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RIKAELY TORRES DE SOUSA

EXCLUSÃO DE AMÔNIO ESTÁ RELACIONADA À SENESCÊNCIA FOLIAR E À PARTIÇÃO DE NITROGÊNIO EM PLANTAS DE ARROZ

FORTALEZA
2018

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Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica da Universidade Federal do Ceará, como requisito parcial para a obtenção do título de Mestre em Bioquímica. Área de concentração: Bioquímica Vegetal.

Orientador: Prof. Dr. Joaquim Albenísio Gomes da Silveira.

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“Comece fazendo o que é necessário, depois o que é possível, e de repente você estará fazendo o impossível.” (São Francisco de Assis).

RESUMO

O nitrogênio (N) é um macronutriente fundamental para plantas e um importante fator que determina a produção de biomassa vegetal. Na faixa de concentração micromolar, as raízes da maioria das espécies preferem a absorção de amônio a nitrato, enquanto que em concentrações na faixa de milimolares, o amônio muitas vezes causa toxidade. O arroz é uma das poucas espécies que conseguem se desenvolverem na presença de altas concentrações de amônio, entretanto, as estratégias de tolerância do arroz ao elevado NH_4^+ ainda não são totalmente conhecidas. Especialmente, a dinâmica da senescência das folhas e a partição de amônio e outras formas de N entre os diferentes tecidos vegetais sob exposição de elevado amônio por longo prazo, são completamente desconhecidas. Visando investigar os mecanismos fisiológicos, morfológicos e bioquímicos de arroz para lidar com o excesso de amônio, plantas de arroz (*Oryza sativa* japonica cv. Nipponbare) foram cultivadas em duas condições contrastantes de fonte de nitrogênio, 15 mM de NO_3^- (referência) ou 15 mM de NH_4^+ por até 56 dias e avaliadas em termos de crescimento, fotossíntese, metabolismo redox, assim como partição de biomassa e partição das diferentes formas de nitrogênio. Plantas cultivadas na presença de alto amônio apresentaram restrição de crescimento de raízes, mas mantiveram inalterados o crescimento de parte aérea e a atividade fotossintética quando comparadas com ao controle. Adicionalmente, plantas de arroz crescidas em elevado amônio apresentam acúmulo da forma tóxica no colmo e folhas senescentes, preservando a raiz e o aparato fotossintético de folhas jovens. Nas raízes, a maior atividade de GPOD foi observada em paralelo ao teor de H_2O_2 inalterado e diminuição dos indicadores de peroxidação lipídica, sugerindo assim uma proteção antioxidativa efetiva nesse tecido, apesar da restrição do crescimento. Os dados apresentados sugerem que a partição de N entre folhas senescentes, colmo e folhas verdes possivelmente representa um importante mecanismo de exclusão de amônio e preservação do aparato fotossintético. Em paralelo, o aumento da atividade de GPOD nas plantas cultivadas em alto amônio pode sugerir um importante papel dessas enzimas na remoção do excesso de EROS (Espécies reativas de oxigênio) e no controle do crescimento de raízes.

Palavras-chave: Mecanismo de tolerância ao amônio. Metabolismo do nitrogênio. Crescimento da parte aérea e radicular. Estresse oxidativo.

ABSTRACT

Nitrogen (N) is a fundamental macronutrient for plants and a determinant factor that determines the production of vegetal biomass. In the micromolar concentration range, the roots of most species prefer the absorption of ammonium to nitrate, whereas in those of millimolar, ammonium often causes toxicity. Rice is one of the species that can be improved in the presence of high concentrations of ammonium, however, as rice tolerance strategies are very important. Especially, the dynamics of leaf senescence and an ammonium partition and other forms of N expression among different ways of projecting themselves to a high degree of long-term exposure are completely unknown. In order to investigate the physiological, morphological and biochemical mechanisms of rice for the excess of plants, rice plants (*Oryza sativa* japonica cv. Nipponbare) were cultivated in two contrasting conditions of nitrogen source, 15 mM NO_3^- (reference) or 15 mM of NH_4^+ during 56 days and evaluated in terms of growth, photosynthesis, redox metabolism, as well as partitioning of biomass and partition of the different forms of nitrogen. Plants cultivated in the presence of high ammonium presented restriction of root growth, but they maintained unchanged shoot growth and photosynthetic activity when compared to control. In addition, rice plants grown in high ammonium present accumulation of the toxic form in culm and senescent leaves, preserving the root and the photosynthetic apparatus of young leaves. In the roots, the highest GPOD activity was observed in parallel to the unchanged H_2O_2 content and a decrease in the indicators of lipid peroxidation, thus suggesting an effective antioxidative protection in this tissue, despite growth restriction. The data presented suggest that the partition of nitrogen between senescent leaves, culm and green leaves possibly represents an important mechanism of exclusion of ammonium and preservation of the photosynthetic apparatus. In parallel, the increase of GPOD activity in plants grown in high ammonium may suggest an important role of these enzymes in the removal of excess EROS (reactive oxygen species) and in the control of root growth.

Keywords: Mechanisms of tolerance to ammonium. Nitrogen metabolism. Roots and shoot growth**.** Oxidative stress.

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LISTA DE ABREVIATURAS

AMT	Transportador de amônio
ATP	Adenosina trifosfato
APX	Peroxidase do ascorbato
CIPK23	Proteína quinase23 de interação de calcineurina b-like
CAP1	Proteína quinase associada $[Ca^{2+}]$cit
CPSII	Carbamoil fosfato sintetase do tipo II
EROS	Espécies reativas de oxigênio
EDTA	Ácido etilenodiamino tetra-acético
Fd	Ferredoxina
GOGAT	Glutamato sintase
GDH	Glutamato Desidrogenase
GS	Glutamina Sintetase
GPOD	Peroxidase do guaiacol
GGH	γ-glutamil hydroxamato
gs	Condutância estomática
Km	Constante de Michaelis – Menten
LATS	Transportadores de baixa afinidade
HATS	Transportadores de alta afinidade
MAPK	Proteína quinase ativada por mitógenos
NAD	Dinucleótido de nicotinamida e adenina
NPQ	Quenching não fotoquímico
OEC	Complexo de evolução de oxigênio
PN	Assimilação líquida de CO_2
PPFD	Densidade de fluxo de fótons fotossinteticamente ativos
PSII	Fotossistema II
TBARS	Substâncias reativas ao ácido tiobarbitúrio
TCA	Ácido tricloroacético
UMAMITs	Transportadores de aminoácidos
Vmax	Valor máximo da velocidade inicial

LISTA DE SÍMBOLOS

CO_2	Dióxido de carbono
Ca^{2+}	Cátion cálcio
C	Carbono
$Ca(NO_3)_2$	Nitrato de cálcio
$FeCl_3$	Cloreto férrico
H_2SO_4	Ácido sulfúrico
HCl	Ácido clorídrico
H_2O_2	Peróxido de Hidrogênio
K^+	Cátion potássio
KNO_3	Nitrato de potássio
KH_2PO_4	Fosfato de potássio monobásico
$MgSO_4$	Sulfato de magnésio
MSO	Metionina sulfoximina
Mg^{2+}	Íon magnésio
N	Nitrogênio
NO_3^-	Nitrato
$(NH_4)_2SO_4$	Sulfato de amônio
NH_4Cl	Cloreto de amônio
NH_4^+	Amônio
NH_3	Amônia
O_2	Oxigênio

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1 INTRODUÇÃO

O nitrogênio (N) é um essencial macronutriente para plantas e que determina o crescimento e produção de biomassa vegetal (LOQUE et al., 2004; TEGEDER et al., 2018). As plantas podem absorver N através de suas raízes em diferentes formas, incluindo nitrato (NO_3) e amônio (NH_4^+), e moléculas orgânicas (ureia, aminoácidos e peptídeos). Na maioria dos solos agrícolas, o nitrato é a principal forma de nitrogênio inorgânico, contudo, em ecossistemas naturais, a forma de nitrogênio pode ser altamente variável, com amônio frequentemente predominante em solos ácidos e/ou alagados (FALKENGREN-GRERUP, 1995; HERRERA-ESTRELLA, 1999). Embora as concentrações absolutas na solução do solo possam ser mais de três ordens de grandeza inferiores às do nitrato (MILLER et al., 2007), a dessorção de amônio da matriz do solo pode preencher rapidamente a associação solúvel na solução do solo, especialmente quando a capacidade de troca de cations é alta (MARSCHNER; RENGEL, 2012). Na faixa de concentração micromolar, as raízes da maioria das espécies de plantas preferem a absorção de amônio em relação ao nitrato (GAZZARRINI et al., 1999), enquanto que em concentrações milimolares, o amônio muitas vezes causa toxicidade (BRITTO; KRONZUCKER, 2002).

Muitas plantas apresentam sintomas de toxicidade quando crescidas com excesso de amônio, esta toxidez tende a ser particularmente pronuciada quando o NH_4^+ é suplementado como única fonte de nitrogênio no solo, ou alternativamente, quando ocorre uma superprodução de NH_4^+ decorrente de uma elevada atividade proteolítica ativada em condições de estresse (SKOPELITIS et al., 2006). No entanto, os mecanismos implícitos à toxidez do NH_4^+ em plantas permaneçam insuficiente claro, no entanto, várias rotas fisiológicas foram encontradas para explicá-lo (GERENDAS et al. 1997; BRITTO; KRONZUCKER 2002; KRUPA, 2003). As típicas respostas de toxicidade do NH_4^+ são a inibição no crescimento da raiz e parte aérea que é associado à clorose foliar, distúrbio no gradiente de pH através da membrana e o alto consumo de carbono na raiz em decorrência do excesso de amônio, na qual poderia explicar os sintomas de toxicidade em plantas superiores (BRITTO; KRONZUCKER, 2002). A ação tóxica do amônio também tem sido associada à alta demanda de energia no bombeamento de amônio para fora das células. Esta descoberta baseia-se nos estudos de espécies domesticadas sensíveis ao amônio e tolerantes ao amônio (cevada e arroz, respectivamente).

Plantas de arroz (*Oryza sativa* L. japonica cv. Nipponbare) são espécies consideradas especialistas em amônio, pois apresenta diversos mecanismos que permitem sua tolerância ao amônio, dentre eles podemos destacar que estas plantas conseguiram evolutivamente adapta-se aos solos alagados, já que nestas condições a concentração de O_2 é menor, proporcionando assim a inibição de microorganismos nitrificantes resultando em alta amonificação (EVINER; CHAPIN, 1997). Isso se deve porque nessas condições a forma protonada da amônia (NH_4^+) predomina (99,4%) em soluções com pH 7,0 e 25 ºC, assim a concentração desse íon é elevada em solos alagados e, consequentemente, nos compartimentos da raiz de arroz (TABUCHI et al., 2007). Além do mecanismo citado anteriormente, outro motivo é atribuído ao eficiente mecanismo de assimilação de amônio, o ciclo GS-GOGAT (ISHIYAMA et al., 2004).

Acumulação de NH_4^+ no tecido é contigente após a excessiva absorção do amônio, e o mecanismo responsável por este processo ainda precisa ser estudado. A conjugação do amônio com ácido glutâmico para formar glutamina e a síntese de ácido glutâmico a partir do ácido 2-oxoglutarato são considerado como as principais vias de assimilação do amônio, e um mecanismo estratégico de proteger a célula da toxidez do amônio. As isoformas citosólicas de GS (GS1) e GOGAT (NADH-GOGAT) são consideradas as principais enzimas envolvidas na rápida assimilação de amônio absorvida do solo (ISHIYAMA et al., 2004; KONISHI et al., 2014). Guan et al. 2016 verificaram que a expressão do gene *AtGLN1;2* (GS1) em *Arabidopsis thaliana*, foi fortemente induzido pelo suprimento de amônio externo. Além disso, esses autores mostraram ainda que mutantes *gln1;2* apresentam baixa atividade de GS, alta acumulação de amônio e inibição do crescimento quando supridas com NH_4^+, sugerindo que essa isoforma é essencial tanto para a assimilação quanto para a desintoxicação de NH_4^+ nas raízes.

A atividade da Glutamina Sintetase (GS) é conhecida por decrescer durante a senescência foliar natural ou induzida pelo escuro. O declínio na atividade da GS em folhas durante a senescência pode resultar, pelo menos em parte, em uma acumulação de NH_4^+. A rápida taxa de senescência foliar de um cultivar de *Nicotiana tabacum* (cv. ZY90) foi associada com baixa atividade de GS, elevada atividade de GDH (glutamato desidrogenase) e alta concentração de amônio do apoplasto, alta emissão de NH_3 e ponto de compensação estomático de NH_3 (WU et al., 2016). Chen et al., 1997 observaram que a acumulação de NH_4^+ em folhas destacadas de arroz poderia está associado ao declínio na atividade de GS e redução de nitrato o que aumenta a sensibilidade por etileno promovendo indução da senescência.

Nas últimas décadas, vários estudos revelaram que o amônio desencadeia múltiplas

respostas fisiológicas e morfológicas, como mudanças específicas na expressão gênica, no metabolismo e na estrutura do sistema radicular (PATTERSON et al., 2010; LI et al., 2010 ; LIMA et al., 2010; FERNÁNDEZ-CRESPO et al., 2015). Portanto, nós hipotetizamos que o arroz, uma espécie tolerante, apresenta mecanismos importantes de proteção contra o excesso de amônio, especialmente relacionadas a alterações da dinâmica da senescência foliar, ativação de um mecanismo de exclusão e redistribuição de amônio dos tecidos fotossintetizantes, direcionando o excesso de NH_4^+ para as folhas senescentes e ativação de uma maquinaria antioxidante efetiva nas raízes.

2 MECANISMOS INTEGRADOS DE PROTEÇÃO DE TOXIDADE DE NH_4^+ EM PLANTAS

Transporte e acumulação de amônio em plantas

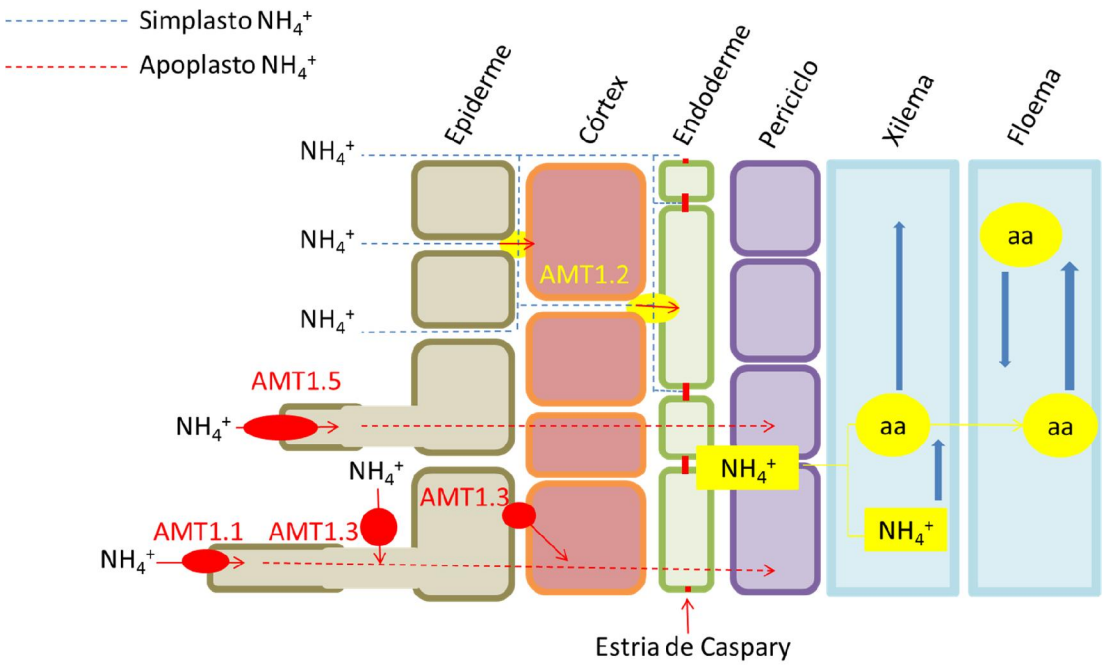
Depois que o NH_4^+ é absorvido pelas raízes, ele é armazenado em vacúolos de raízes, assimilados a aminoácidos, ou transportados para parte aérea. O NH_4^+ pode ser transportado através da planta por caminhos simplásticos e apoplásticos (WANG et al., 2014). Tanto transportadores de alta afinidade (HATS) como os transportadores de baixa afinidade (LATS) para absorção de NH_4^+ são encontrados em raízes de plantas (GLASS et al., 2002). HATS, um sistema de transporte saturável para a captação de amônio, opera em concentrações de amônio inferiores a 0,5 mM (MARSCHNER, 2012). No entanto, o amônio pode ser absorvido pelos LATS, sistema de transporte de alta capacidade, e é dominante em concentrações de amônio acima de 0,5 mM. A absorção de NH_4^+ é mediada por membros da família de transportadores de NH_4^+ (AMT/MEP/Rh) (VON Wiren et al., 2000), que inclui as subfamílias AMT1 e AMT2.

As proteínas AMT1 formam um complexo trimérico, no qual o C-terminal citosólico de cada monómero se liga à região dos poros da subunidade vizinha para trans-ativar o transporte de amônio. A fosforilação de um resíduo de treonina altamente conservado no C-terminal de qualquer monómero individual alivia a interação entre o C-terminal e o poro condutor, mediando a transinativação não apenas do monómero individual, mas também do complexo trimérico completo (LOQUÉ et al., 2007). Isso produz um mecanismo de desligamento rápido e definitivo para a absorção do amônio. De fato, a fosforilação do AtAMT1;1 é rapidamente desencadeada por altas concentrações externas de amônio (LANQUAR et al., 2009). Asim, o próprio AMT1;1 atua como um transceptor e recruta uma quinase do citosol após a ligação de amônio extracelular. CIPK23 fosforila ambos AtAMT1;1 e AtAMT1;2 levando a inibição do transporte de amônio e a modulação do crescimento de plantas sensíveis ao amônio (STRAUB et al., 2017).

A família de transportadores de amônio de alta afinidade contém seis genes de *AMT* em *Arabidopsis* (GAZZARRINI et al., 1999), dez genes em arroz (*Oryza sativa*) (LOQUÉ et al., 2006) e sete genes em pinheiro (*Pinus pinaster*) (CASTRO-RODRIGUEZ et al., 2016). Os transportadores AMT1;1, AMT1;3 e AMT1;5, que são expressos na membrana plasmática de

células de epiderme e pelos radiculares, são responsáveis pela absorção de NH_4^+ via simplástica (Fig. 1., Yuan et al., 2007). Além disso, o NH_4^+ também pode ser transportado nas células da raiz através da via de transporte apoplásico e entrar na raiz via simplasto mediada por AMT1;2 (Fig. 1). Com objetivo de analisar a função de cada um dos genes AMT (transportador de amônio) separadamente em planta, foram realizadas diversas técnicas para a formação de mutantes único, dulpo, triplo e quadruplicado por inserção de T-DNA ou por abordagem de RNAi ou complementando o mutante quádruplo por genes isolados (YUAN et al., 2007). Através destes estudos, foi demonstrada uma contribuição aditiva de cada proteína ao transporte de amônio: *AMT1;1* e *AMT1;3* conferem uma capacidade similar de 30-35%, enquanto a *AMT1;2* conferiu menor capacidade de 18-25% (MASCLAUX-DAUBRESSE et al., 2010). *At*AMT1;1, *At*AMT1;2, *At*AMT1;3 e *At*AMT2;1 são altamente expresso na epiderme da raiz, e promove o crescimento da raiz lateral dependente de amônio (YUAN et al., 2007; LIMA et al., 2010). A expressão de *At*AMT1;1 e *At*AMT1;2 também pode ser encontrada na parte aérea, sendo maior nas folhas maduras.

Figure 1 - Modelo representativo das funções dos transportadores AMT1 de alta afinidade na absorção de amônio nas raízes de plantas.



Fonte: Adaptado de Yuan et al., 2007.

O papel fisiológico dos transportadores de NH_4^+ nas folhas é evidente para importação de NH_4^+ do sistema vascular através da membrana plasmática do mesofilo, porque a concentração de NH_4^+ no xilema pode aumentar para 2.6 mM com fornecimento exclusivo de NH_4^+ ou até mesmo 300 μM em ausência de suplementação de amônio (CRAMER; LEWIS, 1993). Por outro lado, transportadores de NH_4^+ em células do mesofilo podem estar envolvidos na recuperação de NH_3/NH_4^+ fotorrespiratório. Mesmo sob condições ambientais de CO_2, a perda de NH_3 fotorrespiratório nas mitocôndrias (KEYS et al., 1978) pode levar à letalidade ou pelo menos a um aumento dramático nas concentrações foliares se a reassimilação de NH_3 estiver ausente ou inibida, como é o caso de mutantes fotorrespiratórios de cevada e *Arabidopsis* (SOMERVILLE; OGREN, 1980; WALLSGROVE et al., 1987). Como a NH_3 fotorrespiratório provavelmente será reprotonado durante a passagem para o citosol ou quando liberado para o apoplasto da folha, um importador por meio de transportadores de NH_4^+, como a família de genes *AMT1*, pode ser necessária.

Em arroz, OsAMT1s exibem diferentes afinidade para o amônio e afeta o desenvolvimento da raiz (SONODA et al., 2003). Entre eles, o OsAMT1;1, na qual é altamente expresso na epiderme da raiz e cilindro central, na qual contribui com 25% da absorção total do amônio pela raiz e medeia o transporte do amônio da raiz para a parte aérea. Em *Arabidopsis*, ambos mRNA e os níveis de proteínas de AtAMT1s, especialmente para os transportadores de alta afinidade localizada na membrana plasmática, AtAMT1;1, são regulados negativamente pela suplementação de amônio mas regulados positivamente durante a fome de nitrogênio (LOQUÉ et al., 2007). Pelo contrário, transcritos de arroz *OsAMT1;1* e *OsAMT1;2* são transientemente induzido pela suplementação de amônio, e reduzido durante a fome de nitrogênio (SONODA et al., 2003; Li et al., 2016; FERREIRA et al., 2015). Esses resultados sugerem um mecanismo de sensibilidade diferente do AMT em arroz e *Arabidopsis*. A existência de vários genes transportadores de NH_4^+ sugere que o transporte de membrana de amônio é altamente regulado e enfatiza a importância do amônio como principal nutriente mineral de N em plantas de arroz.

Para evitar a toxidade celular, o excesso de moléculas de amônio são compartimentalizadas no vacúolo (LOQUÉ et al., 2004), onde suas concentrações podem atingir até 1 mM para manter as concentrações de amônio citosólicas < 15 μM (ROBERTS; PANG, 1992). Como o pH celular do vácuolo é ácido em comparação ao citosol, o vacúolo pode conter uma concentração cerca de cem vezes maior. O transporte passivo de amônia para o vacúolo é mediado pelas aquaporinas. Em ambiente ácido vacuolar as moléculas são

protonadas e formam amônio, e essas moléculas são compartimentalizadas no lúmem do vaúolo. Geralmente, as concentrações de amônio são mais elevadas em folhas velhas/senescentes e jovens em comparação com folhas maduras, como resultado do catabolismo de aminoácidos e da reciclagem fotorrespiratória, respectivamente (MASCLAUX et al., 2000; DIAZ et al., 2005).

Uma vez que é difícil determinar a concentração de amónio livre, a questão de saber quais as concentrações exatas de amônio em diferentes compartimentos subcelulares ainda permanece sem resposta. Ao utilizar o radioisótopo ^{13}N de curta duração, a absorção e particionamento subcelular de amônio no arroz foi analisado. Na condição de nutrientes de amônio externo de 100 μmol/L, 20% de amônio absorvido foram divididos no vacúolo, 41% no citoplasma e os restantes 19% são metabolizados em 30 min. O sequestro de amônio no vacúolo não só diminui a concentração de amônio no citoplasma, mas também contribui para o ajuste osmótico para manter a absorção de água do solo. A compartimentalização do amônio no vacuolo a partir da sensibilidade ao amônio citosólico tem sido proposta por um receptor ligado a quinase, CAP1. Em *Arabidopsis* mutante *cap1-1* os pelos radiculares na zona de elongação foram altamente sensível ao amônio externo, no qual está ralacionado a um distúrbio citosólico no gradiente de Ca^{+}. Como consequência, os fluxos de amônio através do tonoloplasto de vacúolos isolados de *cap1-1* foram menores, levando a maiores concentrações de amônio citosólico. A autofosforilação da proteína quinase CAP1 asssociada ao Ca^{+} foi demonstrada *in vitro* (BAI et al., 2014), e confirmada por meio de uma abordagem de fosfoproteômica que detectou a fosforilação de CAP1 após a exposição ao amônio (ENGELSBERGER; SCHULZE, 2012). Considerando que CAP1 é expressa em quase todo os tipos de células de raízes, folhas e flores (BAI et al., 2014), sua função na detecção de amônio intracelular e na compartimentalização de amônio não é apenas restrita as células da raiz, mas provavelmente se estende para a maioria dos outros tecidos vegetais.

Efeitos fisiológicos da toxidade de amônio em plantas

A toxicidade por NH_4^+ ocorre tipicamente quando a planta é exposta a um ambiente com alta concentração deste íon (solos alagados), assim como quando ocorre uma produção aumentada de NH_4^+ em condições de estresse devido a uma elevada atividade proteolítica (SKOPELITIS et al., 2006). O excesso de amônio nos tecidos vegetais promove uma série de

mudanças fisiológicas e morfológicas, acarretando principalmente em clorose foliar, restrição do crescimento e até a morte da planta (PURITCH; BARKER, 1967). Os distúrbios causados pela alta concentração de NH_4^+ estão associados direta ou indiretamente com a depleção da disponibilidade de carbono no tecido, danos na estrutura dos cloroplastos, deficiência de cátions minerais, desbalanço da homeostase hormonal e fotossintética, ciclo fútil de amônio transmembranar, aumento do efluxo de prótons, inibição da enzima GDP-manose pirofosforilase, estresse oxidativo, modificação do pH celular e despolarização da membrana (BRITTO; KRONZUCKER, 2002; COSKUN et al., 2013). Esses sintomas dependem da espécie, do estágio de crescimento e das condições ambientais e nutricionais da planta. Assim, para evitar a toxidez, as plantas precisam manter em equilíbrio as taxas de absorção, produção e consumo de NH_4^+.

O papel de desacoplador de prótons do NH_4^+ nas membranas mitocondriais e cloroplásticas foram tidos por muito tempo como o seu principal efeito tóxico na célula vegetal (KROGMANN et al., 1959; MAGALHÃES; HUBER, 1989). Entretanto, alguns estudos demostraram que esse efeito desacoplador de H^+ deve ser considerado apenas em cloroplastos isolados e que os efeitos negativos na fotossíntese e no crescimento são decorrentes de outros fatores acionados pelo excesso de NH_4^+ (ZHU et al., 2000; BRÜCK et al., 2006; ESTEBAN et al., 2016). A restrição do crescimento vegetal sob condições de alta concentração de NH_4^+ também já foi atribuída ao ciclo fútil de NH_4^+/NH_3 transmembranar (BRITTO et al., 2001). Devido à alta taxa de absorção desse íon, um mecanismo de efluxo de NH_4^+ deveria ser acionado à custa de ATP, gerando um alto consumo de energia, para manter a homeostase celular. Entretanto, mais tarde experimentos com isótopos de N não suportaram essa hipótese pelo fato do consumo de O_2 não ter aumentado (COSKUN et al., 2013).

Outro fator relacionado com a toxicidade por NH_4^+ é o desbalanço nutricional que o excesso desse íon pode causar, principalmente por reduzir a absorção de K^+, Ca^{2+} e Mg^{2+} nas raízes. As raízes constituem o primeiro sensor para altas concentrações de NH_4^+ e nessas condições esse órgão sofre severas modificações de sua arquitetura, incluindo raízes primárias mais curtas, inibição da elongação radicular, inserção de raízes laterais na raiz principal e perda do gravitropismo (LI et al., 2010; ZOU et al., 2012; ESTEBAN et al., 2016). A inibição da elongação da raiz primária e lateral é um sintoma comumente observado sintoma de toxicidade de amônio, especialmente quando o amônio é suplementado como única fonte de nitrogênio (BRITTO; KRONZUCKER, 2002; LI et al., 2010; LIU et al., 2013; ARAYA et al., 2016). O amônio inibe o crescimento primário das raízes principalmente pela repressão da divisão celular

no meristema apical da raiz e pela redução do tamanho longitudinal das células (LIU et al., 2013), tomado em conjunto, diversas hipóteses podem explicar os mecanismos subjacentes inibição da elongação da raiz pelo amônio: mudanças de pH extracelular e intracelular (BRITTO; KRONZUCKER, 2002), formação elevada de EROS (espécies reativas de oxigênio) (PATTERSON et al., 2010; XIE et al., 2015), maior efluxo de amônio na zona de elongação da raiz que reforça a inibição da expansão celular longitudinal (LI et al., 2010), ou na diminuição da glicosilação proteica (QIN et al., 2008; BARTH et., 2010; TANAKA et al., 2015). Rogato et al., 2010 e Lima et al., 2010 verificaram que em *L. japonicus* e *Arabidopsis* o transcrito do AMT1 foi induzido pela mesma concentração de NH_4^+ que promoveu modificações fenotípicas na raiz, indicando que a elevada concentração desse íon é percebida localmente e aciona um mecanismo regulatório nesse tecido.

Entre as primeiras repostas fisiológicas desencadeadas por amônio, o EROS pode desempenhar papel de sinalizador secundário amplificando e/ou especificando a resposta de amônio. Em geral, a EROS realiza uma multiplicidade de eventos de sinalização em resposta a estímulos externos (MITTLER et al., 2011). Nas raízes, o suprimento de amônio aumenta os níveis de H_2O_2 (Peróxido de Hidrogênio) tecidual, como mostrado em tomate (FERNÁNDEZ-CRESPO et al., 2015), *Arapidopsis* (PATTERSON et al., 2010) e arroz (XIE et al., 2015). Além disso, o amônio também ativa as enzimas de eliminação de EROS, como catalase, glutationa redutase e peroxidase de guaiacol (PATTERSON et al., 2010; XIE et al., 2015), enquanto a indução de superóxido dismutase indica que os radicais superóxidos podem ser formados. Para investigar se o estresse oxidativo estava associado aos efeitos de fitotoxidade induzido pelo amônio, duas espécies contrastantes ao amônio foram analisadas. Assim, a resposta em espinafre e ervilha, espécie sensível e tolerante ao amônio, respectivamente, não alteraram o estado redox de ascorbato e glutationa, assim como nenhuma alteração nas enzimas antioxidantes. Os autores concluíram que o estresse originado a partir da aplicação de amônio como única fonte de N não é um estresse oxidativo, independente da tolerância ao amônio das espécies de plantas estudadas.

Em geral, altas concentrações de NH_4^+ atingem as folhas apenas após as raízes atingirem sua capacidade máxima de estocagem, preservando assim todo o aparato respiratório e fotossintético na parte aérea. Esse mecanismo de exclusão de íons tóxicos para a parte aérea é bastante conhecido no reino vegetal, principalmente quando se trata de espécies tolerantes ou resistentes (ESTEBAN et al., 2016). Entretanto, em solos pouco aerados, alagados, com baixo pH e temperatura a concentração de NH_4^+ pode atingir cerca de 20 mM ou até 40 mM,

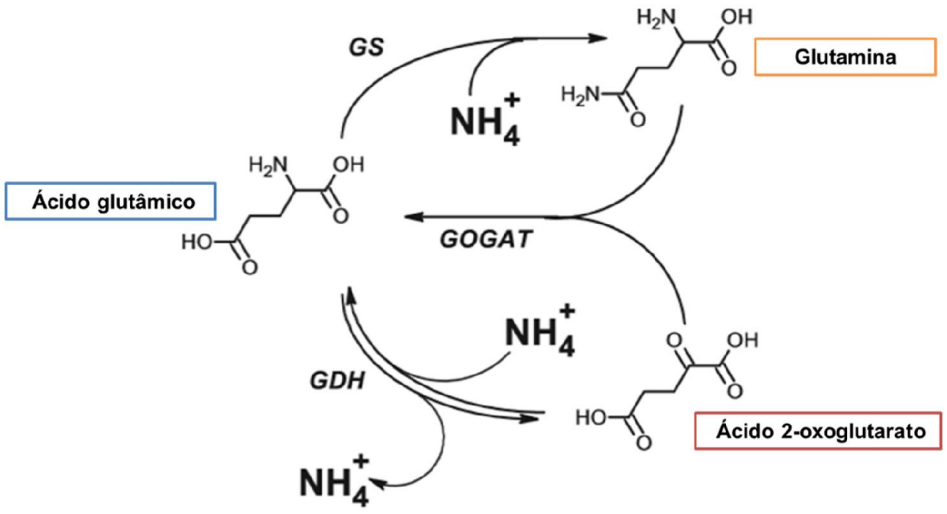
condições que saturam a capacidade de estocagem das raízes e podem facilmente atingir as folhas (WOLT, 1994; KRONZUCKER et al., 2003). Os efeitos tóxicos provocados pelo NH_4^+ no tecido foliar são bastante controversos e com muitas lacunas, mas alguns trabalhos sugerem que esse íon causa deformações na estrutura do cloroplasto, reduz a atividade fotoquímica da fotossíntese e gera estresse oxidativo (PURITCH; BARKER, 1967; PODGÓRSKA et al., 2013; ASKERKA et al., 2015; ALENCAR, 2017). Estudos com células de *Synechocystis* mostraram que a NH_3 se liga em dois sítios do complexo de evolução de oxigênio (OEC) no fotossistema II (PSII) afetando negativamente a fotossíntese (DRATH et al., 2008). Mais recentemente, Alencar, 2017 demonstrou que altas concentrações de NH_4^+ (10 mM) afeta a eficiência quântica do PSII por reduzir o *turnover* da proteína D1 em condições de alta luz (2.000 $\mu mol\ m^{-2}\ s^{-1}$) em plantas de arroz.

Portanto, o excesso de NH_4^+ promove as mais variadas respostas nos vegetais, mas os efeitos tóxicos desencadeados diretamente por esse íon ainda são questionados. Espécies tolerantes ao NH_4^+, como o arroz, se desenvolvem normalmente quando expostos a altas concentrações de amônio, entretanto parte de sua fisiologia e morfologia é modificada por mecanismos ainda pouco conhecidos. Assim, mais estudos são necessários para a compreensão desses processos e melhor aproveitamento de recursos pelos vegetais, envolvendo tanto espécies sensíveis quanto tolerantes.

Mecanismos bioquímicos da desintoxicação de amônio em plantas superiores

Todas as reações de desintoxicação do amônio envolvem a ligação de C-N, e existem várias reações anapleróticas que podem providenciar o carbono necessário para esta transformação. A conjugação do amônio com ácido glutâmico para formar glutamina, e a síntese de ácido glutâmico a partir do ácido 2-oxoglutarato são as principais vias de assimilação do amônio e um mecanismo estratégico para proteger a célula da toxidez do amônio. Estes processos são catalisados pela glutamina sintatese (GS, regulada positivamente pelo amônio), glutamato sintase (GOGAT) localizada nos cloroplastos e a glutamato desidrogenase (GDH) localizada nas mitocôndrias (Fig. 2).

Figure 2 - Atividade das enzimas responsáveis pela assimilação de amônio em planta.



Fonte: Adaptado de Bittsánszky et al., 2015. GS: glutamina sintetase; GOGAT: glutamato sintase; GDH: glutamato desidrogenase.

As isoformas citosólicas de GS (GS1) e GOGAT (NADH-GOGAT) são consideradas as principais enzimas envolvidas na rápida assimilação de amônio absorvida do solo (ISHIYAMA et al., 2004; KONISHI et al., 2014). Guan et al., 2016 verificaram que a expressão do gene *AtGLN1;2* (GS1) em *Arabidopsis thaliana*, foi fortemente induzido pelo suprimento de amônio externo. Além disso, esses autores mostraram ainda que mutantes *gln1;2* apresentam baixa atividade de GS, alta acumulação de amônio e inibição do crescimento quando supridas com NH_4^+, sugerindo que essa isoforma é essencial tanto para a assimilação quanto para a desintoxicação de NH_4^+ nas raízes. A expressão do gene para NADH-GOGAT em *Arabidopsis* também foi intensamente estimulado, enquanto que mutantes *nadh-gogat* exibiram baixa síntese de glutamato e produção de biomassa quando supridos com NH_4^+ (KONISHI et al., 2014).

Uma rota alternativa de assimilação de amônio desencadeada pela glutamato desidrogenase dependente de NADH (NADH-GDH) também deve ser mencionada. Apesar de essa enzima catalisar uma reação reversível (atua tanto como aminante quanto desaminante, dependendo da concentração de nitrogênio) análises de transcritos indicaram que o *GDH2* (α-

subunidade de GDH) serviu como marcador responsivo ao amônio em raízes de *Arabidopsis* (RISTOVA et al., 2016). Após o NH_4^+ ser incorporado em moléculas de aminoácidos, esse último pode ser exportado para outros tecidos diminuindo o efeito tóxico deste íon. Patterson et al., 2010 e Besnard et al., 2016 verificaram que alguns transportadores de aminoácidos (UMAMITs) foram altamente induzidos em plantas após serem supridas com amônio como consequência de sua assimilação. Nesses trabalhos também foi observado que a inibição da atividade de GS por MSO (metionina sulfoximina) inibe a expressão de genes superregulados por amônio, tais como *GDH2* e *UMAMIT14*, sugerindo que as respostas fisiológicas acionadas por amônio dependem de processos ou dos produtos de assimilação, como glutamina e glutamato.

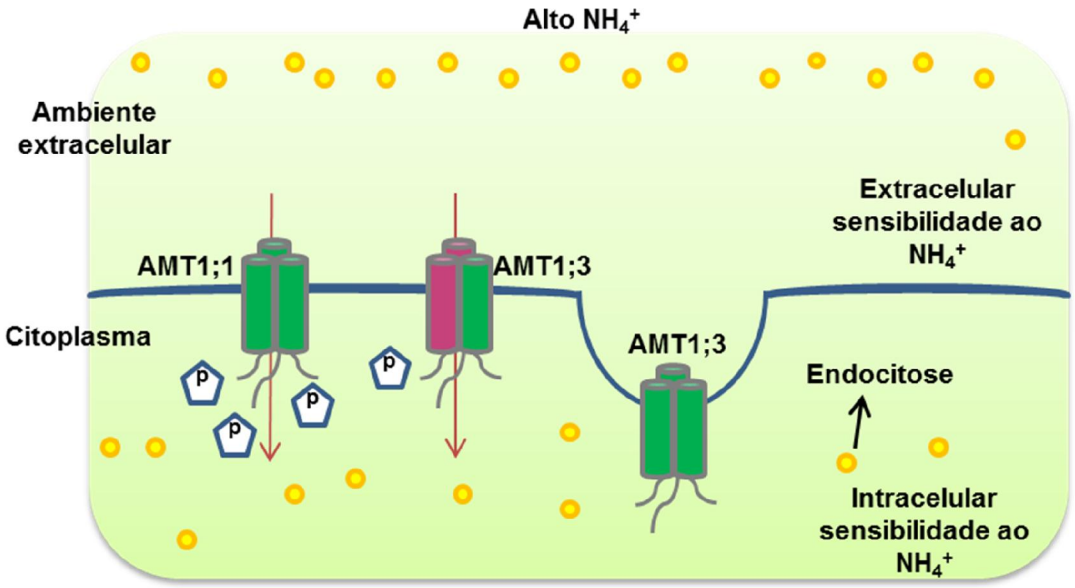
Essas vias de assimilação de NH_4^+ mencionadas acima são consideradas as mais tradicionais, porém em condições de excesso de amônio o ciclo da ureia também pode atuar como uma rota para drenar NH_4^+. A partir da reação catalisada pela carbamoil fosfato sintetase do tipo II (CPSII), o excesso de glutamina proveniente da assimilação de NH_4^+ pode ser convertido em carbamoil fosfato, o qual entra no ciclo da ureia (ZHOU et al., 2000). Diferentemente de animais, plantas superiores não possuem a CPS do tipo I, a qual utiliza diretamente o íon NH_4^+ para produzir carbamoil fosfato, estando presente apenas em alguns eucariotos fotossintetizantes (Rhodophyta e Stramenopila). O ciclo da ureia, além de apresentar grande importância para a distribuição de nitrogênio nas plantas, produz compostos nitrogenados de baixa relação C/N, ureia e arginina, os quais podem ser facilmente estocados nos vacúolos representando um potente estoque compactado de C e N (ESTEBAN et al., 2016). Portanto, apesar da importância da CPSII no metabolismo vegetal ainda não ter sido explorado, a introdução dessa enzima em plantas parece ser um alvo promissor para reduzir consideravelmente a concentração interna de NH_4^+ em condições de estresse.

Além de todas essas rotas mencionadas de assimilação de amônio, um mecanismo adicional para reduzir a concentração desse íon no interior da célula que também pode ser acionado é a emissão de NH_3 na forma de gás pelos estômatos (HERRMANN et al., 2009; WU et al., 2016; ROLNY et al., 2016). A quantidade de amônia emitida pelas folhas depende da concentração de NH_4^+ acumulado no apoplasto das células do mesofilo (HUSTED et al., 2002). Como a maioria das espécies de plantas tenta preservar a parte aérea do excesso de amônio, a liberação de NH_3 pelas folhas representa apenas 1 - 4% da quantidade de N presente nesse órgão (SCHJOERRING; MATTSSON, 2001). Outro mecanismo que pode estar relacionado com a desintoxicação por NH_4^+ é a indução do processo de senescência e o acúmulo do excesso

desse íon nessas folhas. Chen et al., 1997 observaram que a acumulação de NH_4^+ em folhas destacadas de arroz aumenta a sensibilidade por etileno promovendo indução do processo de senescência. A rápida taxa de senescência foliar de um cultivar de *Nicotiana tabacum* (cv. ZY90) foi associada com baixa atividade de GS, elevada atividade de GDH e alta concentração de amônio do apoplasto, emissão de NH_3 e ponto de compensação estomático de NH_3 (WU et al., 2016). Plantas de arroz remobilizam apenas 64% do N total de folhas senescentes, uma pequena parte é volatilizada na forma de NH_3 e o restante é perdido no tecido morto (MAE et al., 1985). Os mecanismos envolvidos nos processos de regulação da emissão de amônia pelas folhas ou o descarte de N pelos tecidos senescentes ainda não está esclarecido e mais estudos são necessários para o melhor entendimento desses processos.

Um mecanismo alternativo induzido por suplementos elevados de amônio foi descoberto. Este mecanismo determina o tempo de permanência de AMT1;3 na superfície da membrana plasmática das células de raiz. Sob condições limitadas de nitrogênio ou de reduzido suplementação de amônio, os oligômeros AMT1;3 mostram uma permanência relativamente longa na membrana plasmática. No entanto, imediatamente após a adição de amônio, as células AMT1;3 se agrupam, desaparecem da membrana plasmática e se internalizam pela via endocítica (Fig. 3). Consistente com um agrupamento acelerado de AMT1;3 no mutante de glutamina sintetase *gln1;2*, na qual o amônio desencadeia a remoção de AMT1;3 da membrana plasmática. Isso pode atuar como um mecanismo de *feedback* para prevenir a toxidade de amônio nas células radiculares (WANG et al., 2014), juntamente com outros mecanismos como assimilação do amônio ou a compartimentalização de amônio no vacúolo (LOQUÉ et al., 2005).

Figure 3 - Aumento dos níveis de amônio citosólico desencadeia a remoção de AMT1;3 da membrana plasmática através de agrupamento de proteínas e endocitose, interrompendo a aquisição de amônio.



Fonte: Adaptado de Liu; von Wirén, 2017.

Arroz como uma espécie tolerante ao amônio

Plantas de arroz (*Oryza sativa*) são consideradas uma das espécies mais tolerantes ao NH_4^+, pois evolutivamente desenvolveram mecanismos eficientes para crescerem em solos alagados (WANG et al., 1993). Nestas condições a concentração de O_2 é baixa e isso inibe a atuação de microrganismos nitrificantes resultando em alta amonificação (EVINER; CHAPIN 1997). Nestas condições os solos possuem pH na faixa de 7,0 e 25 ºC, por isso a forma protonada da amônio (NH_4^+) predomina, assim a concentração desse íon é elevada em solos alagados e, por conseguinte, nos compartimentos da raiz de arroz (TABUCHI et al., 2007). O arroz é uma das poucas espécies que conseguem se desenvolver em solos contendo alta concentração de amônio e isso é atribuído principalmente ao seu eficiente sistema de assimilação de NH_4^+, o ciclo GS-GOGAT (ISHIYAMA et al., 2004a). A isoforma citosólica de GS em arroz é expressa por três genes, dos quais os Os*GLN1;1* e Os*GLN1;2* são abundantemente expressos nas raízes.

As duas isoenzimas de GS (GLN1;1 e GLN1;2) em arroz apresentam alta afinidade para o NH_4^+ com baixo K_m (27 e 73 µM, respectivamente) e alto V_{max} (186.3 e 98.1 nkat/mg proteína, respectivamente) (ISHIYAMA et al., 2004a). Diferentemente do arroz, plantas de

Arabidopsis apresentam duas isoenzimas com baixa afinidade (GLN1;2 e GLN1;3) e outas duas de alta afinidade (GLN1;1 e GLN1;4) também com baixo K_m (< 10 e 48 μM, respectivamente), mas com V_{max} menores do que os encontrados nas isoformas de arroz (ISHIYAMA et al., 2004b). Além disso, a atividade de OsGLN1;1 foi seis vezes maior quando comparado com GLN1;1 de *Arabidopsis* (ISHIYAMA et al., 2004b). Portanto, as propriedades cinéticas das OsGLN1 sugere que as raízes de arroz são capazes de assimilar amônio na faixa de micromolar com alta capacidade de convertê-lo em glutamina, removendo rapidamente o excesso desse íon e evitando seus efeitos tóxicos nas raízes.

O padrão de expressão de Os*GLN1;1* e Os*GLN1;2* na ponta da raiz, região de maior absorção de NH_4^+, ocorre de forma antagônica. Enquanto a expressão de Os*GLN1;1* é induzida por condições de baixo N, a transcrição dos genes de Os*GLN1;2* são abundantemente acumulados após o tratamento com amônio, indicando que essas isoformas são opostamente reguladas por amônio em células específicas da raiz a nível de mRNA (ISHIYAMA et al., 2004a). Portanto, plantas de arroz apresentam maior atividade da GLN1;2, a qual apresenta menor capacidade de assimilação de NH_4^+, em condições de alta concentração desse íon. O genoma de arroz também possui dois genes para NADH-GOGAT e apenas um para Fd-GOGAT, os quais são diferentemente expressos no corpo da planta. Nas raízes a isoforma Os*NADH-GOGAT1* é a mais expressa e atua em plastídios (HAYAKAWA et al., 1999) principalmente de células da epiderme e exoderme após a exposição ao NH_4^+ (ISHIYAMA et al., 2003). Os transcritos de Os*NADH-GOGAT1* começam a se acumular na superfície de raiz após 3-6 h e a quantidade da proteína atinge seu máximo após 12-24 h do suprimento com NH_4^+ (ISHIYAMA et al., 2003).

A isoforma de GOGAT dependente de ferredoxina (Fd-GOGAT), atuante principalmente em cloroplasto com a função de assimilar o NH_4^+ oriundo das reações fotorrespiratórias, também estar presente na zona meristemática, cilindro central e córtex de raízes de arroz (ISHIYAMA et al., 2003). Isso indica claramente que em plantas de arroz existe uma sobreposição da distribuição das isoformas NADH- e FD-GOGAT que atuam mutuamente na assimilação de amônio. A atividade de GOGAT também irá depender da taxa de regeneração do 2-oxoglutarato, o qual é fornecido pela enzima mitocondrial NAD-isocitrato desidrogenase em raízes de arroz (ABIKO et al., 2005). Se existem diferenças nas características cinéticas das isoformas de GOGAT, assim como na de GDH de arroz para lhe conferir tanta tolerância ao amônio em relação às outras espécies ainda não estar esclarecido.

Também tolerância de plantas de arroz a altas concentrações de amônio podem ser atribuídas ao somatório de outras características peculiares. Enquanto a maioria das plantas possui apenas uma camada de estria de Caspary em suas raízes, as de arroz possuem duas camadas, uma entre a exoderme e o esclerênquima e outra na endoderme (MORITA et al., 1996). Dessa forma, a maior parte do NH_4^+ absorvido é assimilada dentro de células da epiderme ou exoderme e apenas seu excesso é transportado para células mais internas da raiz podendo chegar ao sistema vascular e, por último, atingir a parte aérea. A absorção e o transporte de NH_4^+ entre as células é controlado principalmente pelos transportadores de amônio (AMT1;2) (TABUCHI et al., 2007). A expressão dos genes Os*AMT1;3* e Os*AMT1.;1* são regulados negativamente em condições de alta concentração de NH_4^+, enquanto que os transcritos de Os*AMT1;2* são fortemente induzidos por NH_4^+ e glutamina, sugerindo que esse transportar regula ativamente a absorção e transporte célula-célula desse íon (TABUCHI et al., 2007).

Plantas de arroz apresentam mecanismos diferenciados de exclusão de amônio?

Definitivamente, plantas de arroz possuem mecanismos diferenciados e eficientes para a estocagem e assimilação de NH_4^+, entretanto o funcionamento desses processos ainda não está totalmente esclarecido. É sabido que propriedades anatômicas na raiz e a elevada taxa catalítica do ciclo GS-GOGAT em plantas de arroz contribuam para a sua alta tolerância ao NH_4^+ (ISHIYAMA et al., 2004a; TABUCHI et al., 2007). Entretanto, se esses dois fatores sozinhos são suficientes ou se existem outros mecanismos desconhecidos para justificar tanta tolerância é uma questão a ser respondida. Plantas de arroz conseguem se desenvolver normalmente em concentrações muita alta de NH_4^+ (~ 40 mM), concentrações estas altamente tóxicas para a maioria das outas culturas (BRITTO et al., 2014; SUN et al., 2017). Suas raízes e colmos conseguem armazenar grandes quantidades de NH_4^+ sem afetar o seu metabolismo. A maior parte desse amônio pode estar compartimentalizado principalmente no apoplasto e/ou no vacúolo, protegendo as vias metabólicas sensíveis ao excesso de NH_4^+ (WU et al., 2016). Além disso, sob altas concentrações de NH_4^+ as células próximas à exoderme e endoderme (estrias de Caspary) apresenta um espessamento maior de suberina e lignina, os quais promovem redução da permeabilidade de solutos, mas não modifica a condutividade hidráulica (RANATHUNGE et al., 2016).

A modificação do nível de N no solo ativa uma série de processos de sinalização, os quais são percebidos pelas raízes e transmitidos até a parte aérea por meio de sinais específicos, tais como metabolitos secundários e hormônios (SUN et al., 2017; LIU; VON WIRÉN, 2017). Sun et al., 2017 observaram que o excesso de NH_4^+ induz fortemente genes da rota de síntese de ácido abscísico (ABA) e etileno. Esses autores sugerem que o ABA promove ativação da rota de transdução MAPK, a qual aciona um efeito de *feedback* da parte aérea para as raízes. Enquanto que o acúmulo de etileno irá causar um aumento da taxa de senescência. A forte relação entre acúmulo de amônio e aumento da taxa de senescência foliar em arroz já foi percebida há bastante tempo (CHEN et al., 1997), entretanto se isso é uma estratégia utilizada por essa espécie para descartar o excesso de amônio no tecido ainda precisa ser esclarecido.

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3 AMMONIUM EXCLUSION IS RELATED TO LEAF SENESCENCE AND NITROGEN PARTITIONING IN RICE PLANTS

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ABSTRACT – Plants can obtain N as two main forms, nitrate (NO_3^-) and ammonium (NH_4^+). Despite ammonium is well known by its potential toxicity to plants metabolism, several plants have evolved special features to tolerate and increase affinity for ammonium. Among these ammonium tolerant species, rice is a crop with remarkable economic importance. Surprisingly, the physiological mechanisms that confer tolerance to these plants are known in a fragmented manner. For instance, the dynamic of leaf senescence and the partitioning of ammonium and other N-forms under long-term high ammonium exposure are completely unknown to date. We proposed here that rice plants should trigger some effective anatomical and morpho-physiological features to cope with excess ammonium in parallel to an efficient antioxidant mechanism. In order to test this hypothesis rice plants (*Oryza sativa japonica* cv. Nipponbare) were grown in 15 mM NO_3^- or NH_4^+ as the solely N source for up to 56 days and evaluated in terms of growth, photosynthesis, redox metabolism and partition of biomass and different N forms. The obtained results suggest that rice plants exhibiting a complex systemic mechanism of ammonium exclusion, which consisted in accumulate this toxic N form in culms and senescent leaves, protecting roots and photosynthetic apparatus in the young leaves. In roots, the higher activity of GPOD was observed in parallel to unchanged H_2O_2 content and decreased signals of lipid peroxidation, thus indicating an effective antioxidant protection. Nevertheless, the strong impairment in root growth combined with higher induction of leaf senescence in high ammonium-treated rice could also indicates that rice plants are not tolerant to long-term exposure to such high ammonium conditions.

Keywords: Ammonium. N metabolism. NH_4^+ toxicity. Nitrate. *Oryza sativa*. Oxidative stress.

INTRODUCTION

Nitrogen (N) is one of the most important mineral nutrients, which is commonly a limiting factor for the worldwide crop productivity. Plants can obtain this essential resource from two major forms: nitrate (NO_3^-) and ammonium (NH_4^+) (BRITTO; KRONZUCKER, 2013). Different plant species are feasible to be classified according to their affinity for NO_3^- or NH_4^+ as the main N source (BOUDSOCQ et al., 2012). Indeed, native and cultivated plants display great genetic variability for ammonium and nitrate nutrition and these traits might be influenced by environmental factors (BRITTO; KRONZUCKER, 2013). Despite the reasons why plants present different affinity to the contrasting N sources has not been elucidated to date, it has been suggested that ammonium-specialist plants exhibit atrophied nitrate uptake systems in the roots and the nitrate specialist ones are more susceptible to the toxicity mechanisms related to excess NH_4^+ (KRONZUCKER et al., 1997; BRITTO; KRONZUCKER, 2002). Nevertheless, the majority of the cultivated plants absorbs preferentially NO_3^- since this anion is the N-form predominant in these conditions (BRITTO; KRONZUCKER, 2013).

In contrast to NH_4^+/NH_3, nitrate is virtually non-toxic for plants and it can be stored into vacuoles in large amount. Ammonium negative effects on plant growth has been observed for more than one century (DARWIN, 1882). However, the exact molecular and physiological mechanisms of ammonium toxicity in plants remain elusive in nowadays (ESTEBAN et al., 2016; LIU; VON WIRÉN, 2017). As a main mechanism, it has been proposed that nitrate specialist plants exhibit impairment in uptake of potassium, calcium and magnesium in presence of ammonium supplying, which in turns could generate the subsequent deficiency symptoms, remarkably stunted growth (BRITTO; KRONZUCKER, 2002). Indeed, the simultaneous increase of K^+ supplying has been reported as an effective approaching to decrease the NH_4^+ toxicity symptoms in different crops. This response could be related to a relief from the potassium deficiency *per se* as well as by reduced NH_4^+ transport and accumulation involving a competitive mechanism (BRITTO et al., 2014).

Other important ammonium toxicity mechanisms in plants have been widely reported but, intriguingly, the tolerance features are practically unknown. A classical toxic effect that have been proposed many time ago is the potential effect of NH_4^+ as a proton gradient uncoupler, which could generate harmful effects on crucial cellular processes such as NPQ and ATP synthesis during photosynthesis (WRAIGHT; CROFTS, 1970; BRITTO;

KRONZUCKER, 2002). However, despite the potential uncoupler role expected for ammonium *in vitro*, it is astonishing to recognize that there are no evidences for that ammonium uncoupler action in whole plant systems (BENDIXEN et al., 2001). However, several studies have revealed harmful effects related to ammonia (NH_3) binding to the oxygen evolving complex affecting the PSII reaction center efficiency (BECK; BRUDVIG, 1988; DRATH et al., 2008; VINYARD et al., 2016), ammonium toxicity mechanism in plants is still under debate (ESTEBAN et al., 2016).

Regardless the exact molecular mechanism of ammonium toxicity, plants can exhibit several anatomical and morphological differences when supplied with high NH_4^+ levels as exclusive N source (LIU; VON WIRÉN, 2017), especially involving stunted root growth and leaf chlorosis followed by senescence (BRITTO; KRONZUCKER, 2002). More recently, several processes related to ammonium-induced decrease of root growth have been reported, especially regarding changes in gene expression, metabolism, redox status and root-system architecture (LIU; VON WIRÉN, 2017). Several reports evidenced that ammonium is able to inhibiting root elongation, inhibition of lateral root branching and swelling root hairs (LIU; VON WIRÉN, 2017). Root elongation can be impaired by ammonium via repression on both cell division and cell expansion, which might been triggered by signaling transduction pathways involving protein N-glycosylation and reactive oxygen species (ROS) metabolism (PATTERSON et al., 2010).

Arabidopsis, a nitrate specialist species, exhibited increase in H_2O_2 content, lipid peroxidation in the leaf tissue, protein oxidation and decreased APX (ascorbate peroxidase) activity and redox state of ascorbate and glutathione in response to 8 weeks of supplying with 5 mM ammonium (PODGÓRSKA et al., 2013). These effects were observed in rice seedlings just after exposure to 80 mM NH_4^+ during 6 days exhibited a prominent accumulation of H_2O_2 and lipid peroxidation in roots (XIE et al., 2015). However, studies involving oxidative metabolism in ammonium specialist species are scarce. Despite this limitation, a central hypothesis have been raised by some authors, according which, ammonium specialists plants are more prone to avoid over-accumulation of ROS in roots and leaf tissues, and, consequently able to survive under such conditions. Oppositely, nitrate specialist plants supplied with ammonium might present unbalance in redox metabolism, which could contribute to restrict root growth and promote leaf senescence (LIU; VON WIRÉN, 2017).

Interestingly, some NH_4^+-tolerant (rice) and non-tolerant (*Phaseolus sp.*) plants species, when challenged with high ammonium concentrations were able to preserve the shoot

from toxicity, maintaining unchanged growth and photosynthesis (GUO et al., 2007; ZHU et al., 2000). In general, the root growth is more affected than leaf dry mass accumulation, suggesting that the growth partitioning in presence of high ammonium levels could be under phytohormone control. Indeed, some authors have suggested that NH_4^+ might act as a signaling molecule affecting several acclimation mechanisms (LIU; VON WIRÉN, 2017). Recently, studying rice plants exposed to high ammonium concentration (15 mM), Sun et al., 2017 demonstrated that the expression of several genes was affected, especially NH_4^+-transporters and the hormones ethylene and ABA. Moreover, it has been widely reported that NH_4^+ might act as a signaling molecule to trigger senescence in leaves (CHEN et al., 1997; ROLNY et al., 2016; WU et al., 2016) but the physiological significance of this mechanism during ammonium toxicity is still poorly understood.

It is well known that older leaves behave as source tissues during senescence, accumulating mineral N components (NO_3^- and NH_4^+) and progressively loosing carbohydrates and amino acids for sink tissues (MACHADO et al., 1990; SILVEIRA; MACHADO, 1990; MASCLAUX et al., 2000). The N compounds recycling mechanisms during natural and stress-induced senescence are very well known (MACHADO et al., 1990; SILVEIRA; MACHADO, 1990; TEGEDER; MASCLAUX-DAUBRESSE, 2018) but specifically during NH_4^+-toxicity this issue has been neglected. Indeed, under high NH_4^+ supplying in roots (high N-source), associated with high levels of ammonium- transporters, which would be the feedback mechanisms to control and avoid an excess ammonium in tissues? In other words, could the "normal" senescence process predominate in an extreme condition of high ammonium supplying? It has well established that during excess NH_4^+ the detoxification GS/GOGAT cycle system is crucial in the majority of higher plants (BRITTO; KRONZUCKER, 2002). This cycle could avoid ammonium to reach toxic levels and recycle N to other sink tissues and vacuoles (ESTEBAN et al., 2016).

Rice is widely recognized as NH_4^+-tolerant species but, unexpectedly, the physiological mechanisms that confer tolerance to these plants are known in a fragmented manner. For instance, what is the dynamic of the senescence at the whole plant level and which is its consequence for the partitioning of ammonium and other N-forms? Could a high NH_4^+-influx alter the source-sink hierarchy in terms of ammonium distribution? Paradoxically, these relationships have been scarcely studied in order to understand some ammonium toxicity and tolerance mechanisms, especially in a tolerant species such as rice plants. It should be supposed that plants exposed to high concentrations of this toxic molecule must display a myriad of

integrated mechanisms to avoid toxicity. We propose here that a tolerant species should trigger some effective anatomical and morpho-physiological features to cope with excess ammonium in parallel to antioxidant and detoxification processes.

In this study, rice plants were grown in 15 mM NO_3^- or NH_4^+ as the solely N source and evaluated in terms of growth, photosynthesis, redox metabolism and partition of biomass and the different N forms. The obtained results here corroborated partially the hypothesis mentioned above. Indeed, rice plants exhibit evidences for a complex systemic mechanism of ammonium exclusion, which consisted in accumulate this toxic N form in culms and senescent leaves, protecting roots and photosynthesizing young leaves. In roots, the higher activity of GPOD was observed in parallel to unchanged H_2O_2 content and decreased signals of lipid peroxidation, thus indicating an effective antioxidant protection. Nevertheless, the strong impairment in root growth combined with higher induction of leaf senescence in high ammonium-treated rice could also indicates that rice plants are not tolerant to long-term exposure to such high ammonium conditions.

MATERIALS AND METHODS

Plant material and growth conditions

Rice seedlings (*Oryza sativa* L. cv. Nipponbare), 11 days after germination, were transferred to two different hydroponic systems (each 3 L container had 2 plants) with 15 mM NO_3^- or 15 mM NH_4^+, as sole N source, for up to 56 days. The plants were cultivated in a greenhouse under natural conditions as follow: day/night mean temperature of 32/25 °C, mean relative humidity of 65%, maximum photosynthetic photon flux density (PPFD) around 600 µmol m^{-2} s^{-1} at noon, and a photoperiod of 12 h. In these experiments, $(NH4)_2SO_4$ and NH_4Cl salts were utilized as NH_4^+-sources and $Ca(NO_3)_2$ and KNO_3 as NO_3^- sources and all other nutrients were provided in an optimum concentration according to the Hoagland and Arnon's nutritive solution (HOAGLAND; ARNON, 1950). During the growth period, the pH was adjusted every two days to 6.0 ±0.5 and the nutritive solution was completely changed every four days to minimize N concentration oscillations.

Experiments

Aiming to analyse NH_4^+ effects of a long-term ammonium nutrition in growth, biomass, nitrogen metabolism, oxidative stress and photosynthesis rice plants were cultivated with 15 mM NO_3^- (control) or NH_4^+, as a sole N source, by 56 days under greenhouse conditions. During treatments with two source nitrogen, corresponding phenotypes including length of shoot and roots parts, number of leaf and tillers were determined at the indicated time point. Meanwhile, corresponding images were also photographed. Gas exchange parameters were measured every two days and plant material (green leaves, senescence leaves, culm + tillers and root) was harvested to further analyses of root volume, biomass, oxidative stress and metabolism nitrogen (nitrate, ammonium, free amino acid and total N concentration: lyophilized samples were incubated with deionized water at 100 °C for 1 h and filtered to obtain the crude extract).

Phenotype analysis

For ammonium tolerance assay, after the transfer of the plants to the pots, every two days measurement length of root and shoot, number of leaves and tillers were determined. 49 days after ammonium exposure root volume, shoot/root ratios and fresh weight of green leaves, senescence leaves, culm + tillers and root was determinate.

Photosynthetic gas exchange

The net CO_2 assimilation rate (P_N) and stomatal condutance (gs) were measured using a portable infrared gas analyser system (LI-6400XT, LI-COR, Lincoln, NE, USA), equipped with a leaf chamber fluorometer (LI-6400-40, LI-COR, Lincoln, NE, USA). The conditions inside the IRGA's chamber during the measurements were: PPFD of 1,000 μmol photons m^{-2} s^{-1}, 28 °C, air vapor pressure deficit of 1.0±0.2 kPa, and air CO_2 partial pressure of 40 Pa. The amount of blue light was set to be 10% of the PPFD to maximize stomatal aperture (FLEXAS et al., 2008).

Electrolyte leakage (membrane damage) and lipid peroxidation

Membrane damage was measured by electrolyte leakage as describe previously by Blum and Ebercon, 1981. Rice root samples (~500 mg fresh weight of basal part) were placed in tubes containing 10 mL of deionized water. The flasks were incubated for 3 h and electric conductivity in the medium (L1) was measured. The roots were then boiled (98 °C) for 1 h and the electric conductivity (L2) was measured again. The relative membrane damage (MD) was estimated by MD = L1/L2x100. Lipid peroxidation was measured based on the formation of thiobarbituric acid-reactive substances (TBARS) in accordance with (HEATH; PACKER 1991). The concentration of TBARS was calculated using absorption coefficient (155 mM^{-1} cm^{-1}), and the results were expressed as ηmol MDA-TBA g^{-1} FW.

H_2O_2 content determination

An Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Carlsbad, CA, USA) was used to measure H_2O_2 production (ZHOU et al., 1997). For H_2O_2 extraction, 400 mg of fresh roots were ground in liquid N_2, and 1 mL of phosphate butter 100 mM, pH 7,5 was added to frozen tissue. After centrifugation, 100 µL of the supernatant was incubated with 0.2 U mL^{-1} horseradish peroxidase and 100 µL Amplex Red reagent (10-acetyl-3,7-dihydrophenoxazine) at room temperature for 30 min under dark conditions. The absorbance was quantified spectrophotometrically using a wavelength of 560 nm, and the H_2O_2 content was expressed as µmol H_2O_2 g^{-1}FW calculated from a H_2O_2 stand curve according the kit manufacturer's instructions.

Enzymatic activity assays

For enzyme activity measurements, 400 mg of pooled root tissue was frozen in liquid nitrogen, pulverized and added of an extraction buffer containing 100 mM KH_2PO_4 (pH 7.0), 1 mM EDTA and 1 mM ascorbic acid. The suspension was centrifuged at (14,000 x g, 4 °C, 30 min), and the total protein concentration of the supernatant was determined using Bradford assay (Bradford, 1976). The activity of ascorbate peroxidase (APX; EC 1.11.1.1) and guaiacol peroxidase (GPOD) were determined according to previously published protocols (NAKANO; ASADA 1981; Amako et al., 1994), respectively.

Determination of total nitrogen

Plant tissues lyophilized were digested with H_2SO_4, and the concentration of N was determined using the Kjeldahl method (BAETHGEN; ALLEY, 1989).

Nitrate, ammonium and amino acid determination

The nitrate concentration was measured by salicylic acid method, according to Cataldo et al., 1995. Ammonium determination was performed by using the phenol-hypochlorite-ammonia method (FELKER, 1997) and the total free amino acids were measured according to Peoples et al., 1955. The concentration of NO_3^-, NH_4^+ and amino acids were expressed in μmol g^{-1} DW.

Assays of enzyme activity

- *Glutamine synthetase measurement*

Fresh leaves, culm and roots were ground until obtaining a fine powder in presence of liquid N_2, ice-cold 100 mM Tris-HCl buffer (pH 7.6) containing 1 mM EDTA, 1 mM $MgCl_2$ and 10 mM 2-mercaptoetanol. After centrifugation at 14,000 g for 30 min, the supernatant was collected and used as enzymatic extract. All extraction stages were carried out at 4° C and determined by hydroxamate biosynthetic method as described by (HIREL; GADAL, 1980). GS total activity, the assay buffer consisted of 50 mM Tris–HCl buffer, pH 7.8, 5 mM ATP, 12.5 mM $MgSO_4$ and 25 mM Na-glutamate. The reaction was started by the addition of enzymatic extract and 20 mM hydroxylamine hydrochloride neutralized with HCl and the mixture was incubated at 30 °C for 30 min. The reaction was quenched by adding 370 mM $FeCl_3$, 200 mM TCA and 0.67 N HCl solutions. The concentration of the brown complex was determined by measuring the absorbance at 540 nm. The blank consisted of the reaction mixture in the absence of enzymatic extract. A control was performed by omitting hydroxylamine from the reaction mixture. A standard curve was made with γ-glutamyl hydroxamate and the GS activity was expressed as μmol γ-glutamyl hydroxamate (GGH) g^{-1} FW h^{-1}.

Experimental design and statistical analysis

The experiments were arranged in a completely randomized block, with two different nitrogen source and four replicates per treatment. Each replicate was represented by a pot contain 2 plants. The data were subjected to the analysis of variance (ANOVA) and the averages were compared by Tukey at a confidence level of 5% ($p < 0.05$), as referred in figure captions.

RESULTS

Rice plants cultivated exclusively with high ammonium exhibit deep differences in growth and development of shoots and roots as compared to solely nitrate-supplied rice

In order to investigate the effects of high ammonium long-term exposure in rice plants, seedlings at 11 days old were transferred to modified Hoagland & Arnon's nutrient solution containing 15 mM NO_3^- or 15 mM NH_4^+ as exclusive N source, and grown for 56 days under these contrasting conditions. High ammonium-grown plants did not exhibit significant differences regarding shoot length, which varied almost linearly from 9 cm in the onset of treatment to 60 cm after 36 days (**Fig. 4A**). Oppositely, roots elongation was severely affected by ammonium supplying. While plants exhibited approximately 19 cm of root length at the onset of the treatments, nitrate grown plants exhibited an increase of 60% in root length as compared to ammonium-treated plants, after 36 days (**Fig. 4B**). The emergence of leaves and tillers displayed a very similar trend to that observed for shoot length, which is an almost linear increase and absence of significant differences between plants independently of the N source utilized in cultivation (**Fig. 5A,B**).

The **figure 6** corroborates the absence of differences in the shoot elongation of rice plants exposed to NO_3^- or NH_4^+ as exclusive N source. However, despite this similar phenotype, ammonium-treated rice exhibited an earlier and more intense incidence of senescence in the older leaves, as compared to nitrate-grown plants (**Fig. 6**). In parallel, morphological changes in rice roots grown with ammonium corroborated the presence of stunted growth in this plant organs, as compared to rice plants cultivated exclusively with nitrate as N source (**Fig. 7**). Indeed, the NH_4^+ supplying to rice plants induced an intense decrease in root volume and

root/shoot ratios equivalent to 55% and 56%, respectively, as compared to nitrate-grown plants (**Fig. 8A,B**).

Total dry mass in rice plants gown with 15 mM of NH_4^+ or NO_3^- in nutrient solution for 49 days did not displayed significant differences. However the dry mass partitioning among different plant organs exhibited a great contrast between the different N-sourced plants (**Fig. 9**). Nitrate-supplied plants exhibited 21%, 3%, 64% and 12% of dry mass distributed among green leaves, senescent leaves, culm + tillers and roots, respectively. In other hand, ammonium-growth plants exhibited 11%, 15%, 68% and 6% of the total dry mass separated into green leaves, senescent leaves, culm + tillers and roots, respectively. Interestingly, despite the contrasting results concerning green leaf biomass, the ammonium-supplied plants did not exhibited significant differences in the dynamic of specific CO_2 assimilation (P_N) and stomatal conductance (g_S) as compared with nitrate-treated plants (**Fig. 10A,B**).

High ammonium-induced stunted root growth is not correlated with oxidative stress in rice plants

Next, we aimed to understand the possible links between the ammonium-induced decreases in rice roots growth and the generation of unbalances in the root cell redox metabolism. Our central hypothesis was that rice plants exposed to such high ammonium conditions, would suffer over-accumulation of ROS, which could be an important factor to restrict root growth. This hypothesis was refuted. Rice plants exposed to 15 mM of ammonium for 49 days exhibited no significant differences in roots H_2O_2 content and membrane damage, as compared with nitrate-supplied references (**Fig. 11A,B**). Moreover, the content of thiobarbituric acid reactive species (TBARS), important indicator of lipid peroxidation, was slightly lower (13%) in ammonium grown roots, as compared to nitrate-treated references (**Fig. 11C**).

Interestingly, roots from ammonium –treated plants displayed no significant differences in the activity of ascorbate peroxidase (APX) in comparison to nitrate-grown plants (**Fig. 12A**). In contrast, the activity of guaiacol peroxidase (GPOD) was remarkably higher in NH_4^+-supplied plants (75%) than it was in the nitrate-grown references (**Fig. 12B**). Notably, these results are expressed on the basis of fresh mass. In parallel, the content of soluble proteins

increased by 66% in plants exposed to the ammonium treatment, as compared to the nitrate-treated reference plants (**Fig. 12C**).

Ammonium accumulation in senescent leaves and culms represents an exclusion mechanism against toxicity in rice plants

In order to understand the differential partition of the different N forms among the diverse plant organs, we quantified the absolute content of total N, nitrate, ammonium and free amino acids in green leaves, senescent leaves, culm + tillers and roots. We tested the hypothesis that rice plants are able to trigger different compartmentalization of excessive ammonium in order to protect crucial organs, such as green photosynthetic leaves. Nitrate-grown plants exhibited 38%, 3%, 43% and 16% of total N allocated to green leaves, senescent leaves, culm + tillers and roots, respectively. In contrast, the ammonium-supplied plants exhibited 13%, 27%, 54% and 6% of total N portioned into green leaves, senescent leaves, culm + tillers and roots, respectively (**Fig. 12**).

Regarding the nitrate accumulation, the NO_3^--grown plants displayed 5%, 23%, 54% and 18% of total NO_3^- (671.6 μmol) distributed into green leaves, senescent leaves, culm+tillers and roots, respectively. Beside the total NO_3^- has been much lower (85% decrease) in ammonium-grown plants (100.9 μmol), this N form was distributed by 7%, 11%, 80% and 2% by the different plant organs (green leaves, senescent leaves, culm+tillers and roots, respectively). Otherwise, nitrate-plants accumulated much less (96% decrease) NH_4^+ than ammonium-supplied plants. Nitrate-treated references exhibited 16%, 4%, 52% and 28% of total NH_4^+ parted into green leaves, senescent leaves, culm+tillers and roots, respectively, while ammonium-grown plants exhibited 1%, 75%, 23% and 1% of total NH_4^+ (2013.7 μmol) allocated to the same plant organs (**Fig. 12**).

The total free amino acids was allocated by 15%, 3%, 71% and 11% into green leaves, senescent leaves, culm + tillers and roots, respectively, when the plants were cultivated with 15 mM NO_3^-. Nevertheless, plants grown in presence of high ammonium exhibited 3%, 20%, 75% and 2% of total free amino acids distributed into green leaves, senescent leaves, culm +tillers and roots, respectively (**Fig. 12**). Interestingly, despite the ammonium-grown rice plants have exhibited much lower accumulation of NH_4^+ into green leaves, culm + tillers and roots, as compared to senescent leaves (see above), the activity of total glutamine synthetase (GS) in this

plant organs was lower than that found in similar organs from nitrate-supplied plants. Excess ammonium-supplied plants exhibited decreased GS activity by 34%, 54% and 26% in green leaves, culm + tillers and roots, all in comparison to the nitrate-grown plants (**Fig. 13**).

DISCUSSION

In this study was clearly demonstrated that high NH_4^+ supplying to roots of a tolerant rice cultivar induced deep morpho-physiological changes at the whole plant level as compared to NO_3^--supplied plants but, unexpectedly, any signals of oxidative stress were observed. This well-balanced oxidative response is in opposition to other work recently reported for rice seedlings supplied with very high NH_4^+ concentration (80 mM) (XIE et al., 2015) and also for some non-tolerant species exposed to lower concentrations (PODGÓRSKA et al., 2013). Indeed, the most common root response to excess ammonium is a strong reduction in its growth and some authors have associated this consequence to a regulation oxidative (LIU; VON WIRÉN, 2017). Moreover, besides H_2O_2 accumulation, root growth was previously related to increase of GPOD activity, an enzyme directly involved in cell-wall lignification and cell elongation impairment (MAIA et al., 2013).

Several studies have demonstrated the central role of cell wall-GPODs in the regulation of plant cell growth (COSIO; DUNAND, 2009; MARJAMAA et al., 2009; PASSARDI et al., 2004). These enzymes show a dual activity that could favor cell elongation by ROS generation through their hydroxylic cycle, which requires ascorbate as a positive regulator. In opposition, under specific physiological conditions, these enzymes might inhibit cell elongation by increasing lignin synthesis, consuming apoplastic H_2O_2 through their peroxidative cycle (PASSARDI et al., 2004). This late GPOD function commonly occurs under growth restrictive conditions such as roots exposed to salt stress (MAIA et al., 2013). The strong decrease in root growth induced by high NH_4^+ was associated with maintaining of cellular integrity and a slight decrease in lipid peroxidation, suggesting that growth restriction and GPOD activity could have contributed to avoid oxidative stress, as previously observed in cowpea roots exposed to high salinity (MAIA et al., 2013).

Despite the mechanisms involved in root growth restriction in presence of excess ammonium are poorly understood to date, some authors in the past have suggested some explanations. It has been reported previously that excess ammonium might induce a strong

depletion in cytosolic sugars and organic acids from the Krebs cycle, limiting the availability of intermediaries for synthesis of proteins and nucleic acids (ESTEBAN et al., 2016). Interestingly, in spite of 50% a reduction in root growth, rice supplied with high NH_4^+ displayed a similar shoot growth compared to NO_3^--supplied plants, evidencing an effective root-shoot exclusion process in order to restrict toxic ammonium accumulation in green leaves. This ammonium mechanism already has been reported for other plant species, including rice (VON WIRÉN, 2004; TABUCHI et al., 2007; BITTSÁNSZKY et al., 2015; LOQUÉ), involving an effective restriction in root xylem influx associated with an efficient assimilatory system represented by GS/GOGAT cycle in both roots and leaves (BRITTO; KRONZUCKER, 2002).

In this study, the NH_4^+-induced leaf exclusion mechanism allowed the NH_4^+-treated plants to reach very low ammonium content in green leaves, contributing to perform high rates of photosynthesis and shoot growth from 10 to 43 days of exposure, reaching similar levels compared to NO_3^--supplied plants. Thus, the strong root growth restriction induced by excess ammonium in nutrient solution was not limiting for shoot performance of rice plants, as observed by absence of significant differences in photosynthetic CO_2 assimilation. These finds suggest that this exclusion mechanism could be part of a complex systemic avoidance strategy. This remarkable strategy developed by some plant species might have been selected by millions of years of exposure to amply varied, and not limiting, N-source supplying environments (BRITTO; KRONZUCKER, 2013). It is important to highlight here that the Nipponbare rice cultivar utilized in this study displays a good and similar growth and photosynthesis in presence of different NH_4^+:NO_3^- ratios (data not shown). These observations strongly suggest that this rice cultivar is facultative to cope with high supplying of both NH_4^+ and NO_3^-.

Remarkably, NH_4^+-supplied plants displayed strong alterations in their biomass partitioning among roots, senescent leaves, culm + tillers and green leaves, compared to NO_3^- supplying. Indeed, a great fraction of the older leaves (corresponding 60% of total leaves) triggered leaf senescence and progressively died whereas in NO_3^--supplied plants, this phenomenon occurred in a minor extent. It is not clear if this NH_4^+-triggered senescence in leaves is part of a toxicity process or if it is part of a regulated excess ammonium-exclusion mechanism, protecting photosynthesis in the younger leaves. However, it is important to note that despite the green leaves have exhibited similar CO_2 assimilation rates, compared to NO_3^--treated plants, the total number of green leaves in nitrate-grown plants were higher and, as a consequence, the whole photosynthetic capacity (photosynthesis/plant) should be much higher

in NO_3^--treated plants than NH_4^+-supplied plants. Probably, this negative cumulative effect on photosynthesis would occur in the rice yield under aggravated conditions of very high NH_4^+-supplying during, especially for long term.

Recently, rice plants supplied with high ammonium exhibited deep changes in gene expression in both roots and leaves, suggesting that NH_4^+ could have acted as a signaling molecule (PATTERSON et al., 2010; LIU; VON WIRÉN, 2017). Among the several genes that displayed altered expression induced by ammonium exposure, some of them are involved in root ammonium transporters and hormonal regulation, especially related to ethylene and ABA (CHEN et al., 1997; SONODA et al., 2003; PATTERSON et al., 2010; LIU; VON WIRÉN, 2017). Several other reports have evidenced that NH_4^+ ion is a powerful signaling for senescence (CHEN et al., 1997; ROLNY et al., 2016; WU et al., 2016), corroborating the obtained results in this current study, in which excess NH_4^+ is able to induce leaf senescence. Interestingly, NH_4^+ was more accumulated in senescent leaves (by 75%), reaching very high concentrations and, consequently, decreased amounts in all other plant organs. Unexpectedly, senescent leaves also reached a very high total-N content, suggesting that under high NH_4^+-supplying conditions, the dynamics of N-compounds throughout leaf senescence is altered as compared to normal senescence, for example, during the reproductive phase when proteins are hydrolysed and N-soluble compounds are remobilized to sink tissues (MASCLAUX et al., 2000).

A central question that should be raised in this study is: Is the massive amount of ammonium accumulated in older (senescent) leaves a basic consequence of leaf senescence process or part of a NH_4^+ exclusion mechanism? In addition, what is the source of that accumulated ammonium: protein hydrolysis or direct transport from root and/or shoot? In case of an exclusion mechanism, like the salt elimination by some halophytic species, we could be in front to a new physiological feature related to excess ammonium avoidance, associated with tolerance in rice plants. However, further studies are needed in order to answer suitably these important questions. Assays employing the $^{15}NH_4^+$ as a tracer over a long-term exposure, until the end of rice reproductive phase, are essential.

In conclusion, our data evidence that a rice facultative cultivar for nutrition with both nitrate and ammonium, display contrasting morpho-physiological and N-partitioning responses to high supplying of nitrate and ammonia. Despite these two N-source have induced similar shoot growth (leaves and culms) and photosynthesis, NH_4^+ causes strong restriction in

root mass. This decrease is closely associated with increased activity of type III peroxidases (GPOD) but not with oxidative stress. High ammonium supplying induces progressive senescence in older leaves, in parallel to great accumulation of NH_4^+ and free amino acids. The N-partitioning among older leaves, culms and green leaves could be part of ammonium exclusion mechanism for preservation of the photosynthetic apparatus in green leaves.

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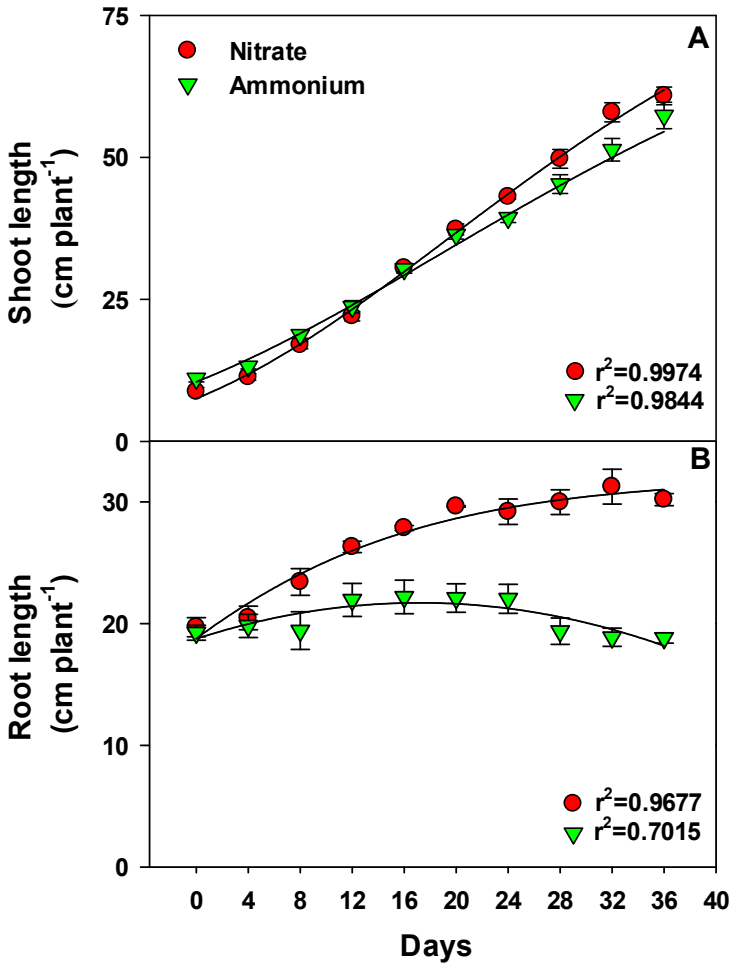

Figure 4 - Changes in absolute (A) shoot and (B) root length of rice plants grown with 15 mM of NH_4^+ (green symbols) and NO_3^- (red symbols) for 36 days in nutrient solution. Seedlings were transferred for treatments 11 days after sowing. The values represent averages from four replicates (n=4) and bars indicate standard error of the mean. Curves were adjusted by Gompertz regression in (A) and by quadratic polynomial in (B).

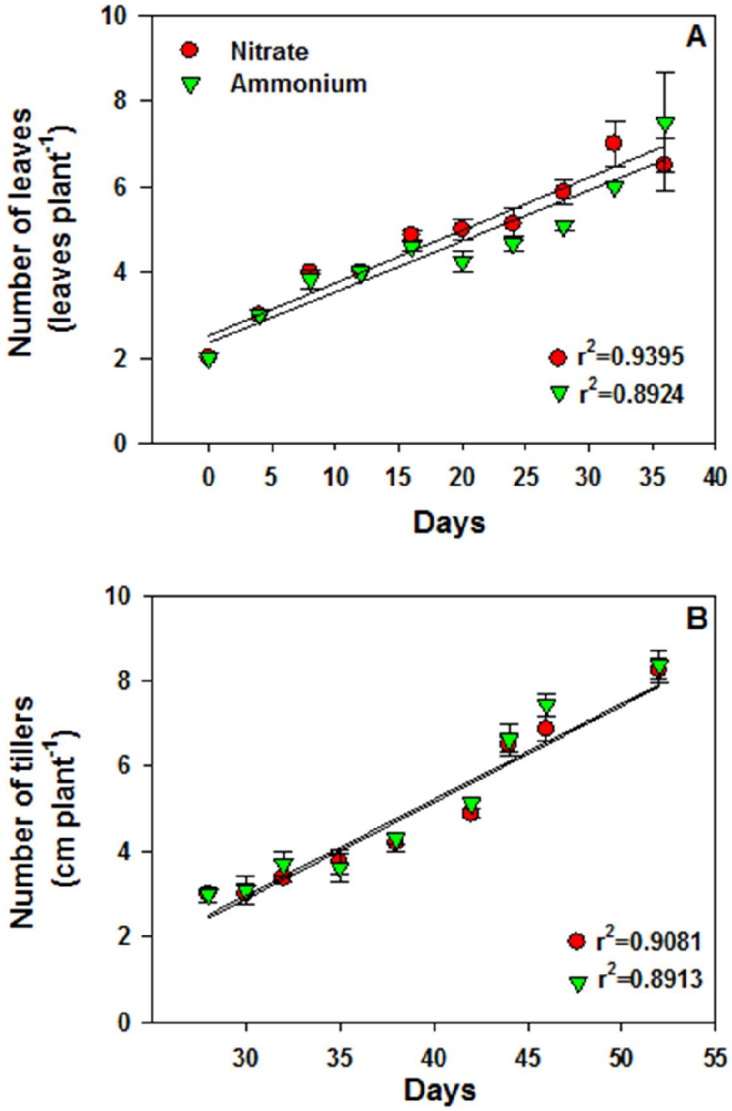

Figure 5 - Changes in (A) number of leaves after 36 days and (B) number of tillers in rice plants grown with 15 mM of NH_4^+ and NO_3^- in nutrient solution after 52 days. Seedlings were transferred for treatments after 11 days after sowing. The values represent averages from four replicates (n=4) and bars indicate standard error of the mean. Curves were adjusted by linear correlation.

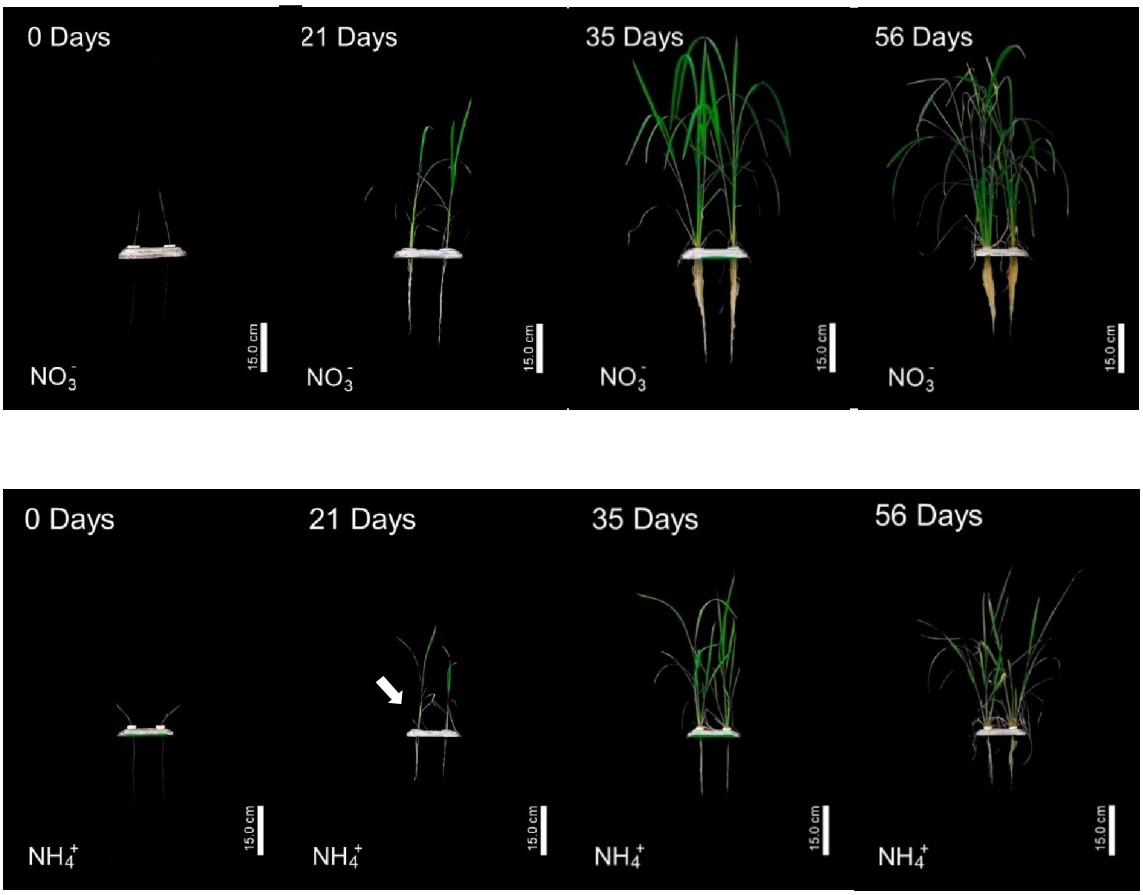

Figure 6 - Morphological changes in roots and shoot of rice plants grown with 15 mM of NH_4^+ and NO_3^- for 56 days in nutrient solution. Seedlings were transferred for treatments after 11 days after sowing (zero time). It is highlighted that NH_4^+-treated roots displayed much lower growth compared to NO_3^--plants. Arrows indicate appearance of senescent leaves, which were strongly aggravated in ammonium-treated plants. Pictures are representative from four independent images.

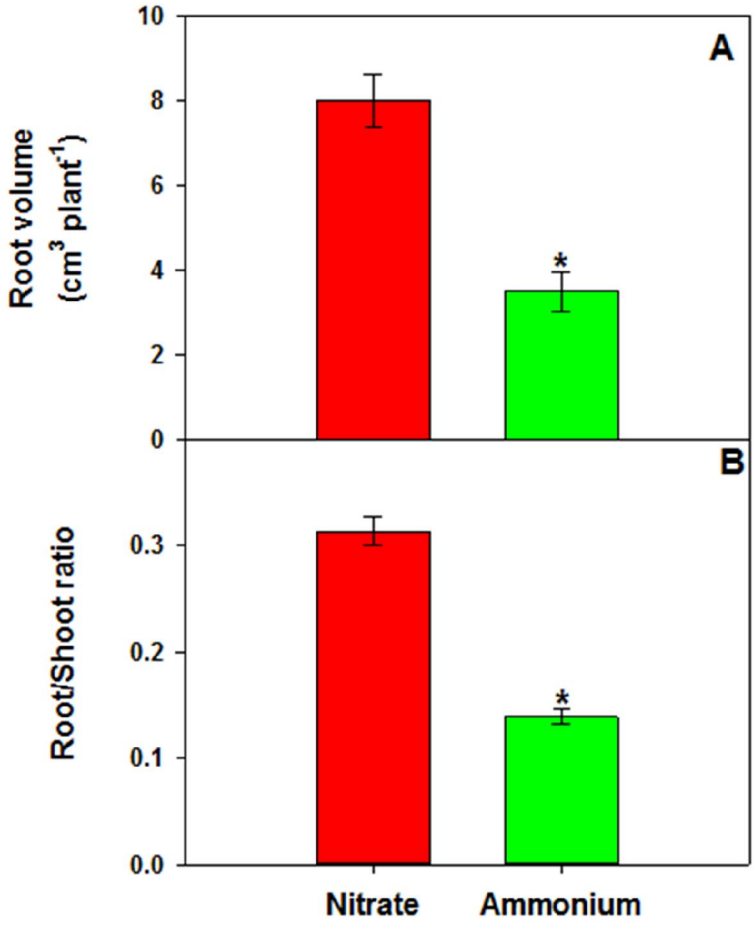

Figure 7 - (A) Root volume and (B) shoot/root ratios in rice plants grown with 15 mM of NH_4^+ or NO_3^- in nutrient solution for 43 days. Seedlings were transferred for treatments after 11 days after sowing. The values represent averages from four replicates (n=4) and bars indicate standard error of the mean. Asterisks (*) represent significant differences at 5% between treatments according to the Tukey test.

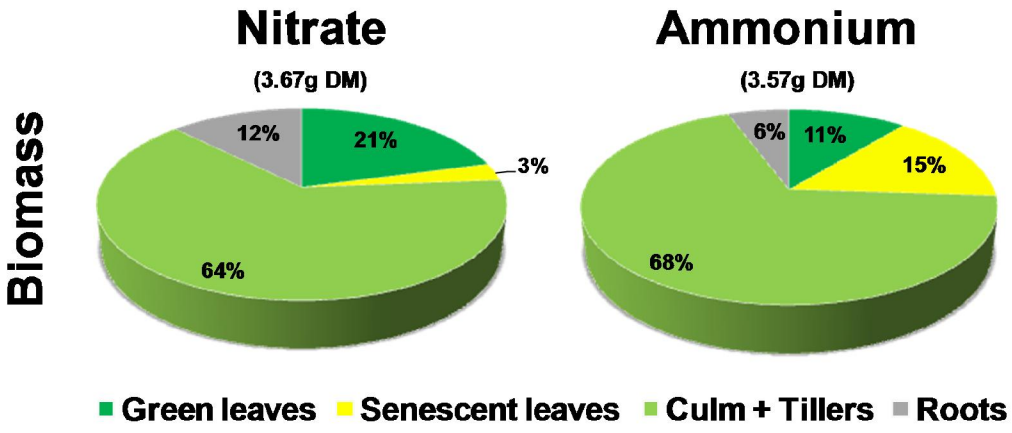

Figure 8 - Dry mass partitioning among green leaves, senescent leaves, culm + tillers and roots of whole rice plants grown with 15 mM of NH_4^+ or NO_3^- in nutrient solution for 43 days. Seedlings were transferred for treatments after 11 days after sowing. The values represent averages from four replicates (n=4).

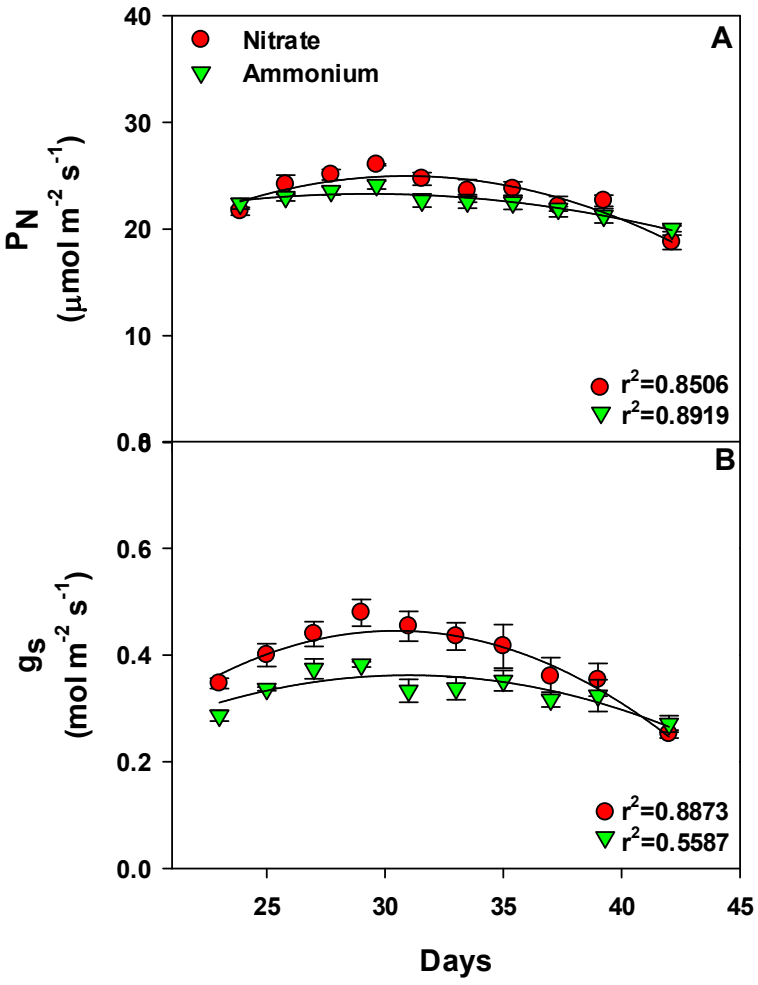

Figure 9 - Changes in (A) CO_2 photosynthetic assimilation and (B) stomatal conductance in rice plants grown with 15 mM of NH_4^+ and NO_3^- in nutrient solution after 43 days. Seedlings were transferred for treatments after 11 days after sowing. The values represent averages from four replicates (n=4) and bars indicate standard error of the mean. Curves were adjusted by quadratic polynomial.

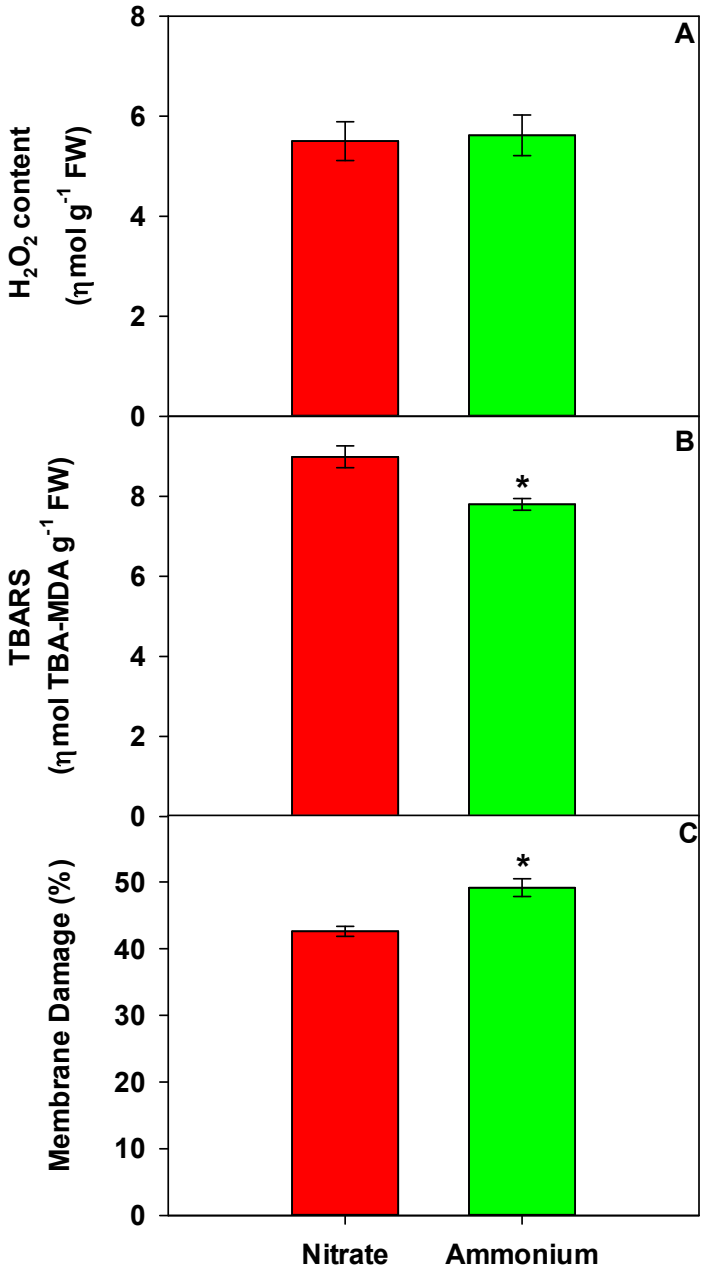

Figure 10 - Contents of (A) hydrogen peroxide, (B) TBARS and (C) membrane damage in rice roots grown with 15 mM of NH_4^+ or NO_3^- in nutrient solution for 43 days. Seedlings were transferred for treatments after 11 days after sowing. The values represent averages from four replicates (n=4) and bars indicate standard error of the mean. Asterisks (*) represent significant differences at 5% between treatments according to the Tukey test.

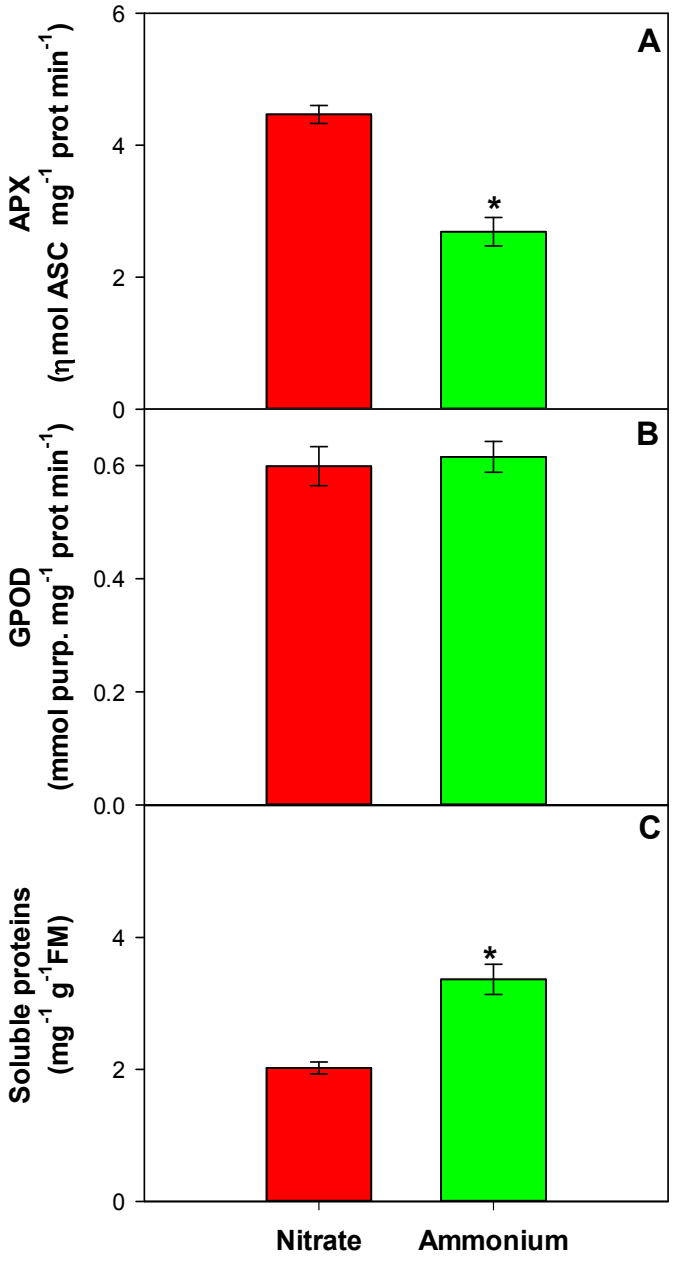

Figure 11 - Activities of (A) ascorbate peroxidases, (B) guaiacol peroxidases and (C) soluble proteins in rice roots grown with 15 mM of NH_4^+ or NO_3^- in nutrient solution for 43 days. Seedlings were transferred for treatments after 11 days after sowing. The values represent averages from four replicates (n=4) and bars indicate standard error of the mean. Asterisks (*) represent significant differences at 5% between treatments according to the Tukey test.

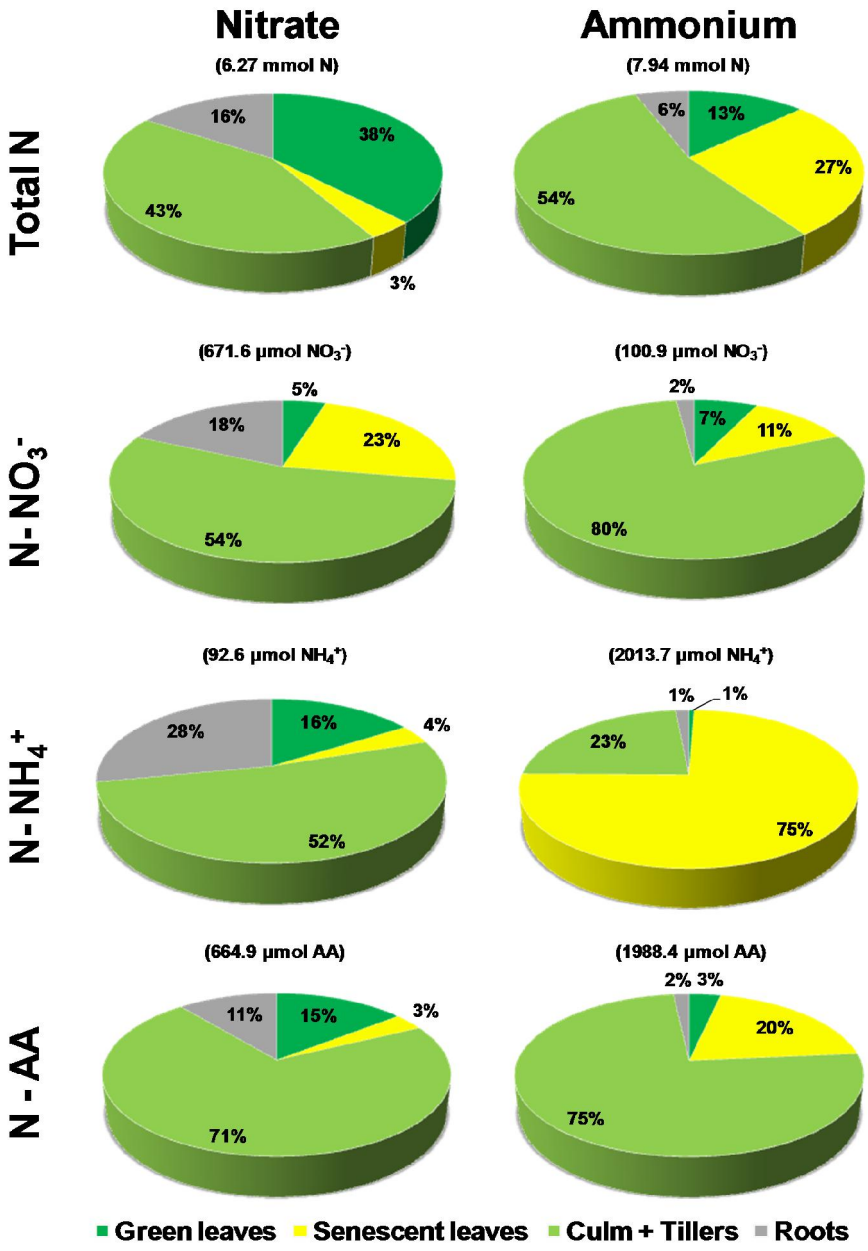

Figure 12 - Partitioning of N-forms (NH_4^+, NO_3^-, free amino acids and total-N) among roots, senescent leaves, culm+tillers and green leaves in rice plants grown with 15 mM of NH_4^+ or NO_3^- in nutrient solution for 43 days. Seedlings were transferred for treatments after 11 days after sowing. The values represent averages from four replicates (n=4). Values in parentheses represent the absolute quantification of each N form per plant. Percentages indicate the absolute fraction of each N form per plant part.

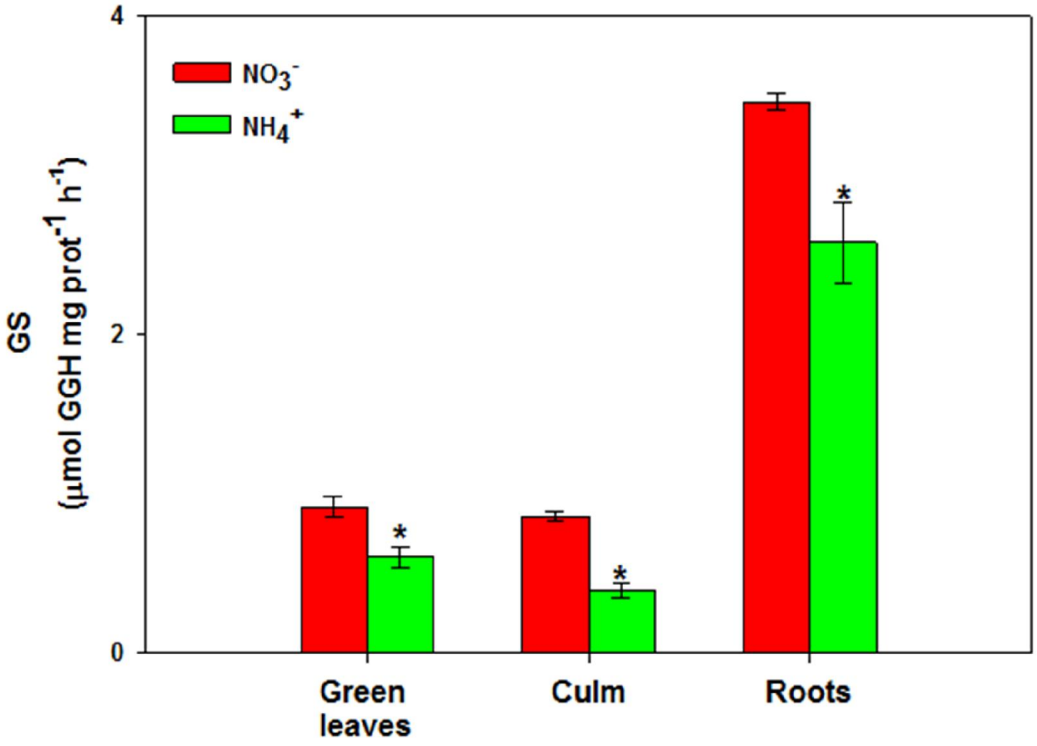

Figure 13 - Changes in gluthamine synthetase activity in green leaves, culm+tillers and roots of rice plants grown with 15 mM of NH_4^+ and NO_3^- in nutrient solution for 43 days. Seedlings were transferred for treatments after 11 days after sowing. The values represent averages from four replicates (n=4) and bars indicate standard error of the mean. Asterisks (*) represent significant differences at 5% between treatments according to the Tukey test

4 CONCLUSÃO

A senescência foliar é um fenômeno muito complexo, perfazendo uma estratégia de desenvolvimento evolutivamente adquirida para se adaptar a fatores internos e externos. Portanto, o acúmulo de amônio em folhas senescentes e colmos representa um mecanismo de exclusão contra a toxicidade em plantas de arroz. Futuros estudos são necessários para elucidar o mecanismo de senescência induzida pelo NH_4^+ e sua relação com a tolerância.

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APÊNDICE A - THE TOTAL N CONCENTRATION IN PLANT PARTS

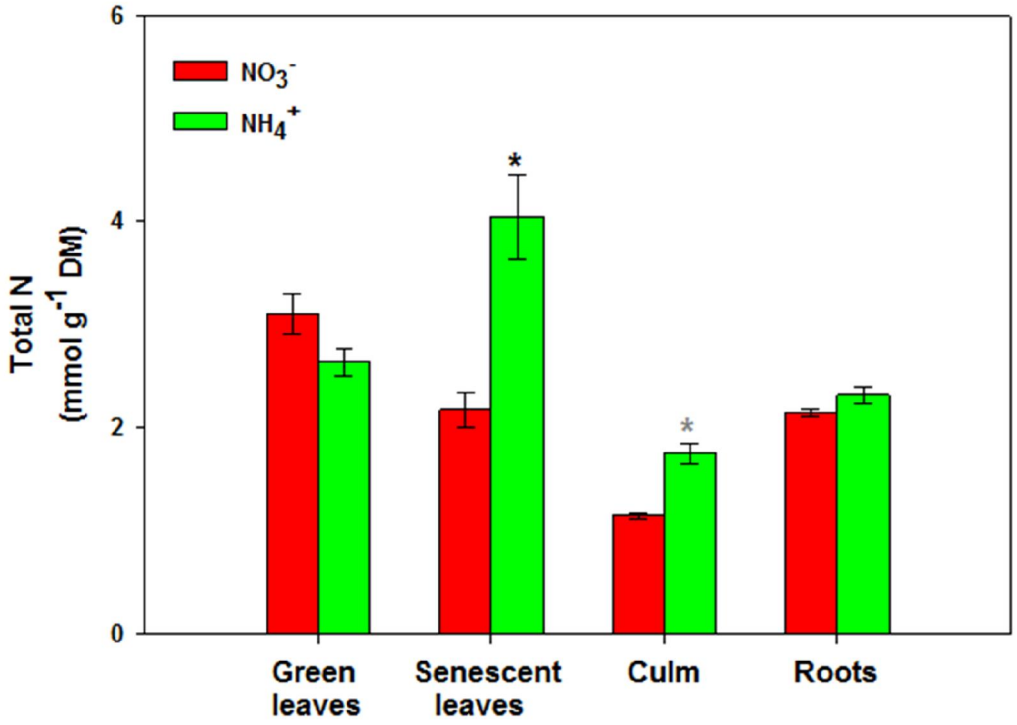

Figure 14 - The total N concentration in plant parts exposed to 15 mM NH_4^+ or NO_3^- as N source. Measurements were made 43 days after transplant under greenhouse conditions. Each measurement is represented by the average of four replicates (±SE). Averages followed by different asterisks indicate significant differences between treatments.

Asterisks (*) indicate Kruskal-Wallis One Way Analysis of Variance on Ranks, followed by Tukey test $P \leq 0.05$.

Asterisks (*) indicate One way ANOVA, followed by Tukey test $P \leq 0.05$.

APÊNDICE B - THE NITRATE CONCENTRATION IN PLANT PARTS

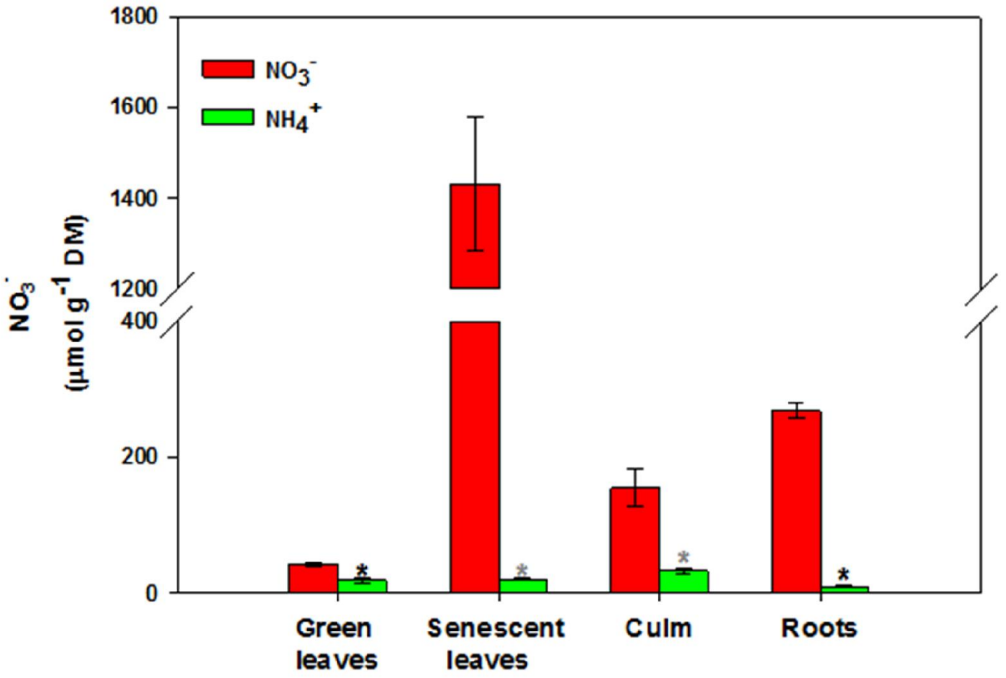

Figure 15 - The nitrate concentration in plant parts exposed to 15 mM NH_4^+ or NO_3^- as N source. Measurements were made 43 days after transplant under greenhouse conditions. Each measurement is represented by the average of four replicates (±SE). Averages followed by different asterisks significant differences between treatments.

Asterisks (*) indicate Kruskal-Wallis One Way Analysis of Variance on Ranks, followed by Tukey test $P \leq 0.05$. Asterisks (*) indicate One way ANOVA, followed by Tukey test $P \leq 0.05$.

APÊNDICE C - THE AMMONIUM CONCENTRATION IN PLANT PARTS

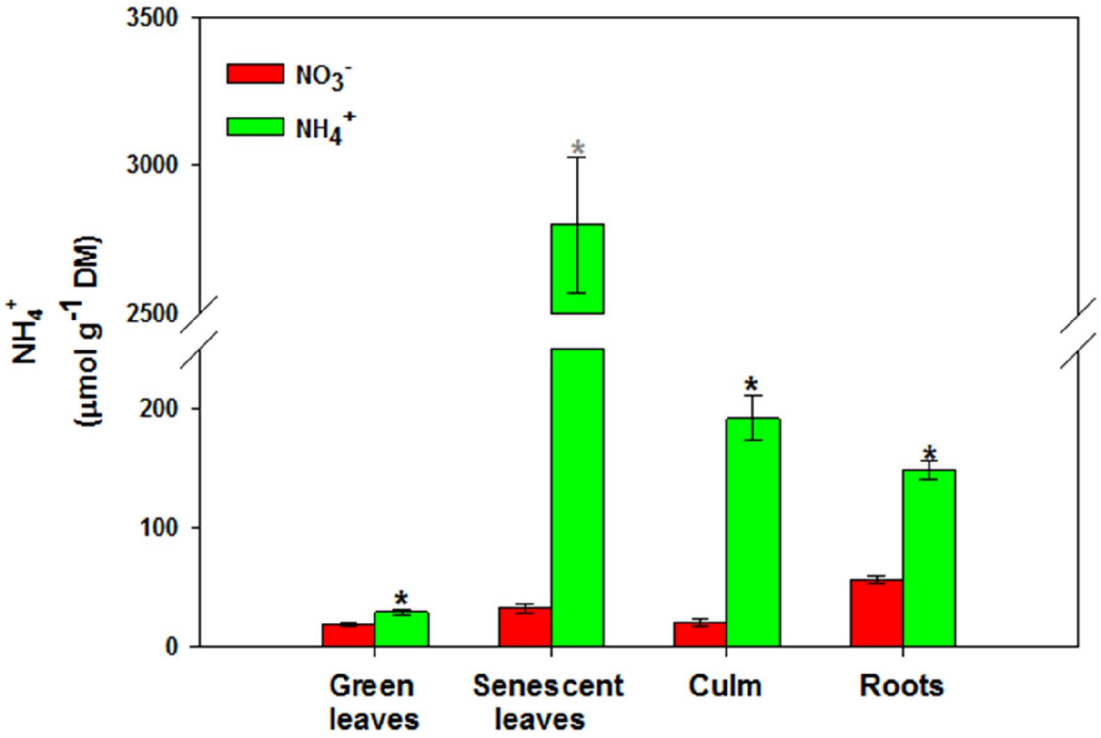

Figure 16 - The ammonium concentration in plant parts exposed to 15 mM NH_4^+ by 43 days under greenhouse conditions. Plants (0 mM NH_4^+) were treated with 15 mM of NO_3^- as N source. Date represent mean values (n=4) ± standard error. Averages followed by asterisks indicate significant differences between treatments.

Asterisks (*) indicate Kruskal-Wallis One Way Analysis of Variance on Ranks, followed by Tukey test $P \leq 0.05$. Asterisks (*) indicate One way ANOVA, followed by Tukey test $P \leq 0.05$.

APÊNDICE D - THE FREE AMINO ACID CONCENTRATION IN PLANT PARTS

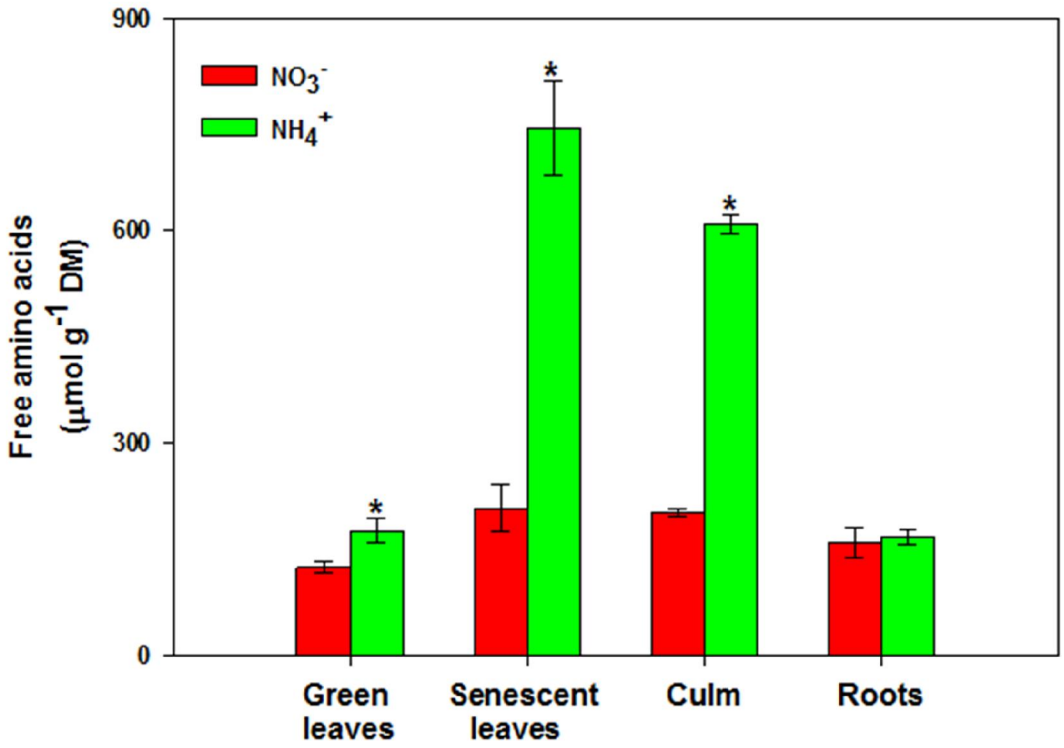

Figure 17 - The Free amino acid concentration in plant parts exposed to 15 mM NH_4^+ by 43 days under greenhouse conditions. Plants (0 mM NH_4^+) were exposed to 15 mM NO_3^- as N source. Each measurement is represented by the average of four replicates (±SE). Averages followed by different asterisks indicate significant differences between treatments. Asterisks (*) indicate One way. ANOVA, followed by Tukey test $P \leq 0.05$.

APÊNDICE E - PARTITIONING OF N-FORMS (NH_4^+, NO_3^-, FREE AMINO ACIDS AND OTHER N FORMS)

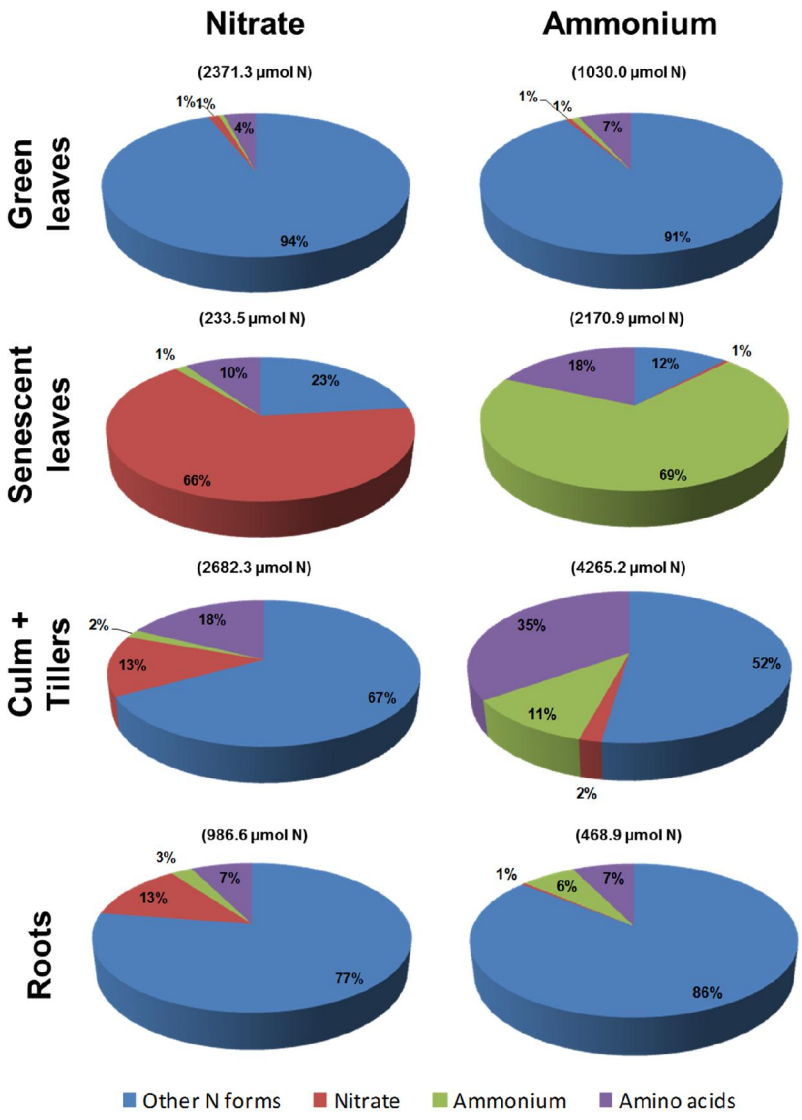

Figure 18 - Partitioning of N-forms (NH_4^+, NO_3^-, free amino acids and other N forms) among roots, senescent leaves, culm+tillers and green leaves in rice plants grown with 15 mM of NH_4^+ or NO_3^- in nutrient solution for 43 days. Seedlings were transferred for treatments after 11 days after sowing. The values represent averages from four replicates (n=4). Values in parentheses represent the absolute quantification of each N form per plant. Percentages indicate the absolute fraction of each N form per plant part.

APÊNDICE F - RADICULAR ANATOMY OF RICE

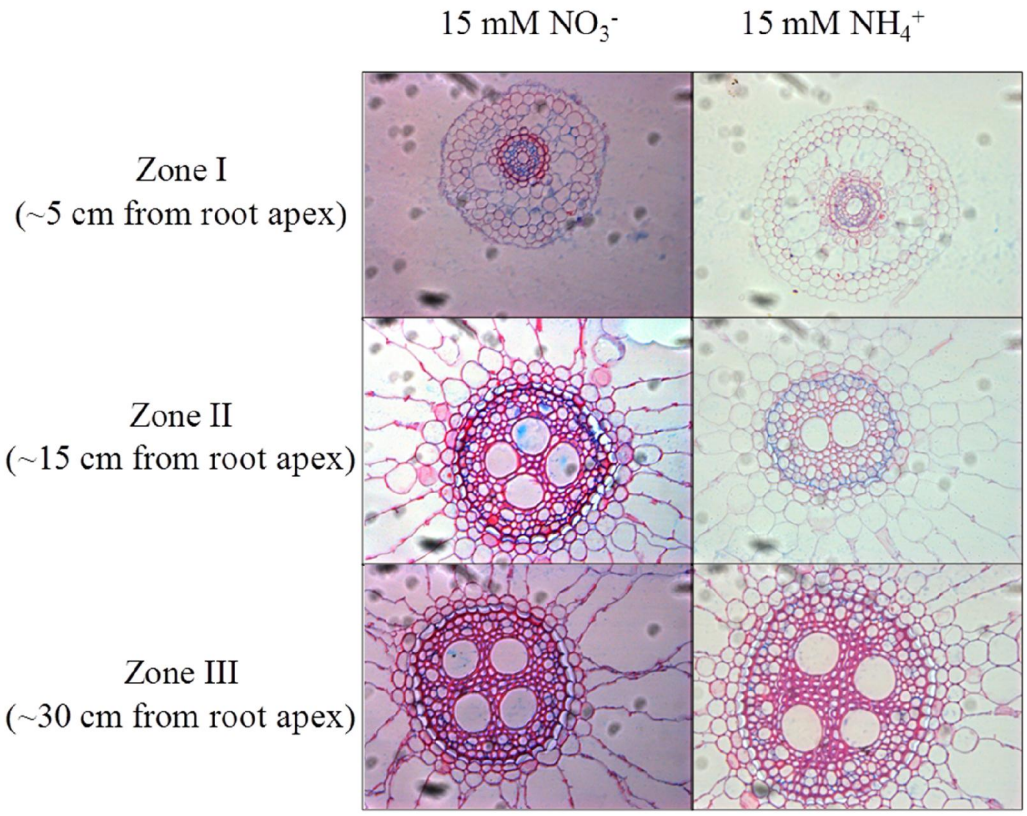

Figure 19 - Radicular anatomy of rice cultivated by 43 with 15 mM NH_4^+ or 15 mM NO_3^-.

APÊNDICE G - DIAMETER OF THE VESSEL ELEMENT AND THE CENTRAL CYLINDER OF THE RADICULAR SYSTEM

	Vessel element Diameter (µm)		Central cylinder Diameter (µm)	
	NO_3^-	NH_4^+	NO_3^-	NH_4^+
Zone I (~5 cm from root apex)	17.412	22.914	50.142	61.282
Zone II (~15 cm from root apex)	31.618	32.842	130.350	107.480
Zone III (~30 cm from root apex)	36.860	34.571	139.224	135.710

Table 1 - Diameter of the vessel element and the central cylinder of the radicular system of rice cultivated by 43 with 15 mM NH_4^+ or 15 mM NO_3^-.