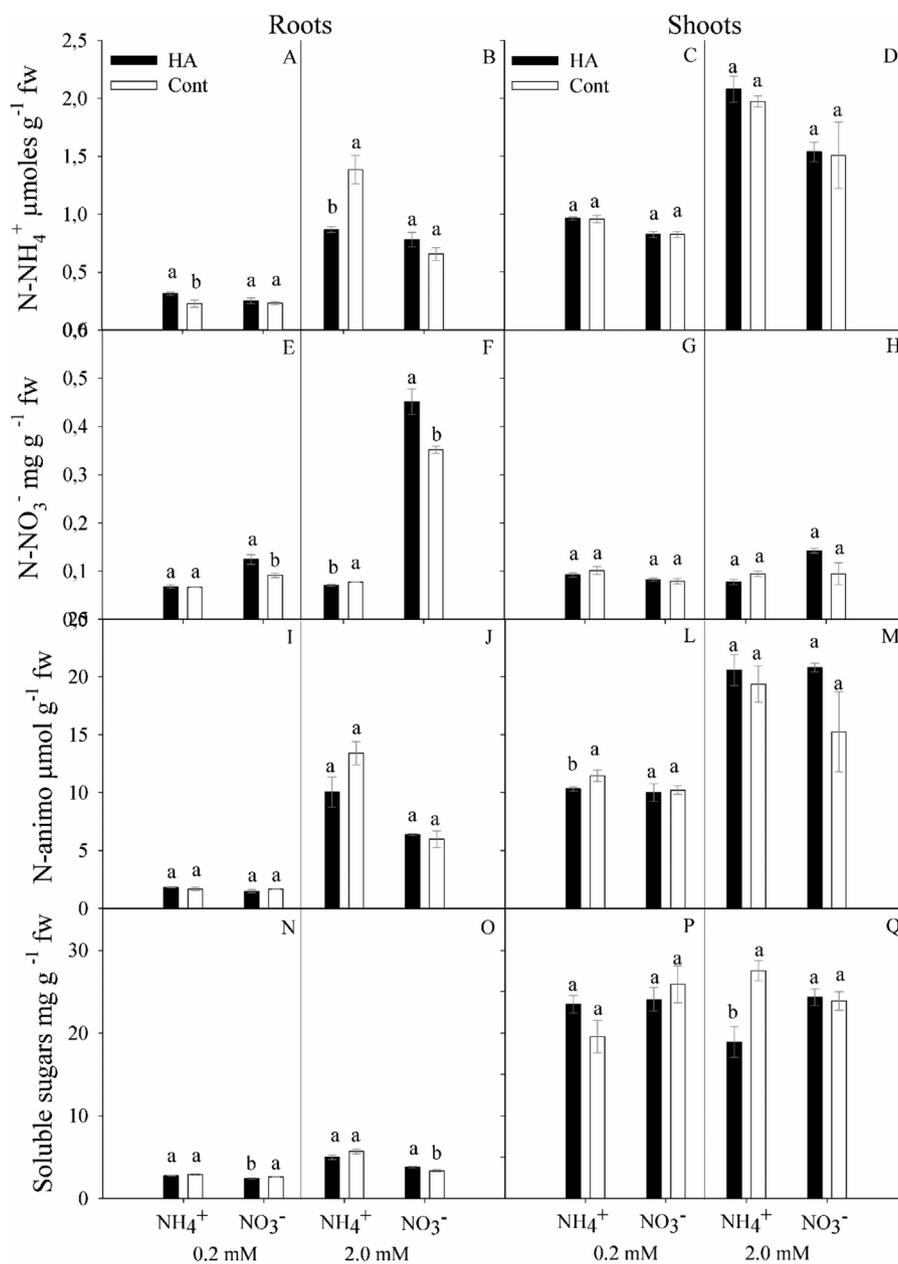
Fig. 4. NH_4^+, NO_3^-, N-Amino and soluble sugars contents in the root (A, B, E, F, I, J, N, O) and shoot (C, D, G, H, L, M, P, Q) in rice plants with or without humic acid (HA) pretreatment in the nutrient solution, followed by replenishment of two levels of $N-NO_3^-$ or $N-NH_4^+$ (0.2 and 2.0 mmol N L^{-1}). Means followed by different lowercase letters are statistically different by the F test ($p < 0.05$). Bars = standard error.

Although the net influx curve at low N (0.2 mmol) was apparently higher than the HA curve, the trend shows an inverse pattern in Fig. 1.

3.3. Metabolite contents in the plant tissue

There were higher levels of NH_4^+ in the roots ($p < 0.05$) of plants subjected to resupply in the experiment with 0.2 mmol NH_4^+ L^{-1} pretreated with HA (Fig. 4A). However, there was a reduction in the levels of NH_4^+ in the roots of plants subjected to HA pretreatment and resupplied with 2.0 mmol NH_4^+ L^{-1} (Fig. 4B). There were no differences in the experiments where the plants were resupplied with $N-NO_3^-$. However, there were higher levels of NH_4^+ ($p < 0.05$) in the shoots (Fig. 4C and D) than in the roots when resupplied with $N-NH_4^+$ or $N-NO_3^-$ (Fig. 4A and B). NH_4^+ and NO_3^- in the roots can be translocated to the shoots for further NO_3^- reductions and assimilation of both compounds.

There were relatively high levels of NO_3^- in the roots ($p < 0.05$) of plants subjected to resupply in the experiment with 0.2 and 2.0 mmol NO_3^- L^{-1} pretreated with HA (Fig. 4E and F), but there were no differences detected in the shoots (Fig. 4G and H).

Rice plants under the 2.0 mmol $N-NH_4^+$ L^{-1} treatment had relatively high N-amino contents ($p < 0.05$) in the roots and shoots (Fig. 4J, M), and 0.2 mmol $N-NH_4^+$ L^{-1} resupply without HA addition promoted N-amino accumulation in the shoots (Fig. 4L). Higher contents of soluble sugars were also observed in the shoots (Fig. 4P, Q) in comparison to the roots (Fig. 4N, O), especially when the plants were treated with 2.0 mmol L^{-1} of $N-NH_4^+$ without HA pretreatment (Fig. 4Q). Differences in sugar contents ($p < 0.05$) were detected in the roots (Fig. 4N, O), and these differences were relatively greater with the addition of HA under 2.0 mmol $N-NO_3^-$ L^{-1} (Fig. 4O).
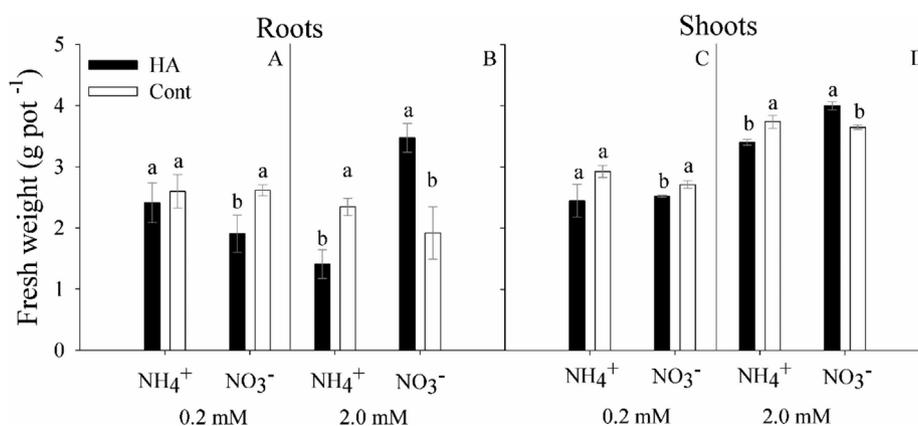
Fig. 5. Root and shoot fresh weight of the Piaui rice variety, with or without humic acid (HA) pretreatment in the nutrient solution, followed by replenishment of two levels of N-NO$_3^-$ or N-NH$_4^+$ (0.2 or 2.0 mmol N L^{-1}). Means followed by different letters are statistically different by the F test ($p < 0.05$). Bars = standard error.

3.4. Fresh weight of rice plants

Rice plants produced more fresh weight, both in the roots and shoots, when pretreated with humic acid and later grown in a solution containing 2.0 mmol N-NO$_3^-$ L^{-1} ($p < 0.05$) (Fig. 5B, D). Unexpectedly, the plants pretreated with HA and a 2.0 mmol N-NO$_3^-$ L^{-1} resupply produced less root and shoot fresh weight ($p < 0.05$) (Fig. 5A, C). On the other hand, plants presented relatively low root fresh weight and shoot fresh weight ($p < 0.05$) when pretreated with humic acid and later grown in the presence of 2.0 mmol N-NH$_4^+$ L^{-1} (Fig. 5B, D).

3.5. Pearson's correlations and multivariate analysis

The results of the correlation analysis of the fresh weight and metabolites in the different parts of the plants resupplied with 0.2 mmol N L^{-1} and 2.0 mmol N L^{-1} are presented in Fig. 6A and B. In the plants under 0.2 mmol N L^{-1}, the soluble sugar content in the shoots was not related to any other variable. On the other hand, the soluble sugars in the roots had significant positive associations ($p < 0.05$) with NH$_4^+$, NO$_3^-$ and N-amino compounds in the shoots (r = 0.6, 0.7 and 0.6, respectively), although it had a negative association with NO$_3^-$ in the

roots (Fig. 6A). The amino acid content in the shoots had a significant positive relationship ($p < 0.05$) with NO$_3^-$ in the shoots (r = 0.7), and the amino acid content in the roots had a positive relation with NH$_4^+$ in the roots (r = 0.7). The NO$_3^-$ content in the shoots had a significant positive relation with NH$_4^+$ in the shoots (r = 0.6), and NO$_3^-$ in the roots had a significant negative relation with NH$_4^+$ in the shoots (r = −0.7). The root/shoot ratio showed a positive relationship with fresh root weight (Fig. 6A).

In the plants resupplied with 2.0 mmol N L^{-1}, the soluble sugar content in the shoots was not related to any other variable (Fig. 6B), as occurred for 0.2 mmol N L^{-1} (Fig. 6A). On the other hand, the soluble sugar content of the roots had a significant positive association ($p < 0.05$) with NH$_4^+$ in the roots and shoots and NO$_3^-$ and amino acid contents in the shoots (r = 0.8, 0.7 and 0.9, respectively), although it had a negative association (r = −0.8) with NO$_3^-$ in the roots (Fig. 6B), as occurred for 0.2 mmol N L^{-1} (Fig. 6A). The amino acid content in the shoots was not related to any other variable. However, the amino acid content in the roots had a significant positive relationship with NH$_4^+$ in the roots and shoots (r = 0.8 and 0.7), although it had a negative association (r = −0.8) with NO$_3^-$ in the roots (Fig. 6B). The NO$_3^-$ content in the shoots had a significant positive relationship ($p < 0.05$) with the
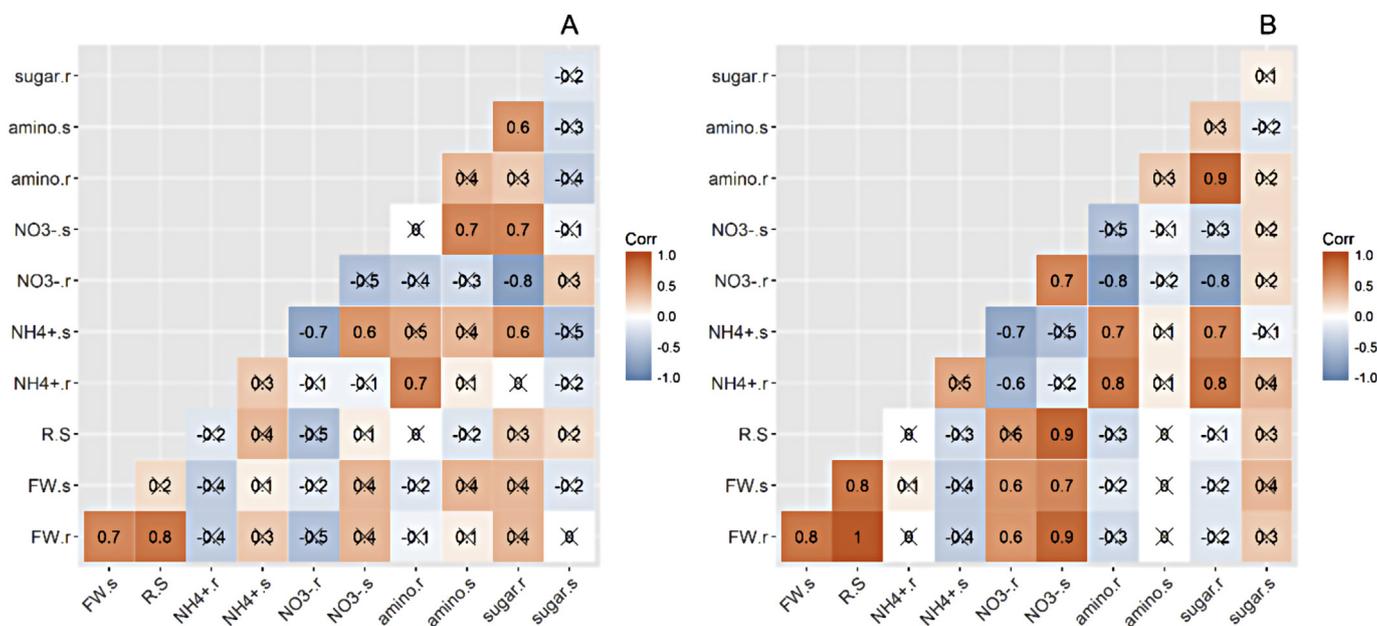

Fig. 6. Pearson correlation matrix for soluble metabolites and root (r) or shoot (s) fresh weight of rice plants, with or without humic acid (HA) pretreatment in the nutrient solution followed by replenishment of two levels of N-NO$_3^-$ or N-NH$_4^+$ form and 0.2 mmol N L^{-1} (A) or 2.0 mmol N L^{-1} (B). T test ($p < 0.05$) for significant correlations.
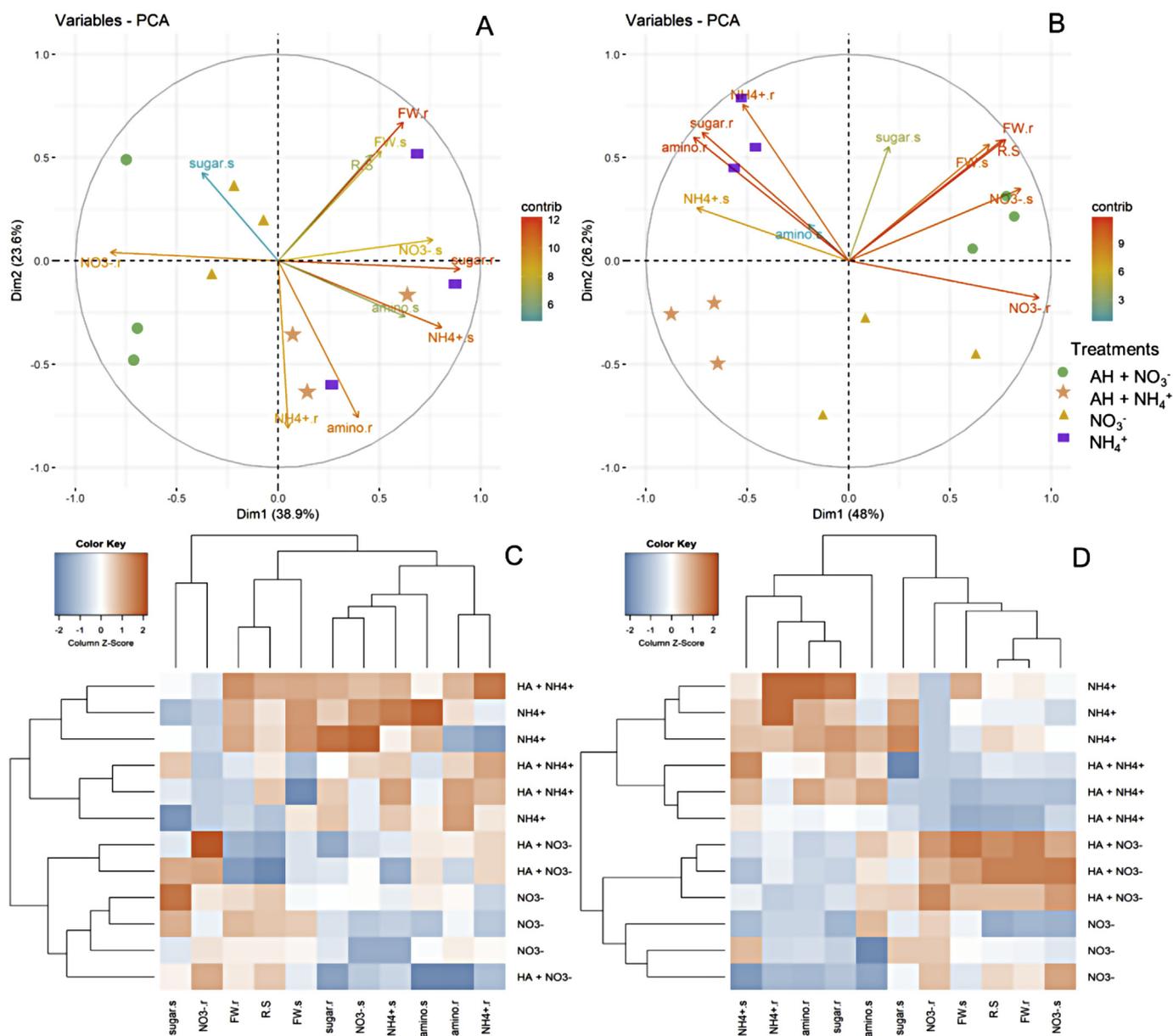
Fig. 7. Principal components analysis (PCA) and heatmap of metabolites and shoot (s) or root (r) fresh weight and the root/shoot ratio of rice plants with or without addition of humic acid (HA) in a nutrient solution, followed by replenishment of N. (A, C) 0.2 mM N-NO$_3^-$ or N-NH$_4^+$; (B, D) 2.0 mM N-NO$_3^-$ or N-NH$_4^+$.

root and shoot fresh weight, the root/shoot ratio (r = 0.9, 0.7 and 0.9, respectively), and NO$_3^-$ in the roots (r = 0.7). Moreover, the NO$_3^-$ content in the roots had a significant positive relation with the root and shoot fresh weight and a significant negative relation with the NH$_4^+$ content in the roots and shoots (r = −0.6 and −0.7). The root/shoot ratio showed a positive relationship with the root fresh weight (r = 1.0) and shoot weight (r = 0.8).

Principal component analysis (PCA) of plants pretreated with HA under 0.2 mmol L^{-1} nitrate or ammonium explained 62.5% of the total variation in the first two components (Fig. 7A), with 38.9% of the variation explained by CP1 and 23.6% explained by CP2. CP1 was explained by the positive contributions of the NH$_4^+$ and NO$_3^-$ contents in the shoots, fresh weight, and root amino acid and sugar contents associated with the treatment with 0.2 mmol L^{-1} of ammonium; these findings were confirmed by a heatmap that separated the plants treated with ammonium and nitrate into two large groups (Fig. 7C). In contrast, the NO$_3^-$ content in the roots extends negatively at CP1 (as observed by the negative correlation between the nitrate and sugar contents in the roots), which is associated with treatment with 0.2 mmol L^{-1} nitrate (Fig. 7A).

The PCA of the plants under 2.0 mmol L^{-1} of nitrate or ammonium explained 74.2% of the total variation in the first two components (Fig. 7B), with 48% of the variation being explained by PC1 due to the N sources (NO$_3^-$ or NH$_4^+$), which were positively bound to root and shoot fresh weight, and due to the NO$_3^-$ in the roots and shoots, which were significantly correlated (Fig. 6B); these findings were associated with the HA and nitrate treatments. In contrast, the NH$_4^+$, free amino acid and soluble sugar contents in the roots extended negatively along CP1, with significant correlations (Fig. 6B), in the treatment with 2.0 mmol L^{-1} of ammonium as the lone source of N. This findings were confirmed by a heatmap that separated the plants resupplied with ammonium and nitrate into two large groups, followed by plants pretreated with HA and control plants (Fig. 7D). The positive contributions (red color) to the formation of the groups of NO$_3^-$-treated plants involve high levels of NO$_3^-$ and root and shoot fresh weight. On the other hand, the positive contributions to the formation of the groups of ammonia-treated plants involve high levels of NH$_4^+$ and free amino acid and soluble sugar contents in the roots and shoots.

4. Discussion

pH variations as a function of different sources of N (NO_3^- or NH_4^+) have already been known for some time (Marschner, 2012). These responses were observed for NO_3^- by Santos et al. (2011) in a study evaluating the kinetic parameters of NO_3^- uptake (0.2 or 2.0 mmol L^{-1}) in rice plants. This phenomenon is most likely due to the process of nitrate absorption, which occurs through a symporter with along with two protons ($NO_3^-/2H^+$) (Xuan et al., 2017; Wang et al., 2018). This cotransport of protons is proportional to the amount of NO_3^- absorbed (Glass, 2003; Santos et al., 2011; Rocha et al., 2014). Thus, protons are removed from the external solution into the cell, leading to an increase in the pH value of the nutrient solution. On the other hand, the absorption of each mole of NH_4^+ takes place by a uniport mechanism. In response, pumping of one mole of H^+ occurs to the outside of the cell, maintaining the balance of charges and thus reducing the pH value of the nutrient solution. Interestingly, HA promoted a pH value reduction in relation to the control under ammoniacal nutrition ($p < 0.05$), especially at high concentrations of that ion (Fig. 1). This phenomenon may have had a negative influence on plant nutrient uptake, which was manifested by the relatively weak growth of the root and shoot system (Fig. 3A and B). Plants can perfectly withstand pH values between 4.5 and 7.5 without great physiological effects (Bugbee, 2003; Epstein and Bloom, 2005); however, in hydroponic cultivation, a pH between 5.5 and 5.8 is recommended to allow for maximum availability of nutrients in general (Bugbee, 2003). Furthermore, the pH value of the nutrient solution can be strongly influenced by N form in response to differences in the uptake rate of cations and anions, N assimilation and cell pH value stabilization (Marschner, 2012).

Nitrate is considered the most important source of mineral N for the growth of plants in well-aerated soils (Wang et al., 2018). Once inside the cell, NO_3^- can be reduced to nitrite (NO_2^-) in the cell cytosol and then to NH_4^+ in plastids or stored in vacuoles and later exported via conducting vessels to the shoots (De Angeli et al., 2006; Santos et al., 2011; Wang et al., 2018). The first step in the assimilation of NO_3^- occurs in the cytosol by the nitrate reductase action (NR), which catalyzes the reduction of NO_3^- to NO_2^- driven by reducing power such as nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH). In the plastids, like chloroplasts, NO_2^- is then reduced to NH_4^+ by the action of NiR (nitrite reductase), utilizing reduced ferredoxin. NH_4^+, resulting from the NO_3^- reduction and/or NH_4^+ uptake, is then incorporated into carbon skeletons by the GS/GOGAT (glutamine synthetase/glutamate synthase) cycle, and asparagine synthetase (AS), a crucial step for converting inorganic nitrogen into organic nitrogen in plants (Tegeder and Masclaux-Daubresse, 2018; Wang et al., 2018). Glutamate can undergo transamination by the action of aminotransferases, giving rise to other amino acids that in turn give rise to proteins (Tischner, 2000; Wang et al., 2018).

Pretreatment of plants with HA promoted increases in NO_3^- net influx over the entire concentration range studied (Fig. 2A and B). It has been reported that in experiments of NO_3^- uptake with wheat seedlings induced by low-molecular-weight humic fractions (LMS) (Cacco et al., 2000), there were increases in Vmax and reductions in *Km* by 60% when compared to the integer humic treatment. In another study, a nutrient solution containing 1.0 mmol L^{-1} NO_3^- plus water-extracted HS (WEHS) treatment caused a faster induction of a higher capacity to take up nitrate in maize roots, increasing the activity of the high-affinity nitrate-uptake system. In addition, genes involved in nitrate transport and assimilation were strongly modulated by WEHS (Zanin et al., 2018). In a study with maize seedlings pretreated with LMS for 48 h and after being transferred to 1.5 mmol L^{-1} NO_3^-, a higher NO_3^- influx (70%) was observed compared to that of control plants (Quaggiotti et al., 2004). This effect was attributed to an increase in transporter activity or an increase in the electrochemical potential gradient, as verified in maize seedlings exposed to NO_3^- and WEHS,

which showed a higher NO_3^- uptake and hydrolytic activity of PM H^+-ATPase (Pinton et al., 1999; Quaggiotti et al., 2004; Mora et al., 2010). Humic acids from various sources have also been shown to induce increased activity of the PM H^+-ATPase (Zandonadi et al., 2007), which may lead to an increased proton motive force for NO_3^- uptake.

Interestingly, most studies on the action of HS on NO_3^- uptake involve the low-affinity transport system of absorption or doses greater than 1.0 mmol L^{-1} NO_3^-. In this work, the rice seedlings after pretreatment for 48 h with HA and subjection to 2.0 mmol L^{-1} NO_3^- exhibited a significant increase in Vmax and a significantly reduction in Km under 0.2 mmol L^{-1} NO_3^-, indicating an increase in the affinity of NO_3^- transporters, which led to a higher net influx of NO_3^- in both conditions (0.2 and 2.0 mmol L^{-1}). The results in this study suggest that HA promotes higher NO_3^--uptake efficiency both at high and low availability of this ion (Fig. 2A and B). This behavior might be due to HA themselves, which act as biostimulants of nitrate acquisition in crop species such as maize, cucumber, rapeseed (Zanin et al., 2018; Mora et al., 2010; Jannin et al., 2012) and rice. In addition to ion uptake, HS have been shown to promote nitrogen assimilation, promoting the increase in nitrate reductase enzyme activity and the GS/GOGAT pathway (Mora et al., 2010; Jannin et al., 2012; Zanin et al., 2018).

Interestingly, there are few reports involving the action of HA on the NH_4^+ nutrition of plants. In this study, the net uptake rate in rice treated with 2.0 mmol NH_4^+ L^{-1} was stimulated by HA pretreatment, leading to a significant reduction in the Km (Fig. 3B), while the uptake rate in plants treated with 0.2 mmol NH_4^+ L^{-1} showed no differences between treatments (Fig. 3A). These results indicate that pretreatment of plants with humic acids possibly increases the uptake efficiency of NH_4^+ via the LATS or under conditions of high availability of this ion (Fig. 3B). However, a high NH_4^+ supply can affect the growth of plant root systems (Fig. 4A), compromising their development. Ammonium acts by dissipating transmembrane proton gradients, which are necessary for electron transport in photosynthesis and respiration processes as well as for the capture of metabolites in vacuoles and the transport of nutrients through biological membranes (Taiz and Zeiger, 2017).

When the contents of nitrogen metabolites in the plant tissues were evaluated, plants treated with 0.2 and 2.0 mmol NO_3^- L^{-1} had a relatively high NO_3^- content in the roots when pretreated with HA (Fig. 4E and F), but there were no differences in the shoots (Fig. 4G and H), as observed in rapeseed treated with 1 mmol KNO_3 plus HA (100 mg organic carbon L^{-1}) (Jannin et al., 2012). However, contrasting results were observed by Quaggiotti et al. (2004), as maize seedlings treated with LMS showed 50% more NO_3^- in the leaves compared to control seedlings.

Absorbed NO_3^- is preferentially reduced and assimilated in the shoots, while NH_4^+ is toxic and should be rapidly assimilated into carbon skeletons and transported as amino acids to the shoots ($p < 0.05$) (Britto and Kronzucker, 2013). In view of this, a higher NH_4^+ (Fig. 4C and D) amino content (Fig. 4L, M) was observed in the shoots in comparison to the roots (Fig. 4A, B, I, J) upon resupply with nitrate or ammonium.

The plants treated with 2.0 mmol $N-NH_4^+$ L^{-1} presented relatively low root and shoot fresh weights when pretreated with humic acid. According to Souza and Fernandes (2006), the reduction under conditions of exposure to high doses of ammonium occurs due to the assimilation of NH_4^+ basically in the roots, which requires a high supply of carbohydrates to avoid toxic effects of NH_4^+ (Taiz and Zeiger, 2017). In addition, NH_4^+ efflux to the external medium is often stronger than that of NO_3^- and can be quite considerable, equivalent to up to 80% of the inflow. The high energy consumption associated with NH_4^+ efflux by roots was proposed to be one of the main reasons for the manifestation of NH_4^+ toxicity at high concentrations (Britto and Kronzucker, 2013). The relatively high N-uptake capability for ammonium may explain these results, which would be an advantage in physiological terms. Soluble sugar levels in plants are indicators of energy readily available for cellular metabolism (Rocha et al., 2014), and these levels

were relatively low in the shoots of plants pretreated with HA and re-supplied with 2.0 mmol N-NH$_4$$^+$ L^{-1}. In addition to an energy source, soluble sugars are carbon skeletons used in the synthesis of organic acids, such as 2-oxoglutarate (2-OG), generated from the tricarboxylic acid cycle (TCA) and required for the assimilation of NH$_4$$^+$ into glutamine (Souza and Fernandes, 2006; Bloom, 2015). Once assimilated into glutamine and glutamate, N is incorporated into other amino acids via transamination reactions. N-amino acids then are incorporated into other organic nitrogen compounds, such as purines and pyrimidines (Bloom, 2015).

The PCA results for 0.2 mmol N L^{-1} show that rice plants grow better under conditions of low NH$_4$$^+$ resupply because they grew less under low doses of NO$_3$$^-$, which can be explained by their greater need for energy uptake (symporter + 2H$^+$) and the reduction of NO$_3$$^-$ to NH$_4$$^+$ [NAD(P)H; 6 Reduced Ferredoxin] (Bloom, 2015).

The PCA and heatmap results for 2.0 mmol N L^{-1} show that high NH$_4$$^+$ and high amino-N in the roots and shoots promote reductions in the fresh weight of both tissues. Normally, high NH$_4$$^+$ values are rarely found in plant tissues due to their rapid assimilation, since high concentrations of this ion in tissues can cause symptoms of toxicity (Rocha et al., 2014). According to Taiz and Zeiger (2017), plants assimilate ammonium near the region of uptake or production, and any excess is quickly stored in the vacuoles, avoiding toxic effects on the membrane and cytosol. High levels of NH$_4$$^+$ favor the synthesis of the amides asparagine and glutamine, which can account for more than 80% of the total free N-amino content, promoting a free N-amino/N-amide ratio up 10 to 20 times greater as a response to NH$_4$$^+$ toxicity (Souza and Fernandes, 2006). Therefore, some strategies used by plants to decrease the amount of free NH$_4$$^+$ in tissues are related to increased assimilation of NH$_4$$^+$ (Britto and Kronzucker, 2013).

In studies by Fernandes (1983), rice plants had a lower fresh weight when treated with NH$_4$$^+$ and then submitted to low light and high temperature, causing greater stress in the plants. However, when the plants were grown under NO$_3$$^-$ conditions, the fresh weight was relatively high, which is a favorable condition for growth. It was also demonstrated that rice plants under conditions of excess NH$_4$$^+$ led to NH$_4$$^+$ accumulation in the tissues and presented a positive correlation with free N-amino contents (Fernandes and Souza, 2006), as observed in our results. NH$_4$$^+$ toxicity occurs because the assimilation of NH$_4$$^+$ in the roots requires large amounts of carbohydrates (Souza and Fernandes, 2006) and promotes disturbances in pH regulation, photophosphorylation dissociation, deficiencies in N-protein glycosylation, and increases in the production of reactive species of oxygen (Liu and Von Wirén, 2017) and the futile cycle (energetically intense transmembrane ion NH$_4$$^+$ cycle) (Britto and Kronzucker, 2013).

On the other hand, under conditions of excess NO$_3$$^-$, this ion can be absorbed and accumulated in the vacuoles (as a reserve pool) and is later metabolized by the growing plant (Fernandes and Souza, 2006; Souza and Fernandes, 2006). Under normal conditions, rice plants can accumulate large amounts of NO$_3$$^-$ as a reserve pool while keeping the pool of free N-amino compounds under control, maintaining normal N metabolism (Fernandes and Souza, 2006; Santos et al., 2011). As shown in this study, plants had relatively high root and shoot growth were pretreated with HA followed by resupply with 2.0 mmol NO$_3$$^-$ L^{-1}.

The use of HA extracted from vermicompost may constitute a sustainable alternative that could be part of a biotechnology package for the purpose of increasing the nitrogen nutrition of rice plants.

5. Conclusion

Humic acid applications to rice plants increase the uptake of N in both the N-NO$_3$$^-$ and N-NH$_4$$^+$ forms. Preinduction of plants with vermicompost humic acid in the nutrient solution promotes a relatively high net nitrate uptake in Piaui rice plants via the low-affinity transport system, improving the efficiency of nitrate uptake and biomass production. The supply of high-dose ammonium, after previous humic acid

induction of the plants, provides increased ammonium uptake but is harmful to rice plants, causing a decrease in fresh weight production. These results indicate that humic acids are a natural compound that could be a valuable tool to improve seedling rice nitrogen-use efficiency, especially in adverse environmental conditions. Nevertheless, other studies are necessary to better understand the capability of humic acids to potentially cause possible N-NH$_4$$^+$ toxic effects described in this work for the first time.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101226.

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