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VICENTE THIAGO CANDIDO BARROS ALENCAR

TOLERÂNCIA AO EXCESSO DE AMÔNIO E FOTOSSÍNTESE EM PLANTAS DE ARROZ

FORTALEZA
2017

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Dissertação apresentada ao Curso de Mestrado em Ciência do Solo do Departamento de Ciências do Solo da Universidade Federal do Ceará como requisito parcial para a obtenção do título de mestre em Ciência do Solo. Área de concentração: Nutrição mineral de plantas.

Orientador: Prof. Dr. Joaquim Albenísio Gomes da Silveira.
Coorientadora: Dra. Ana Karla M Lobo

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BANCA EXAMINADORA

_____

Prof. Dr. Joaquim Albenísio Gomes da Silveira (Orientador)
Universidade Federal do Ceará (UFC)

_____

Prof. Dr. Márcio Cleber de Medeiros Corrêa
Universidade Federal do Ceará (UFC)

_____

Dra. Ana Karla Moreira Lobo
Universidade Federal do Ceará (UFC)

_____

Dr. Fabricio Eulálio Leite Carvalho
Universidade Federal do Ceará (UFC)

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aos meus avós Geraldo e Marilaque, meus pais Fatima e Alencar, meus irmãos Flavio e Flavia e meus sobrinhos Leobides e Iara.

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RESUMO

Plantas superiores utilizam nitrato (NO_3^-) ou amônio (NH_4^+) como principal fonte de N. Mesmo assim, a acumulação de NH_4^+ na célula apresenta efeitos potenciais prejudiciais ao metabolismo. Tais como, desacoplador do gradiente de prótons, diminuição da fotossíntese, crescimento atrofiado, decréscimo de produtividade e ocasionalmente levando a morte das plantas. Portanto, plantas que utilizam preferencialmente NH_4^+, como planta de arroz (*Oryza sativa ssp japonica* cv. Nipponbare) tiveram que desenvolver vários mecanismos para lidar com exposição às altas concentrações externas de amônio. Entretanto, mecanismos de toxidade, bem como, estratégias de tolerância do arroz ao NH_4^+ não são totalmente conhecidos. Visando investigar esses fenômenos plantas intactas e segmento foliar de arroz foram expostas a 10 mM de NO_3^- ou 10 mM de NH_4^+ sob 400 μmol m^{-2} s^{-1} (LM) ou 2000 μmol m^{-2} s^{-1} (LA) de luminosidade. Plantas intactas de arroz apresentaram alta acumulação de NH_4^+ radicular associado com atividade de GS/GOGAT inalterados e baixa translocação de amônio via xilema. Esta estratégia de restrição do transporte amônio provavelmente permitiu a proteção do aparatos fotossintéticos, assimilação de CO_2 e eficiência quântica PSII e PSI (ΦPSII, ΦPSI). Apesar da atividade semelhante de ΦPSII, plantas intactas expostas a NH_4^+ exibiram um pequeno retardo na cinética de relaxamento do PSII quando adaptado ao escuro, indicando a existência de evidências precoces para a toxicidade de amônio nas folhas de arroz. Em oposição, segmentos de folhas expostas a 10 mM NH_4^+ exibiu um acúmulo muito alto de amônio, especialmente em AL associado com a alta indução da atividade de GS_1. Esses segmentos exibiram também, um grande retardamento na PSII na recuperação a adaptação ao escuro, sendo agravado por AL, e baixa máxima eficiência quântica máxima do PSII (Fv/Fm) que são evidências de fotoinibição. A maior limitação do PSI do lado aceptor (ΦNA), sobe tais condições, corrobora a existência de indução dos efeitos negativos na recuperação do PSII. Assim, propomos que o mecanismo de toxidez de NH_4^+ nas folhas envolve a restrição no reparo do PSII no escuro e a resistência em plantas de arroz em termo de manutenção da fotossíntese, é dependente dos níveis de amônia que alcançam o aparato fotossintético.

Palavras chave: Alta luz. toxidez de NH_4^+. metabolismo de nitrogênio. *Oryza sativa.*

ABSTRACT

Land plants can utilize nitrate (NO_3^-) or ammonium (NH_4^+) as preferably N source. Nevertheless, cellular NH_4^+ accumulation presents potential harmful effects for metabolism, such as proton gradient uncoupling, photosynthesis constraining, growth stunting, decreased productivity and occasionally leading to plant death. Therefore, plants that are preferably NH_4^+ users, such as rice (*Oryza sativa japonica* cv. Nipponbare) had to evolve several mechanisms to cope with high ammonium concentrations exposure. However, toxicity mechanisms as well as rice tolerance strategies to NH_4^+ are not completely understood. Aiming to investigate this phenomenon, intact rice plants and leaf segments were exposed 10 mM NO_3^- or 10 mM NH_4^+ in presence of 400 μmol m^{-2} s^{-1} (ML) and 2000 μmol m^{-2} s^{-1} (HL). Intact rice plants exhibited high NH_4^+ accumulation in roots associated with unchanged GS-GOGAT activities and low ammonium export *via* xylem. This avoidance strategy probably allowed photosynthetic apparatus protection and absence of differences in CO_2 assimilation and PSII and PSI quantum efficiency (ΦPSII, ΦPSI). Despite similar ΦPSII activity, intact plants exposed to NH_4^+ exhibited slight delay in PSII dark-recovery, which indicates the existence of early evidences for ammonium toxicity in rice leaves. Oppositely, leaf segments exposed to 10 mM NH_4^+ exhibited very high ammonium accumulation, especially at HL, associated with great GS1 activity induction. These segments exhibited also a greater delay in PSII dark-recovery, which was aggravated by HL, and lower maximum quantum efficiency of PSII (Fv/Fm), which are evidences of photoinhibition. The greater PSI limitation at donor side (ΦNA), under such conditions, corroborates the existence of ammonium-induced negative effects on PSII recovery. Thus, we propose that NH_4^+ toxicity mechanism in leaves involves constraint in the PSII repair in the dark and resistance in rice plants, in terms of photosynthesis maintenance, is dependent on the ammonium levels that reach photosynthetic apparatus.

Key-words: High light. NH_4^+ toxicity. N metabolism. *Oryza sativa*.

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LISTA DE ABREVIATURAS

$(NH_4)_2CO_3$	Carbonato de amônio
$(NH_4)_2SO_4$	Sulfato de amônio
ABA	Ácido abscísico
ADP	Adenosina difosfato
AMT	Transportador de amônio
AQP	Aquaporinas
ATP	Adenosina trifosfato
C	Carbono
Ca^{2+}	Cátion cálcio
Ci	Pressão parcial interna de CO_2
$CO(NH_2)_2$	Ureia
CO_2	Dióxido de carbono
CTE	Cadeia transportadora de elétrons
CV	Cultivar
E	Taxa de transpiração
EPR	Ressonância paramagnética eletrônica
EROS	Espécie reativa de oxigênio
Fd	Ferredoxina
Fv/Fm	Eficiência quântica máxima do fotossistema II
Fv′/Fm′	Eficiência quântica máxima do fotossistema II folhas aclimatadas a luz
GDH	Glutamato dehidrogenase
GLN	Glutamina
GLU	Glutamato
GOGAT	Glutamato sintase
GS	Glutamina sintetase
g_S	Condutância estomática
H^+	Cátion de hidrogênio
H^+ - ATPase	Bomba de prótons
H_2O	Água
K^+	Cátion potássio
K_m	Constante de Michaelis – Menten
LA	Luz alta
LM	Luz moderada
Mg^{2+}	Cátion magnésio

mM	Milimolar
N	Nitrogênio
N_2	Nitrogênio gasoso
NAD	Dinucleótido de nicotinamida e adenina
NH_3	Amônia
NH_4^+	Amônio
NH_4Cl	Cloreto de amônio
$NH_4H_2PO_4$	Fosfato de amônio
NH_4NO_3	Nitrato de amônio
NO_2^-	Nitrito
NO_3^-	Nitrato
NPQ	Quenching não fotoquímico
OEC	Complexo evolução de oxigênio
OH^-	Hidroxila
pH	Potencial de hidrogênio
Pi	Fosforo inorgânico
pKa	Potencial de constantes de ionização
P_N	Assimilação liquida de CO_2
PSI	Fotossistema I
PSII	Fotossistema II
qE	Componente térmico do NPQ
RCII	Centro de reação do PSII
RN	Redutase do nitrato
RNi	Redutase do nitrito
Rubisco	Ribulose -1,5 – bisfosfato carboxilase/oxigenasse
ΔpH	Variação de H^+
ΦNA	Limitação do lado aceptor
ΦND	Limitação do lado doador
ΦPSI	Eficiência quântica real do fotossistema I
ΦPSII	Eficiência quântica real do fotossistema II

SUMÁRIO

1 INTRODUÇÃO

O nitrogênio (N) é o elemento mineral mais requerido pelas plantas, pois é componente primário de nucleotídeos e proteínas, sendo, portanto, o principal fator limitante para seu crescimento (LEA; AZEVEDO, 2006). Por esta razão, dar-se-á necessidade de aplicações intensivas de fertilizantes nitrogenados no solo, uma vez que a disponibilidade de N no solo irá determinar diretamente a qualidade e a taxa de produtividade das culturas (LEMAÎTRE et al., 2014). A aplicação de fertilizantes nitrogenados no solo tem sido realizada de forma frequente, os quais apresentam altas concentrações de amônio e nitrato a fim de atender as necessidades das culturas. As principais formas inorgânicas de N utilizadas nos fertilizantes são os sais nitrato de amônio (NH_4NO_3), sulfato de amônio ($(NH_4)_2SO_4$) e fosfato de amônio ($NH_4H_2PO_4$), além destes sais, a ureia ($CO(NH_2)_2$) e/ou o amoníaco anidro também podem ser aplicados na superfície do solo (BITTSÁNSZKY et al., 2015).

As plantas em geral absorvem preferencialmente os íons amônio (NH_4^+) e nitrato (NO_3^-) como fontes de N (SARASKETA et al., 2016). A proporção de cada um no solo dependerá dos fatores ambientais, das características químicas e físicas do solo (pH e concentração de matéria orgânica), das taxas de nitrificação e amonificação no solo e do seu uso (MARINO et al., 2016).

Em solos bem aerados, geralmente a concentração de nitrato é maior do que a de amônio, já em cultivos de alagados, ocorre o contrário, isso devido a permaneça de microrganismos aeróbicos facultativos e anaeróbicos, em solos alagados, que obtém energia através da oxidação da matéria orgânica e ao invés de utilizarem o oxigênio como aceptor final de elétron da cadeia respiratória , eles transferem esses elétrons dos compostos orgânicos para outros compostos oxidados, tais como, o nitrato que é transformado em gás nitrogênio (N_2) que é facilmente volatilizado e dando estabilidade a outros compostos nitrogenados como o íon amônio (MEURER, 2010). Essa modificação da disponibilidade das formas de N mineral no solo promove uma série de respostas diferentes no metabolismo dos vegetais (MARSCHNER, 1995).

Segundo Novais (2007), com o aumento na disponibilidade de N, as proteínas sintetizadas a partir dos aminoácidos promovem um crescimento do desenvolvimento

da parte aérea, aumentando a superfície fotossintética, enquanto que a deficiência proporciona menor síntese de clorofila.

A absorção do íon NH_4^+ implica em custos energéticos mais baixos do que o íon NO_3^- (MEHRER; MOHR, 1989). Após absorvido, o nitrato é reduzido a nitrito que por sua vez é reduzido a amônio, esse processo utiliza alta energia, um consumo equivalente a 12 ATP (BLOOM et al., 1992).

Independentemente de como as plantas adquirem o nitrogênio, ele somente é assimilado, ou seja, incorporado em biomoléculas, a partir do NH_4^+. Entretanto, o amônio torna-se um paradoxo, uma vez que elevadas concentrações desse íon no interior das células causa fitotoxidez (LI et al., 2014).

A susceptibilidade á toxidez por amônio tem sido descrita para *Arabidopsis thaliana* e plantas da família Brassicaceae que geralmente apresentam redução da biomassa e diminuição no crescimento da parte aérea, clorose foliar, raiz atrofiada quando expostas a altas concentrações desse íon (SARASKETA et al., 2014; LI et al., 2014).

Entretanto, outras espécies são consideradas tolerantes a altas concentrações de NH_4^+, um exemplo seria a planta de arroz (*Oryza sativa*. L) que geralmente é cultivada em ambientes inundados, os quais apresentam em predominância o amônio como fonte primaria de N (WANG et al., 1993a, b; BRITTO; KRONZUCKER, 2002; CRUZ et al., 2006; ESTEBAN et al., 2016). Então, por crescer em condições de elevadas concentrações de N-amoniacal, o arroz de alagado é considerado uma das espécies mais tolerantes ao NH_4^+ (WANG et al., 1993a, b).

Os sintomas mais comuns de toxidez por amônio em plantas incluem, redução do crescimento, modificações da arquitetura do sistema radicular, diminuição da absorção de cátions, acidificação do pH extracelular, inibição da respiração radicular, clorose foliar, diminuição da fotossíntese e estresse oxidativo (BRITTO et al., 2002; BITTSÀNSZKY et al., 2015).

Alguns autores afirmam que um dos principais efeitos tóxicos do amônio em plantas é a despolarização das membranas, a qual promove redução da síntese de ATP e de várias rotas metabólicas, incluindo a fotossíntese (BITTSÁNSZKY et al., 2015). Acredita-se que os efeitos deletérios do NH_4^+ no aparato fotossintético estejam

relacionados com a redução da atividade do complexo de evolução de oxigênio e do gradiente de prótons na membrana dos tilacoides (PEREZNAVARRO et al., 2013; OYALA et al., 2015). Entretanto, alguns estudos demostraram que o aumento da concentração de amônio não causa grandes mudanças no potencial eletroquímico citoplasmático e, portanto este efeito não seria a causa primária da inibição da fotossíntese e de outros processos metabólicos (BITTSÁNSZKY et al., 2015; ESTEBAN et al., 2016).

Vários estudos vêm sendo aplicados a fim de compreender os efeitos tanto de toxidez quanto de tolerância. Para explicar os efeitos que levam a toxidez de NH_4^+ nas plantas, algumas hipóteses foram propostas, como a redução de carbono radicular induzida pela assimilação de NH_4^+ (FINNEMANN; SCHJOERRING, 1999), deficiência de minerais (SIDDIQI et al., 2002) e distúrbio na N-glicosilação de proteínas aumentando a sensibilidade a esse íon (BARTH et al., 2010) e principalmente o ciclo fútil do NH_4^+ (BRITTO et al., 2001; KRONZUCKER et al., 2001; BRITTO; KRONZUCKER, 2002; SZCZERBA et al., 2008). Alguns estudos indicam que a tolerância a elevadas concentrações de amônio está relacionada com a maior atividade de glutamina sintetase (GS) e menor acúmulo de NH_4^+ livre nos tecidos vegetais (MAGALHÃES; HUBER, 1991; BALKOS; BRITTO; KRONZUCKER, 2010). O aumento da assimilação de NH_4^+ e do transporte desse íon para o apoplasto (efluxo) e/ou para o vacúolo também tem sido relatado como um mecanismo que algumas plantas realizam para evitar os danos causados pelo acúmulo excessivo de NH_4^+ (BRITTO et al., 2001;SZCZERBA et al., 2008). Embora o entendimento da toxidez e/ou sensibilidade ao NH_4^+ tenha melhorado nas últimas décadas, os mecanismos envolvidos neste processo ainda não foram totalmente esclarecidos (ESTEBAN et al., 2016).

2 Toxidez de amônio e fotossíntese em plantas cultivadas: uma visão integrativa

Mecanismos de toxidade do amônio e tolerância em plantas

Absorção de NH_3/NH_4^+, transporte de longa distância e redistribuição nas plantas

Na natureza existe duas formas de nitrogênio (N) amoniacal, o íon amônio (NH_4^+) ou a forma neutra amônia (NH_3). Dependo de fatores externos, principalmente do pH, pode se ter predominância de umas dessas duas formas nitrogenadas. O valor de pKa para que haja uma desprotonação do íon NH_4^+ para que ocorra a mudança para NH_3 é de 9,24. Portanto, valores de pH maiores que 9,25 encontra-se em predominância a forma nitrogenada neutra (HOWITT; UDVARDI, 2000; BITTSÁNSZKY et al., 2015). Ambas as formas de N amoniacal podem ser absorvidas pelas plantas, porém qual entre as duas formas predomina na absorção pelas plantas ainda não está totalmente esclarecido (KRONZUCKER; SIDDIQI; GLASS, 1996; YUAN et al., 2007; COSKUN et al., 2013).

A absorção líquida de NH_4^+/ NH_3 pelas raízes das plantas, assim como para qualquer outro íon, é dada pela diferença entre as taxas de influxo e efluxo (VON WIRÉN et al., 2000). Vários estudos correlacionam a absorção líquida de NH_4^+ com a cinética enzimática de Michaelis-Menten (KRONZUCKER; SIDDIQI; GLASS, 1996;YUAN et al., 2007;COSKUN et al., 2013.), portanto o influxo que ocorre em raízes intactas, depende da concentração externa, ocorre numa cinética bifásica. Para concentração externa de amônio $\leq$ 1 mM, a cinética de influxo se assemelha com de Michaelis-Menten, entretanto sob condições de concentração externa $\geq$ 50 μM, a cinética de influxo é linear (VON WIREN et al., 2000).

A absorção de amônio em raízes de plantas é realizada através de proteínas transportadoras integradas a membrana que são classificadas por famílias de Transportadores de Amônio (AMTs) (LI et al., 2016). Em geral, nas plantas os AMTs são divididos em duas principais famílias distintas, AMT1 e AMT2, e suas quantidades dependem da espécie estudada (LOQUÉ; VON WIRÉN, 2004). Ninnemann et al., 1994, foram os primeiros a isolar um transportador de amônio AtAMT1.1 de *Arabidopsis thaliana*, referida como planta modelo, e desde então vários autores vêm isolando e caracterizando transportadores de outras espécies vegetais, tais como *Lycopersicon esculentum*, *Lotus japonicus* e *Oryza sativa* (SONODA et al., 2003a;

LUDEWIG; NEUHÄUSER; DYNOWSKI, 2007; ROGATO et al., 2010; GU et al., 2013)

Em *Arabidopsis thaliana* foram codificados seis genes para AMT (LI et al., 2009). Já em arroz foram codificados dez genes, os quais foram divididos em: *OsAMT1.1*, *OsAMT1.2*, *OsAMT1.3*, *OsAMT2.1, OsAMT2.2, OsAMT2.3, OsAMT3.1, OsAMT3.2, OsAMT3.3 e OsAMT4.1*, sendo classificados em quatro subfamílias (SUENAGA et al., 2003). Os membros de AMT1 são os mais estudados por serem caracterizados como transportadores de alta afinidade (HATS). Esses transportadores ganham grande importância por serem responsáveis pela captação de nitrogênio quando a concentração externa de amônio é muito baixa, evitando assim a deficiência de N pelas plantas (VON WIREN et al., 2000). Assim, esse sistema de transportadores saturam com k_m sobre uma concentração externa submilimolar (D'APUZZO, 2004). Enquanto que os das outras famílias (OsAMT2, OsAMT3 e OsAMT4) estão associadas a baixa afinidade pelo amônio (LATS) (LOQUÉ; VON WIRÉN, 2004; SONODA et al., 2003), os quais atuam principalmente quando as concentrações de amônio são elevadas.

Estudando leveduras Peña et al. (1987) propuseram um modelo de sistema de absorção ativa secundário de amônio. De acordo com este modelo, quando há o influxo do íon amônio ocorre também o efluxo de um próton. Dessa forma, para que o amônio entre na célula da raiz, é realizado um transporte de H^+ contra o gradiente eletroquímico através da atividade da H^+-ATPase da membrana plasmática, a qual serve para compensar a despolarização do potencial de membrana causado pelo influxo do íon NH_4^+ (KRONZUCKER et al., 2001). Assim, a H^+-ATPase é estimulada levando um alto turnover de ATP no interior da célula (Figura 1).

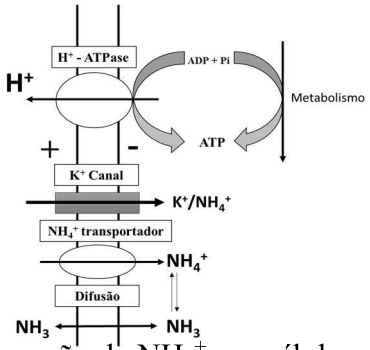

Figura 1: Mecanismo de absorção de NH_4^+ em células de raízes de plantas. K^+ canal: canal de potássio (K^+), NH_4^+ - transportador, difusão transmembranar de amônia (NH_3), H^+ - ATPase impulsiona próton (H^+) para o meio externo da raiz para que ocorra absorção de NH_4^+ pelas raízes. Adaptado de Ninnemann, Jauniaux e Frommer (1994).

Alguns estudos evidenciam que NH_4^+ pode ir para o interior das células das raízes das plantas através de múltiplos canais, tais como canais não seletivos de cátions e canais específicos de K^+ (BALKOS; BRITTO; KRONZUCKER, 2010; COSKUN et al., 2013). Por outro lado, outros trabalhos demonstram que as plantas absorvem o N amoniacal não na forma de íon NH_4^+, mas sim na forma neutra de NH_3. A amônia possui propriedades físico-químicas e estrutura molecular similar a molécula de água (H_2O) e, por isso, o seu transporte para dentro das células da raiz pode ocorrer tanto por difusão quanto através de aquaporinas (COSKUN et al., 2013;BITTSÁNSZKY et al., 2015;ESTEBAN et al., 2016) (Figura 2).

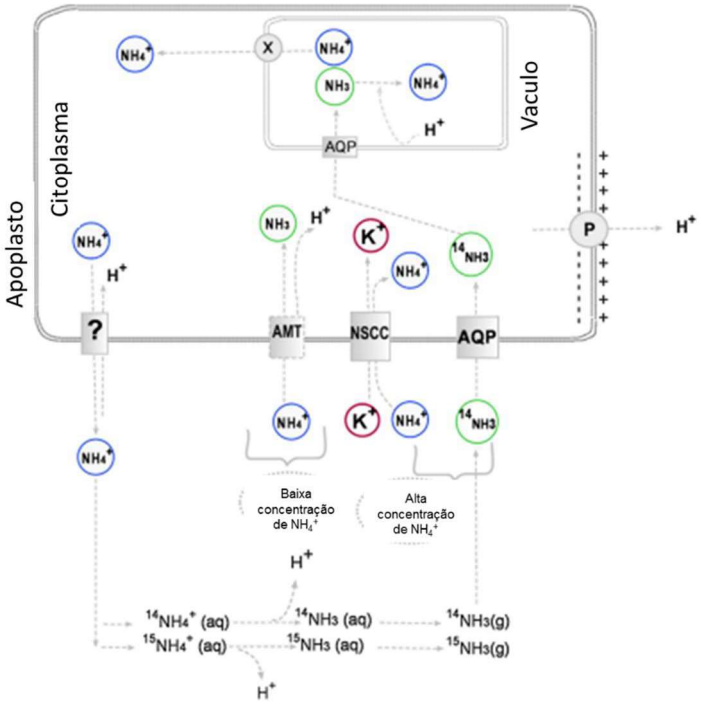

Figura 2 Absorção do amônio pela célula vegetal. Transportadores de amônio: AMT; Canal de cátions não específicos: NSCC; aquaporinas: AQP, canal de prótons: P; amônio: NH_4^+; potássio: K^+. Adaptado de Esteban et al. (2016)

Em concentração externa moderada (submilimolar) o amônio uma vez que entrar no interior das células das raízes normalmente é assimilado diretamente a aminoácidos, os quais podem ser transportados pelo xilema através de transportadores específicos para outros tecidos (TEGEDER, 2014; HACHIYA; SAKAKIBARA, 2016).

Quando a concentração interna de amônio ultrapassa a capacidade das enzimas assimiladoras (glutamina sintase – GS e glutamato sintetase – GOGAT), esse íon é transportado via xilema para outros tecidos. Existe uma hipótese de que o amônio é

translocado para a parte aérea através do apoplasto, uma vez que na seiva do xilema as concentrações de amônio no apoplasto de todos os órgãos da planta é similar em condição de alta concentração externa desse íon (SCHJOERRING et al., 2002). Apesar da abundância de trabalhos envolvendo a absorção e o transporte de amônio no interior do vegetal na literatura, os mecanismos moleculares relacionados com a presença do amônio no xilema permanecem pouco entendidos.

Mecanismos fisiológicos, bioquímicos e biofísicos da toxidade de NH_3/NH_4^+

A toxidez causada pelo NH_4^+ não é um assunto novo, esse fenômeno foi percebido por Charles Darwin em 1882 que demostrou que plantas de *Euphorbia peplus* tiveram inibição em seu crescimento depois de serem cultivadas com o sal carbonato de amônio ($(NH_4)_2CO_3$). Desde então, vários estudos vêm sendo aplicados a fim de esclarecer os danos gerados por esse íon. Como foi relatado em uma revisão crítica sobre toxidez por amônio, Britto e Kronzucker (2002) listaram plantas susceptíveis e tolerantes.

Dentre os sintomas notáveis atribuídos à fitotoxidez do amônio em plantas, estão mudanças bioquímicas e fisiológicas, tais como: alterações do pH intracelular; alcalinização intracelular e acidificação extracelular; desequilíbrio osmótico; indução de deficiências nutricionais causada pela inibição na absorção de cátions (K^+, Mg^{2+} ou Ca^{2+}); inibição na respiração da raiz e estimulação da fotorrespiração; interferência na atividade fotossintética (menor taxa de assimilação de CO_2, condutância estomática e transpiração), aumento no conteúdo de H_2O_2 causando estresse oxidativo e diminuição dos teores de clorofila *a* e *b* e carotenóides; alteração nas atividades de enzimas relacionadas com a assimilação do NH_4^+; e alto custo energético para manter baixos níveis de amônio no citosol (GERENDÁS et al., 1997; CRUZ et al., 2003; DOMÍNGUEZ-VALDIVIA et al., 2008; ARIZ et al., 2010; CRUZ et al., 2011; SHENGQI et al., 2012; BORGOGNONE et al., 2013; BITTSÁNSZKY et al., 2015).

Os principais sintomas visuais característicos da toxidez ao amônio são raiz atrofiada e clorose nas folhas (BRITTO; KRONZUCKER, 2002; LI et al., 2014). A toxidez pelo amônio torna-se típica, normalmente, quando as plantas são expostas a elevadas concentrações exógenas ou quando as células vegetais estão sob condições de

estresses ambientais, nas quais ocorrem alta produção de amônio através do aumento das atividades proteolíticas e da fotorrespiração (SKOPELITIS et al., 2006). Em um estudo com $^{13}NH_4^+$, Britto et al (2001) estudaram o influxo e efluxo de amônio em cevada (susceptível) e em arroz (tolerante) e demonstraram que na espécie susceptível a taxa de efluxo se torna maior do que a de influxo, evidenciando um ciclo fútil de influxo e efluxo, tendo em vista que essas plantas assimilam pouco essa fonte de nitrogênio. Com o aumento do efluxo, o meio externo e intracelular é modificado e, normalmente, ao absorver amônio a rizosfera se torna ácida enquanto o citosol se alcaliniza (MARSCHNER, 2012).

Já em plantas tolerantes como o arroz, foi observado que a partir de 1 mM de NH_4^+ ocorre a formação de um potencial transmembranar, permitindo um fluxo alto de amônio (BRITTO et al., 2001). Acredita-se que as concentrações de amônio nos tecidos vegetais saudáveis permanecem sempre baixas e nestas condições é sugerido que o NH_4^+ absorvido ou gerado nas raízes seja rapidamente assimilado, não sendo translocado para a parte aérea (TOBIN; YAMAYA, 2001).

Mecanismo bioquímico de assimilação e desintoxicação de NH_3/NH_4^+

Após ser absorvido, o amônio, por ser tóxico quando acumulado na célula, é rapidamente assimilado, primeiramente através do ciclo GS/GOGAT. A primeira enzima combina NH_4^+ com glutamato para formar glutamina, hidrolisando uma adenosina trifosfato (ATP) (FORDE; LEA, 2007), gerando glutamina, adenosina difosfato (ADP) e fosfato inorgânico (Pi) (TAIZ; ZEIGER, 2013). Nas plantas superiores há duas isoformas dessa enzima, sendo uma predominante nos cloroplastos (GS_2) e a outra citosólica (GS_1) (SWARBRECK et al., 2011). A GOGAT catalisa a conversão, dependente de NADH ou ferredoxina reduzida (Fd_{red}), da glutamina e 2-oxoglutarato em duas moléculas de glutamato. A NADH - GOGAT está localizada nos plastídios (raiz), enquanto que a Fd - GOGAT se encontra nos cloroplastos (folhas) (ANDREWS; RAVEN; LEA, 2013) (Figura 3).

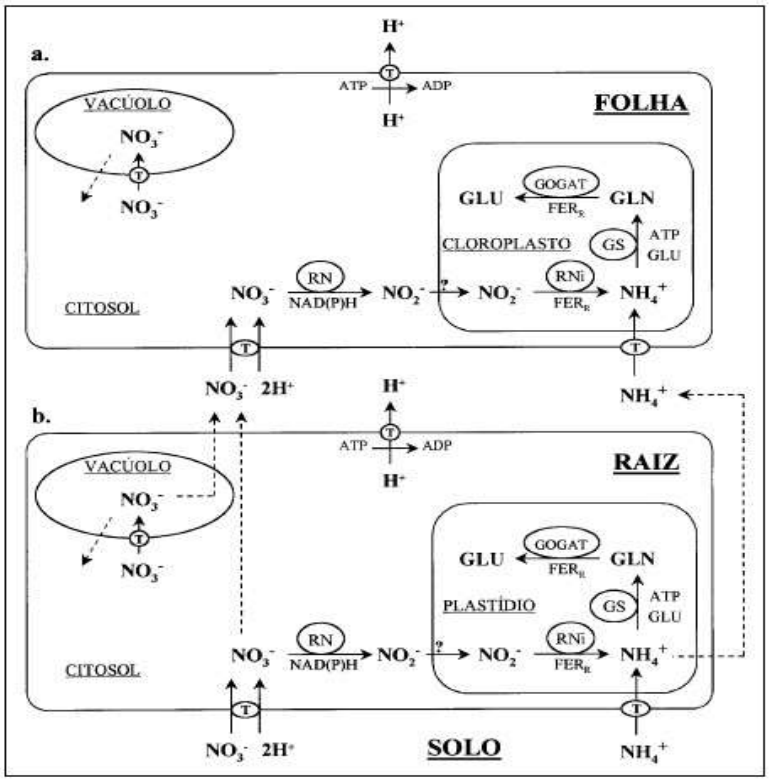

Figura 3 Representação esquemática da rota de assimilação do nitrogênio nas raízes e folhas de plantas. (NO_3^- nitrato; NO_2^- nitrito; NH_4^+ amônio; GLN: glutamina; GLU: glutamato; RN: redutase do nitrato; RNi: redutase do nitrito; GS: sintetase da glutamina; GOGAT: sintetase do glutamato; T: transportador). Fonte: Bredemeier e Mundstock (2000).

Uma via alternativa para assimilar o amônio é catalisada pela glutamato desidrogenase (GDH), a qual catalisa a reação reversível com 2-oxoglutarato para sintetizar ácido glutâmico (BITTSÁNSZKY et al., 2015). Embora os papéis fisiológicos exatos desta enzima no crescimento e no desenvolvimento da planta não estejam claros, sabe-se que ela é ativada por altas concentrações de amônio (HIREL et al., 2007; RUZICKA et al., 2010), sugerindo que essa enzima tenha um papel fundamental na desintoxicação de amônio (LASA et al., 2002). Duas formas dessa enzima são relatadas nos vegetais, uma com predominância na mitocôndria (NADH-GDH) e uma outra ocorrendo nos cloroplastos (NAD(P)H - GDH) (CAMMAERTS; JACOBS; BRUSSEL, 1983). No entanto, a GDH possui atividade reversível promovendo tanto a assimilação de amônio, quanto a decomposição do glutamato (NH_4^+ + α - cetoglutarato ↔ glutamato + H_2O) (FERRARO et al., 2015).

Outro mecanismo de assimilação de amônia pelos vegetais ocorre a partir de associação com bactérias fixadores de N atmosférico (N_2), tais como a associação

simbiótica que ocorre entre plantas leguminosas e bactérias do gênero *Rhizobium* (ANDREWS; RAVEN; LEA, 2013; ESTEBAN et al., 2016). Para que essa interação simbiótica ocorra, as bactérias induzem a formação de nódulos (bacteróides) especializados na fixação de N_2 próximo a região dos pelos radiculares (FIGUEIREDO et al., 2008). No interior dos nódulos, o nitrogênio gasoso é convertido em amônia (NH_3) pelo complexo da enzima nitrogenase, que em seguida é convertida em NH_4^+ no ambiente aquoso do citoplasma dos bacteróides (FRANCHE et al., 2009; SPRENT, 2009). Posteriormente, esse amônio é convertido a amidas e em aminoácidos através da ação de rotas de assimilação do amônio na raiz (TAIZ; ZEIGER, 2013). Essa simbiose entre raiz de leguminosas e bactéria *Rhizobium* ocorre numa troca do nitrogênio fixado por fonte de carbono (VANCE; HEICHEL, 1991).

Um mecanismo endógeno de produção de amônio também deve ser mencionado, como é o caso da fotorrespiração, que pode ser considerada uma das vias mais importantes de produção de amônio endógeno em condições de estresse (FERNIE et al.,2013). Esta reação é responsável por gerar até 20 vezes mais amônio do que o ciclo do nitrogênio, resultante da redução de nitrato (CANVIN, 1990; GUO et al., 2007). A fotorrespiração ocorre a partir de reações de oxigenação catalisada pela Rubisco (Ribulose-1,5-bifosfato carboxilase/oxigenase), na qual o subproduto 2-fosfoglicolato é regenerado em 3-fosfoglicerato com liberação de CO_2 e NH_4^+ na mitocôndria (LEEGOOD et al., 1995). Esse amônio é então liberado e lançado para os cloroplastos, onde é reassimilado pela ação do ciclo GS/GOGAT ou pela GDH. A reassimilação desse amônio gerado a partir da fotorrespiração é realizada principalmente pela GS_2 (PÉREZ-DELGADO et al., 2015).

Como foi relatado anteriormente, o amônio é tóxico para as células das plantas e para evitar estresse relacionado a esse íon, as plantas o convertem, assim como qualquer composto tóxico ou poluente, em derivados não tóxicos (aminoácidos) (BITTSÁNSZKY et al., 2015). Em geral para ocorrer uma desintoxicação o amônio é ligado ao carbono através de várias reações anapleróticas (GARC; MORAN, 2012). Nesse contexto, entra em ação as principais enzimas envolvidas na assimilação do amônio (GS/ GOGAT e GDH) que operam de maneira coordenada para diminuir as concentrações internas de amônio através da síntese do ácido glutâmico (BITTSÁNSZKY et al., 2015) (Figura 4)

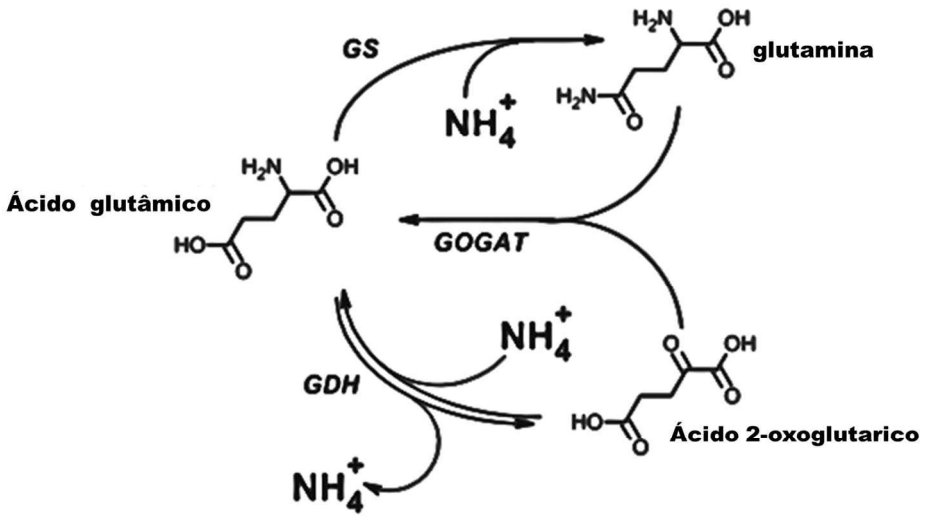

Figura 4 Esquema de desintoxicação de amônio em plantas. GS: Glutamina Sintetase; GOGAT: Glutamato Sintase; GDH: Glutamato Dehidrogenase; NH_4^+: amônio. Adaptado de Bittsánszky et al. (2015).

Mecanismos de tolerância NH_3/NH_4^+

Em geral, vários estudos mostram que plantas tolerantes suportam concentrações elevadas de amônio externo atribuídas a atividades das principais enzimas envolvidas com a assimilação primaria desse íon, principalmente de GS (CRUZ et al., 2006; SARASKETA et al., 2014: EL OMARI et al., 2010). Omori et al. (2010), realizaram experimentos com híbrido de sorgo e atribuíram a tolerância ao amônio a alta atividade da enzima GS na raiz. Em plantas de arroz e outas espécies, a alta tolerância ao amônio também foi correlacionada com uma elevada atividade de GS radicular (MAGALHÃES; HUBBER, 1989; MAGALHÃES et al. 1992; ISHIYAMA et al., 2004b; GLEVAREC et al. 2004).

Nem sempre o acúmulo de amônio em órgãos vegetais tais como raiz ou folha significa que a planta esteja sob toxidez de amônio, por outro lado essa pode ser uma estratégia de mecanismo de tolerância (SARASKETA et al., 2014a). Lasa et al. (2002) afirmam que o local de maior assimilação do amônio está diretamente relacionado com o mecanismo de susceptibilidade ou tolerância para este íon e que, geralmente, plantas tolerantes possuem alta assimilação de NH_4^+na raiz (LASA et al., 2002).

Atualmente sabe-se que vários fatores podem influenciar e conferir o estado de toxidez ou tolerância das plantas ao amônio, tais como o aumento da concentração de CO_2 e intensidade de luz (BIVER et al., 2008; ARIZ et al., 2010; ARIZ et al., 2013; VEGA-MAS et al., 2015). Em condições de alto CO_2, disponibilidade de esqueletos de C na raiz, alta concentração de K^+ e regulação do pH extracelular os efeitos tóxicos do NH_4^+ podem ser aliviados (SZCZERBA et al., 2008; BRITTO; BALKOS; BRITTO; KRONZUCKER, 2010;.KRONZUCKER,2013; ZHENG et al., 2015). Outro mecanismo adotado pelas plantas para evitar a toxidez por amônio é a compartimentalização de todo o seu excesso nos vacúolos, principalmente de raízes, tornando as principais reações do metabolismo da planta (fotossíntese e respiração) livre do excesso desse íon (LASA et al., 2002; SARASKETA et al., 2014b). Como mencionado anteriormente, o amônio pode causar uma série de distúrbios no metabolismo vegetal, entretanto os danos causados nas reações do cloroplasto e mitocôndrias parecem ser os mais relevantes (SETIÉN et al., 2013).

Toxicidade por amônio e seus efeitos negativos na fotossíntese

Distúrbios no aparato fotoquímico induzido por NH_3/NH_4^+

Dentre as formas de N mineral utilizados pelas plantas, nitrato (NO_3^-) e amônio (NH_4^+), a partir de diferentes pressões evolutivas cada espécie desenvolveu independentemente a preferência por uma fonte em particular, convencionalmente denominada β (BOUDSOCQ et al., 2012). Entretanto o amônio pode desencadear respostas deletérias em muitas espécies vegetais como mencionados acima. Logo, plantas com elevados valores de β, as quais apresentam elevada afinidade por NH_4^+ em comparação ao NO_3^- precisaram desenvolver ao curso da evolução diferentes mecanismos de proteção, os quais podem garantir seu crescimento (BRITTO; KRONZUCKER, 2002).

A fotossíntese consiste em um dos principais processos metabólicos que determinam o crescimento vegetal (KRAUSE et al., 2013). Por meio das reações fotoquímicas efetivadas nas cadeias de transporte de elétrons das membranas dos tilacóides (CTE) e das reações redutoras, referentes ao ciclo de assimilação de CO_2, do ciclo de Calvin-Benson, a planta produz todo o seu poder redutor e moléculas de alto

potencial energético (HILL; BENDALL, 1960; CALVIN, 1962). Essas rotas metabólicas são cruciais para o provimento da energia necessária para suprir a demanda metabólica inerente ao crescimento da planta. O papel do NH_4^+ como desacoplador do potencial de membranas (GARCÍA-MENDOZA; COLOMBO-PALLOTTA, 2007) tem sido relacionado como um importante efeito deletério na produção de ATP a partir da CTE, haja visto que o complexo sintetase de ATP é dependente da força próton-motriz criada a partir do transporte de elétrons na CTE (ZHU et al., 2000).

Conforme essa hipótese, a redução na síntese de ATP poderia afetar os demais processos metabólicos redutores essenciais para o crescimento da planta entretanto, esse mecanismo hipotético de toxidade causada pelo NH_4^+ tem encontrado grande oposição. Plantas submetidas a elevadas irradiâncias luminosas e alta concentração de NH_4^+, exibem sintomas visuais de toxicidade, mas não apresentam redução no componente térmico do NPQ (qE), o qual é fundamentalmente dependente de ΔpH (ZHU et al., 2000; RUBAN, 2016). Esse resultado aparentemente se contrapõe ao clássico efeito químico do NH_4^+, comumente relatado em ensaios envolvendo microalgas e cloroplastos isolados em suspensão (GARCÍA-MENDOZA; COLOMBO-PALLOTTA, 2007). De fato, plantas intactas consistem em um modelo muito mais complexo do que organismos unicelulares, ou cloroplastos em suspensão. Processos de compartimentalização (BRITTO et al., 2002), fluxo fútil de NH_4^+ através da membrana plasmática, *cross-talking* energético envolvendo cloroplastos e mitocôndrias e o controle do transporte xilemático raiz-folha, poderiam, ao menos em parte, justificar as respostas ao NH_4^+ contrastantes entre esses modelos.

Durante a década de 1980, pesquisadores americanos utilizando de técnicas de espectroscopia de ressonância paramagnética eletrônica de baixa temperatura (EPR) descobriram que membranas de tilacoides isolados de espinafre tratados com 100 mM de NH_4Cl apresentavam alterações de sinais compatíveis com a ligação de NH_3 ao estágio S2 do complexo de evolução do oxigênio (OEC) do fotossistema II (PSII), consequentemente, o NH_4^+ poderia estar associado a um efeito deletério da atividade do PSII por consequência direta da interação com o centro de reação do PSII (RCII), causando, portanto, quedas no rendimento quântico efetivo (ΦPSII) e déficits energéticos. Essa hipótese é corroborada pela maior sensibilidade ao NH_4^+ apresentada por cianobactérias deficientes na proteína ftSH2, uma proteína chave no processo de *turnover* da proteína D1 e reparo do RCII (DRATH et al., 2008).

Embora as evidências para a ação direta de fotoinibição gerada por excesso de NH_4^+ em microrganismos seja bastante plausível, o mecanismo inerente a sua toxidade em plantas intactas novamente se encontra obtuso. Isso se deve ao fato de que plantas de feijão intactas expostas a 5 mM de amônio não apresentam indícios de fotoinibição (Citação), novamente, como argumentado anteriormente, processos de evitamento poderiam estar sendo ativados de tal modo a não possibilitar a ação tóxica direta do amônio nos cloroplastos de plantas intactas. Alternativamente, processos como desacoplamento de gradiente de pH e ligação direta ao RCII poderiam não ser os principais atores do processo inerente à toxidez do NH_4^+ em plantas.

O ciclo de reparo do RCII em plantas superiores é um processo crucial e finamente regulado. Esse processo envolve o desmonte do supercomplexo do PSII danificado, fosforilação das proteínas D1 e encaminhamento para sua degradação (Citação), síntese *de novo* da proteína D1 e remontagem do aparato fotossintético esse evento, as espécies reativas de oxigênio (EROS) têm sido frequentemente relacionadas como inibidores do processo de síntese *de novo* da proteína D1, por meio de sua ação inibitória da proteína de alongamento fator G (NISHIYAMA; ALLAKHVERDIEV; MURATA, 2011).

Plantas de *Arabidopsis thaliana* cultivadas por oito semanas com NH_4^+ como sua única fonte de N apresentam forte restrição do crescimento, aumento da relação $NAD(P)H/NAD(P)^+$, acúmulo de EROS e acúmulo de proteínas oxidadas (PODGÓRSKA et al., 2013).

A utilização de plantas capazes de tolerar altas concentrações de amônio, em particular, se apresenta como modelo promissor de estudo. Apesar desse fato, estudos aprofundados acerca dos detalhes fotoquímicos e de assimilação de CO_2 em resposta ao excesso de amônio nessa espécie tolerantes, como o arroz, são praticamente inexistentes.

Perturbação nas reações do ciclo de Calvin-Benson induzida por NH_3/NH_4^+

As reações fotossintéticas e as de absorção e assimilação de N devem ser finamente orquestradas para não causar nenhum distúrbio nos vegetais, tendo em vista que o metabolismo de N é considero um dreno forte de esqueletos de C e de poder redutor (BLOOM, 2015). As consequências da utilização do amônio como única fonte de N na assimilação de CO_2 depende da concentração do íon, tempo de exposição, espécie e estágio de desenvolvimento. Claussen e Lenz (1995) verificaram que clorose foliar e redução da fotossíntese foram inexistentes quando plantas de berinjela foram expostas a 10 mM de NH_4^+ por 10 dias no estágio reprodutivo em comparação com o vegetativo. Já plantas de mirtilo apresentaram maior taxa fotossintética e produção de massa seca na presença de NH_4^+ (6 mM) por 6 semanas quando comparada com as plantas nutridas apenas com NO_3^- (CLAUSSEN; LENZ, 1999). Em oposição, plantas de morango e framboesa nestas mesmas condições apresentaram clorose, redução de fotossíntese e biomassa.

Em *Phaseolus vulgaris* sob condições de NH_4^+ (5 mM), a assimilação de CO_2 não foi alterada, contudo o ponto de compensação aparente de CO_2 (Γ^*_{app}) reduziu e a respiração mitocondrial aumentou em comparação com as plantas nutridas com NO_3^- (GUO et al., 2005). O maior Γ^*_{app} nas plantas tratadas com NO_3^- demonstra que a assimilação de CO_2 não fotorrespiratório é dependente de alta luz, enquanto que alta concentração de amônio promove aumento da taxa respiratória para suportar a assimilação deste íon em aminoácidos. Em um experimento com plantas de arroz sob condições de 3 mM de NH_4^+, Guo et al. (2007) mostraram que os parâmetros de trocas gasosas não foram modificados e que a eficiência de carboxilação de Rubisco aumentou 23% quando comparadas com as plantas nutridas com NO_3^- (3 mM).

Apesar de poucos trabalhos abordarem a influência do NH_3/NH_4^+ na fotossíntese de planta, no geral quando em excesso nas folhas, este íon modifica diretamente as reações mitocondriais e em último caso as reações fotoquímicas da fotossíntese, tendo pouca influência direta no ciclo de Calvin-Benson (GUO et al., 2007). Alguns autores afirmam ainda que a reserva de carboidratos na raiz e alta taxa respiratória suportam um aumento da assimilação de NH_4^+ em plantas supridas apenas com esta fonte de N, promovendo assim maior síntese de proteína, principalmente de

Rubisco, as quais promovem estímulo da fotossíntese e de produção de biomassa (BRÜCK; GUO, 2006; GUO et al., 2007).

A Rubisco representa o maior dreno de N foliar, uma vez que esta enzima representa cerca de 50% da proteína solúvel total do vegetal (PARRY et al., 2013). Assim, sob condições de alta concentração de NH_4^+ a síntese de aminoácidos é estimulada, a qual pode favorecer a síntese de Rubisco e de outras proteínas da fotossíntese a fim de suportar o requerimento de C na assimilação desta fonte de N (TERCÉ-LAFORGUEA et al., 2004; GUO et al., 2007). Entretanto, mais estudos são necessários para avaliar mais detalhadamente o papel da alta concentração de amônio nas reações fotossintéticas de plantas superiores.

Interação mitocondrial e cloroplástica associado a toxidade NH_3/NH_4^+

As plantas que crescem e se desenvolvem em um ambiente sem estresse biótico e/ou abiótico têm interação entre cloroplasto e mitocôndria, na troca de metabolitos energéticos, esqueleto de carbono e principalmente redutores equivalentes como ATP e NAD(P)H (HOEFNAGEL; ATKIN; WISKICH, 1998).

Uma boa parte desses redutores originados do cloroplasto e da mitocôndria são utilizados em várias reações metabólicas localizadas no citosol que reduz NAD^+ a NADH, como na glicólise e na redução do nitrato. (PODGÓRSKA et al., 2013).

Para absorção de nitrogênio as plantas necessitam de mais de 25% desses redutores provenientes da fotossíntese para poder usar na redução do nitrato vindo do solo (NOCTOR; FOYER, 1998).

Por tanto, quando uma planta é suprida somente de nitrato, como fonte de nitrogênio, a redução desse íon nas folhas compete com a reação de Mehler por elétrons (e^-) a partir do fotossistema I (PSI) (GERENDÁS et al., 1997)(Figura 5). Por outro lado, em plantas supridas somente de amônio como fonte nitrogênio esse fenômeno, ocorre livremente.

Conhecido como reação de Mehler, é ocasionado quando há uma diminuição por concorrentes pelos redutores formados pela fotossíntese, assim como ocorre na redução do nitrato no citosol. Quando acontece isso, há o transporte de elétrons na membrana dos tilacoides durante a fotossíntese, podendo levar a fotorredução do oxigênio produzindo o radical superóxido (O_2^-). Então, ocorre a dismutação do O_2^- através da

enzima peroxido dismutase (SOD) que tem como produto o peroxido de hidrogênio (H_2O_2) em seguida a reação de H_2O_2 com O_2^- resulta na formação do radical hidroxil (HO^-) que é altamente reativo (GERENDÁS et al., 1997).

Então, a partir disso podem ocasionar a danos tais como, a inativação de enzimas, peroxidação lipídica e degradação proteica (HYDROPEROXIDEN; HZEL; SPITELLER, 1995).

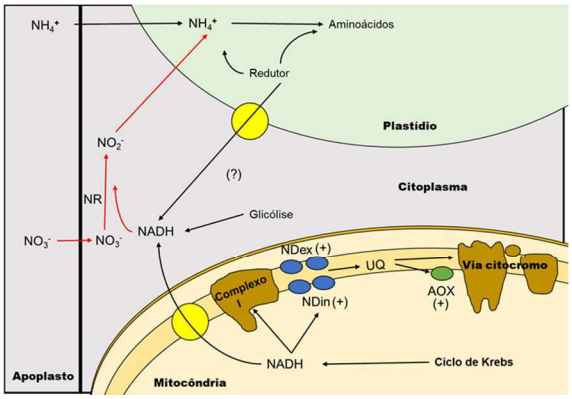

Figura 5 Um modelo para balanceamento redox pelos caminhos alternativos da cadeia respiratória mitocondrial em resposta à imposição de nitrogênio (N) mudanças na demanda redutora. Rotas do nitrogênio e elétrons reduzidos sob nitrato (NO_3^-) e amônio (NH_4^+). Sob nutrição com nitrato (setas vermelhas), sua redução constituirá um grande dreno de NADH redutora citoplasmático e plastídicos (NADPH ou ferredoxina fornecida pela via de fosfato de pentose ou pela fotoquímica). O NADH citoplasmático pode ser derivado da glicólise ou redox vindos de mitocôndrias ou plastídios. (Nas folhas, a fotorrespiração irá, além disso, produzir redutores nas mitocôndrias e consumi-lo nos peroxissomos e pela re-assimilação de amônio no cloroplasto.) Sob nutrição com amônio, ocorre uma indução de vias respiratórias alternativas, incluindo internas (NDin) e externas (NDex) tipo II NADH desidrogenases e Oxidases alternativas (AOXs). Este caminho pode oxidar o excesso de NADH que não é mais necessário para redução do nitrato. Portanto, as vias alternativas da cadeia respiratória podem ter um papel funcional em mantendo a homeostase redox em resposta a variações nas fontes N disponível para raízes no solo [uma extensão das hipóteses anteriores propostas por (LAMBERS, 1980 e BARNEIX; BRETELER; VAN DE GEIJN, 1984) . Abreviações: NR, nitrato redutase; UQ, ubiquinone

Então, para evitar esse estresse nas folhas a mitocôndria pode estar envolvido na regulação do estado redox celular (NOCTOR; PAEPE; FOYER, 2007). Isso é percebido em plantas crescidas com amônio como única fonte de nitrogênio onde foi observado um aumento na respiração (ESCOBAR; GEISLER; RASMUSSON, 2006).

Com a fotossíntese em steady-state, mais de 50 % de NADH pode ser exportado para o citosol (KRÖMER; HELDT, 1991; KROMER, 1995). Na mitocôndria existe uma “lançadeira de malato” que durante a fotorrespiração aumenta a taxa NADH/NAD

no citosol elevando ainda mais o nível de redutores que serveriam para a redução do nitrato no citosol (RACHMILEVITCH; COUSINS; BLOOM, 2004; BLOOM et al., 2010).

Então, se em condições de absorção de nitrogênio ocorrer através do nitrato como fonte de N, uma regulação baixa de um NAD(P)H dehidrogenase tipo II volta para matriz e várias AOXs podem efetivamente diminuir reoxidação respiratória da matriz produzindo NADH durante a síntese de 2 – oxoglutarato (ESTEBAN et al., 2016) (Figura 6).

Porém, uma planta suprida com apenas amônio como fonte de N haverá rotas alternativas da cadeia respiratória que serão super regulados. E na ausência da redução do nitrato NADH citosolico pode se acumular permitindo ativação de NAD(P)H dehidrogenase tipo II e AOX induzido por esse íon (ESTEBAN et al., 2016). Assim, equivalentes redox poderão ser lançados da cadeia transportadora estabilizando o nível redox citoplasmático (GARDESTRÖM; IGAMBERDIEV; RAGHAVENDRA, 2002; KROMER, 1995)(Figure 6).

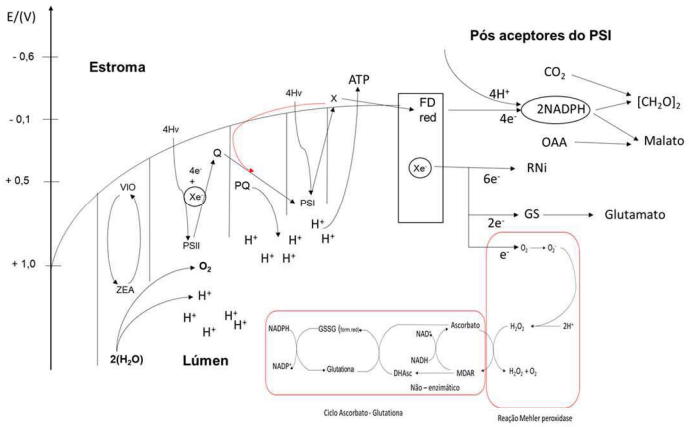

Figura 6 A cadeia de transporte de elétrons e a ação de enzimas de eliminação de radicais de oxigênio (Reação Mehler-peroxidase, ciclo ascorbato-glutationa): 1 superóxido dimutase, 2 ascorbato peroxidase, 3 monodehidroascorbate redutase, 4 Dehidroascorbate redutase, 5 glutationa redutase, 6 Ciclo de zeaxantina, VIO violaxanthine, ZEA Zeaxantina, FD red. ferredoxina reduzida, DHAsc dehidroascorbate, radicais MDAR monodehidroascorbato. (modificados de MARSCHNER, 1995 e POLLE, 1996).

A assimilação primaria do NH_4^+ ocorre principalmente na raiz, podendo assim evitar o aumento de rotas de capacidade alternativa respiratória na mitocôndria que pode também resultar em uma maior oxidação respiratória da matrix e pouca exportação de equivalentes redox ao citosol (ESTEBAN et al., 2016). Deste modo, o

metabolismo do nitrogênio está interligado com o metabolismo do carbono através de metabolitos anapleróticos provenientes da fotossíntese nos cloroplastos e metabolismo do ciclo ácido tricaborxilico e glicólise na mitocôndria (YANG et al., 2016).

O metabolismo de N na mitocôndria é conhecido no contexto da suplementação de esqueleto de carbono para assimilar o amônio (SZAL; PODGÓRSKA, 2012). No cloroplasto o ciclo GS/GOGAT, responsável pela assimilação de amônio, necessita de 2-oxoglutarato (esqueleto de carbono) que vem da mitocôndria (TCHERKEZ et al. 2009; GAUTHIER et al. 2010). Assim, a interação dessas duas organelas sob efeitos tóxicos causados pelo amônio é bastante requisitada pelas plantas.

Perspectivas para melhoramento em produtividade vegetal sob condições de alto níveis de NH_3/NH_4^+

No âmbito do uso e eficiência do nitrogênio, alguns estudos vão ganhando cada vez mais importância no quadro mundial, a medida que essa problemática torna – se global com o uso intensificado de fertilizantes agrícolas (ESTEBAN et al., 2016) que tem como finalidade atender ao crescimento populacional. Estima-se que em 2050 um aumento de 70-100% na produção agrícola mundial será necessário para sustentar uma população mundial de nove milhões pessoas (GODFRAY,2010).

Na agricultura, o uso e eficiência do (N) é notoriamente pobre, uma vez que, mais de 50% do fertilizante aplicado no campo não é absorvido pelas plantas, mas perdido para o ambiente na forma de gás amônia, nitrato, que é facilmente lixiviado, e óxidos nítricos (O_2H), um dos principais gases do efeito estufa sendo 300 vezes mais intensificador do que o dióxido de carbono (COSKUN et al., 2016).

Fertilizantes baseados em amônio (ureia, NH_3 anidro, sulfato de amônio ($(NH_4)_2SO_4$) e nitrato de amônio (NH_4NO_3) compreendem comumente formas de N mais usadas na agricultura (PALM et al., 2014). Enquanto que, alguns solos podem reter eletrostaticamente uma variedade de cátions tais como NH_4^+ por esses solos terem cargas negativas na superfície de suas partículas, depronação desse íon produz gás amônia que é volatilizado e perdido à atmosfera em grande quantidade (18%) (PAN et al., 2016). Para evitar algumas perdas expressivas de nitrogênio, os agricultores utilizam inibidores de nitrificação sintetizados juntos com os fertilizantes (ABALOS et al., 2014). Algumas plantas, tais como, o arroz, sorgo e o trigo, têm exsudados de suas raízes que são inibidores naturais de nitrificação, conhecido coletivamente como

inibidores biológico de nitrificação (BNIs) (SUBBARAO et al., 2009; COSKUN et al., 2016).

Assimilação do nitrogênio inorgânico, na forma de íon NH_4^+, em moléculas orgânicas é um processo metabólico crucial ao crescimento das plantas, podendo até ser um fator limitante para seu crescimento (LEARY; PLAXTON, 2014), tanto na falta quanto em excesso. Futuramente o íon amônio poderá ganhar grande importância como fertilizante nitrogenado na agricultura frente a mudanças climáticas. Já que, à medida que vai aumentando a concentração do CO_2 atmosférico, vai se dando a importância da utilização desse íon. Uma vez que para assimilação de amônio é necessário esqueleto de carbono, então, a medida que aumenta o dióxido de carbono atmosférico, ocorre a inibição da absorção de nitrato (J. BLOOM et al., 2014).

Com isso conhecer quais mecanismos estão envolvidos no processo de tolerância ao amônio serão necessários para se obter um melhoramento vegetal e assim produzir nesse âmbito global, uma vez que, a grande maioria das espécies vegetais são susceptíveis ao estresse causado pela toxidez ao amônio, levando em queda consideravelmente a produção vegetal (KRONZUCKER et al., 2001;BITTSÁNSZKY et al., 2015).

Nem sempre na agricultura o amônio é tido como agente causador de danos, pois seu metabolismo em plantas pode também alterar a tolerância das plantas em outros estresses ambientais (BITTSÁNSZKY et al., 2015). Em alta irradiação a tolerância ao amônio da planta de ervilha (*Pisum sativum*. L) foi aumentada devido ao aumento de assimilação do carbono total, proporcionando assim um suporte de energia para manter um baixo teor de amônio no interior do tecidos (ARIZ et al., 2010).

Contudo, níveis de amônio nos tecidos vegetais é utilizado como um índice indicativo para avaliar os estresses induzidos pelo ambiente em plantas (BARKER, 1999). GUO et al. (2007) sugerem que plantas de arroz cultivadas apenas com amônio, sobe estresse hídrico, apresentam uma melhoria na fotossíntese. Isso devido às altas taxas fotossintéticas dessas plantas que estão relacionadas com os altos custos enérgicos da assimilação do amônio (RAVEN; FARQUHAR, 1990) e de atividades enzimáticas (RAAB; TERRY, 1994).

Em efeito de estresse hídrico e sobre nutrição com amônio como única fonte de nitrogênio DING et al., (2016) mostraram que as plantas de arroz tiveram acumulo de

ABA na raiz levando ao aumento de expressão genica de *OsTIPs* e *OsPIPs,* que são genes que regulam a atividade das aquaporinas, aumentando assim condutância hídrica na raiz e que também a longo tempo de exposição dessas plantas em estresse hídrico e nutrição de amônio diminuiria o pH da seiva xilemática fazendo com que o ABA nas folhas ficassem na forma inativa e melhorasse a condutância estomática mantendo assim a fotossíntese dessas plantas.

Com isso novos estudos referentes a tais mecanismos envolvidos nessa aliviação de estresse ambiental junto com os de tolerância ao amônio são necessários para se obter um melhor aproveitamento nutricional de nitrogênio.

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3 How tolerant is rice plants to ammonium excess? New insights from photochemical responses under two contrasting light regimes

ABSTRACT – Land plants can utilize nitrate (NO_3^-) or ammonium (NH_4^+) as preferably N source. Nevertheless, cellular NH_4^+ accumulation presents potential harmful effects for metabolism, such as proton gradient uncoupling, photosynthesis constraining, growth stunting, decreased productivity and occasionally leading to plant death. Therefore, plants that are preferably NH_4^+ users, such as rice (*Oryza sativa japonica* cv. Nipponbare) had to evolve several mechanisms to cope with high ammonium concentrations exposure. However, toxicity mechanisms as well as rice tolerance strategies to NH_4^+ are not completely understood. Aiming to investigate this phenomenon, intact rice plants and leaf segments were exposed 10 mM NO_3^- or 10 mM NH_4^+ in presence of 400 μmol m^{-2} s^{-1} (ML) and 2000 μmol m^{-2} s^{-1} (HL). Intact rice plants exhibited high NH_4^+ accumulation in roots associated with unchanged GS-GOGAT activities and low ammonium export *via* xylem. This avoidance strategy probably allowed photosynthetic apparatus protection and absence of differences in CO_2 assimilation and PSII and PSI quantum efficiency (ΦPSII, ΦPSI). Despite similar ΦPSII activity, intact plants exposed to NH_4^+ exhibited slight delay in PSII dark-recovery, which indicates the existence of early evidences for ammonium toxicity in rice leaves. Oppositely, leaf segments exposed to 10 mM NH_4^+ exhibited very high ammonium accumulation, especially at HL, associated with great GS_1 activity induction. These segments exhibited also a greater delay in PSII dark-recovery, which was aggravated by HL, and lower maximum quantum efficiency of PSII (Fv/Fm), which are evidences of photoinhibition. The greater PSI limitation at donor side (ΦNA), under such conditions, corroborates the existence of ammonium-induced negative effects on PSII recovery. Thus, we propose that NH_4^+ toxicity mechanism in leaves involves constraint in the PSII repair in the dark and resistance in rice plants, in terms of photosynthesis maintenance, is dependent on the ammonium levels that reach photosynthetic apparatus.

Key-words: High light. NH_4^+ toxicity. N metabolism. *Oryza sativa*. Photosynthesis.

INTRODUCTION

Plants require large amounts of nitrogen (N), which is the most essential nutrient and the major limiting factor to plant growth and production. Nitrate (NO_3^-) and ammonium (NH_4^+) are the main inorganic N source used by plants. However to rice plants grown in paddy soils, NH_4^+ is the prevailing N source available (Tabuchi et al., 2007). Despite of NH_4^+ be a cheap N source, in terms of fertilization and plant metabolic costs, its use as a sole N source and/or in high concentrations could trigger many physiological disorders in plants (Britto and Kronzucker, 2013; Li et al., 2012). Reduced growth, changes in root architecture, rhizosphere acidification, leaves chlorosis, increase oxidative stress and modifications in the mitochondrial and chloroplast reactions are some of NH_4^+ toxicity symptoms (Cruz et al., 2011; Ariz et al., 2011, Bittsánszky et al., 2015). In general, plants that have high NH_4^+ preference or tolerance developed efficient mechanisms to avoid toxicity in root and leaf tissues.

Rice is considered the most tolerant plant to NH_4^+ (Wang et al., 1993) and this ability is attributed to its high activity of GS/GOGAT (glutamine synthetase/glutamate synthase) cycle in roots, preventing the toxic effects of this ion in plant growth (Ishiyama et al., 2004; Balkos et al., 2010). Therefore, the distribution of N to long-distance occurs main in the glutamine (Gln) form produced by GS (Yamaya and Oaks, 2004). Ammonium transporters (AMTs), GS mRNA and activity are induced by external NH_4^+ mainly in the root tip region (Ishiyama et al., 2004). In plants, two GS isoforms acting in the ammonium assimilation, GS_1 and GS_2 located in two different subcellular compartments, cytosol and plastid/chloroplast respectively. GS_1 has an important role in sacavenging NH_4^+ taken up from soil or provided by protein proteolysis, whereas GS_2 is essential to reassimilate NH_4^+ from photorespiration cycle (Lea and Miflin, 2003).

In rice roots tow GS isoenzymes, OsGLN1;1 and OsGLN1;2, promote Gln synthesis and both are categorized as high-affivity enzymes with very high V_{max} (186 nkat/mg protein) (Ishiyama et al., 2004). Indeed, GS activity is crucial for the maintance of plant metabolism under elevated NH_4^+ concentration, however the interection with other relvant mechanisms should not be disregarded to rice tolerance, such as NH_4^+ compartimentalization and efflux to the external medium (Li et al., 2012). These processes help to keep low amount of free NH_4^+ in the cell, but when in excess this ion

could be translocated to shoot cells and compromises many metabolic reactions, mainly respiration rate and photosynthesis (Britto and Kronzucker, 2002). The classical NH_4^+ toxic effect in plant cells are the H^+-uncoupler, from NH_4^+/NH_3 interconversion, in mithocondria and chloroplast, however this hypothesis is controvertial (Esteban et al., 2016a).

Acting as an H^+-uncoupler, NH_4^+ should decrease ΔpH and, consequently, respiration rate and photochemical reactions in the thylacoid membrane, specially NPQ (non-photochemical quenching). Nevertheless, some studies releved that excess of NH_4^+ did not decrease qE (NPQ thermal component) and respiratory rate in *Phaseolus vulgaris* and *Arabidopsis thaliana*, respectively (Bendixen et al., 2001; Podgórska et al., 2013). Futhermore, it is hypothesized that NH_4^+ can inhibits photosynthesis through the binding of NH_3 in two sites of oxygen-evolving complex (OEC) of photosystem II (PSII) (Drath et al., 2008; Askerka et al., 2015). NH_3-OEC binding does not completely block O_2 evolution, suggesting that water molecules can still bind and react at the OEC in the presence of NH_3 (Askerka et al., 2015). However, these effects of NH_4^+ on photosynthesis are not the same and depend on vegetal species and NH_4^+ concentration and exposure time (Esteban et al., 2016a).

Podgórska et al. (2013) demonstrated that the impairment of NH_4^+ long-term nutrition in *Arabidopsis thaliana* was mainly associated with redox reactions in the mitochondria by increasing reactive oxygen species (ROS), but did not impair photosynthetic capacity. In addition, NH_4^+ nutrition caused increase of lipid peroxidation and glutatione-ascorbate cycle enzyme activity, as a consequence of greater ROS production in NH_4^+ sensitive plants (Zhu et al., 2000; Patterson et al., 2010). In contrast, in relative NH_4^+ tolerant plants ammonium nutrition does not induce oxidative stress (Domínguez-Valdivia et al., 2008; Esteban et al., 2016b), suggesting no agreement on the main cause of NH_4^+ toxicity in plants. Furthermore, the key traits that confer plant tolerance to ammonium and the interaction with abiotic stress are missing so far. Thus, the elucidation of the mechanisms involved with NH_4^+ toxic effects and tolerance in plants are crucial to improve crop production using other low cost N source (Podgórska et al., 2013).

In this study, we investigated growth, photosynthesis and N metbolism in rice plants exposed to NH_4^+ excess under moderate and high light. In this condition, rice

plants showed a intense influx of NH_4^+, however only low concentrations of this ion was translocated to shoot via xylem. CO_2 assimilation in NH_4^+ supplied plants did not change, but the recovery of photochemic activity was affected by this ion. The straightforward NH_4^+ effect in leaf under two different lights was also explored and suggest that rice tolerance, in terms of photosynthesis maintenance, is dependent on the ammonium levels that reach photosynthetic apparatus.

MATERIALS AND METHODS

Plant material and growth conditions

Rice seedlings (*Oryza sativa* L. cv. Nipponbare), 10 days after germination, were transplanted in a 3 L plastic pots filled with quarter-strength Hoagland-Arnon's nutritive solution (Hoagland and Arnon, 1950). The pH was daily adjusted to 6.0 ±0.5 and the nutritive solution was changed every 7 days and it reached the full-strength from the third week. The plants were cultivated for 35 days 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.

Experiments

Aiming to analyse NH_4^+ uptake and metabolism, an ammonium flux experiment was performed with three-day N-deprivation rice plants. Thus, these plants were exposed to nutritive solution containing 10 mM of NO_3^- (control) or NH_4^+, as a sole N source, by 24 h. Afterwards, N influx rate and xylem flux and the concentration of nitrate and ammonium in leaves and roots were measured. Further, to verify the effects of a long-term ammonium nutrition in photosynthesis, growth and ammonium metabolism, rice plants were cultivated with 10 mM NO_3^- (control) or NH_4^+, as a sole N source, by 21 days under greenhouse conditions. In the experimental period, the pH was adjusted to 6.0 ±0.5 daily and the nutritive solution was changed every four days to keep the N concentration. Gas exchange parameters were measured and plant material (leaf and root) was harvested to further analyses of amino acid content and activities of GS and NADH-GOGAT.

A third and fourth experiment were performed to study the influence of high light and ammonium and their combination in both leaf segments and whole plant. For these experiments, the plants were transferred to growth chamber with the following controlled conditions: 28/24 °C day/night temperature, 60% relative humidity, PPFD of 400 μmol m^{-2} s^{-1} and 12 h photoperiod. After an acclimation of three days inside the growth chamber, the treatments were applied. For the leaf segment experiment, 2 cm length segments from full expanded and health leaves were incubated in a reaction medium containing 10 mM NO_3^- (control) or NH_4^+ dissolved in 10 mM Hepes buffer and 0.01% (v/v) Triton X-100 by 8 h under PPFD of 400 μmol m^{-2} s^{-1} (moderate light – ML) or 2,000 μmol m^{-2} s^{-1} (high light – HL). A previous 5 min vacuum was applied to allow a more effective infiltration of the solutions into the leaf tissues. At the end, photochemical parameters measurements, electrolyte leakage, NO_3^- and NH_4^+ content and cytosolic and chloroplast GS activity were assessed. For the whole plant experiment, plants with three-day N-deprivation were exposed to 10 mM NO_3^- (control) or NH_4^+, as a sole N source, by 7 days and in the last day the plants were divided in two groups, which the first one remained under moderate light (400 μmol m^{-2} s^{-1}) and the second one was subjected to high light (2,000 μmol m^{-2} s^{-1}) conditions by 8 h. At the end, photochemical parameters measurements were performed.

Gas exchange and chlorophyll a fluorescence measurements

The leaf CO_2 assimilation (P_N), stomatal conductance (g_S), intercellular CO_2 partial pressure (C_i) and transpiration rate (E) were measured by using a portable infrared gas analyzer 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 chamber during the measurements were: PPFD of 1,500 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).

In vivo chlorophyll *a* fluorescence was measured using a Dual-PAM 100 (Walz, Germany). The light curves were performed with increasing light intensities from 0 to 2000 μmol m^{-2} s^{-1}, with 5 min of exposure at each light intensity. For induction/recovery kinetics, 2000 μmol m^{-2} s^{-1} of PPFD was employed for 5 min

(induction, followed by 5 min dark (recovery) and subsequently 5 min at 2000 μmol m^{-2} s^{-1} (re-induction). The fluorescence parameters were measured using the saturation pulse method (Schreiber et al., 1994) and leaves were previously acclimated to dark for 30 min. The intensity and duration of the saturation pulse were 8000 μmol m^{-2} s^{-1} and 0.6 s, respectively. The following parameters were assessed: the maximum quantum yield of PSII [Fv/Fm = (Fm−Fo)/Fm] and the effective quantum yield of PSII [ΦPSII = (Fm − Fs)/Fm']. The non-photochemical quenching coefficient was calculated as [NPQ = (Fm/Fm')-1], whereas Fm was determined in the onset of light curve or induction kinetics. The actual flux of photons from the PSII was calculated as ETR = (ΦPSII × PPFD × 0.5 × 0.84). The redox state of the PSI primary donor, P700 was measured and the following parameters were assessed: the photochemical quantum yield of PSI [ΦPSI = 1 – Φ(ND) – Φ(NA)] and the estimated electron transport rate of PSI [ETRI = ΦPSI x 0PPFD x 0.5 x 0.84]. The donor side limitation of PSI was calculated as [$\Phi(ND) = 1 - P700_{red}$] and the acceptor side of PSI limitation as [Φ(NA) = (Pm-Pm')/Pm]. The relative cyclic electron flow (CEF) was estimated by the ETRI/ETRII ratios according to Yamori et al. (2011).

Electrolyte leakage (membrane damage)

Electrolyte leakage (EL) was measured as described previously by Lima Neto et al. (2014). Leaf segments 5 cm length were placed in tubes containing 10 mL of deionized water and incubated in a shaking water bath at 25°C for 24 h. After, the electric conductive in medium (L1) was measured. Next, the segments were boiled at 95 °C for 1 h, cooled to 25 °C, and the electric conductivity (L2) was measured and the EL was calculated using the following equation: EL = (L1/L2) x 100.

Nitrogen influx and xylem flux quantification

The nitrogen influx quantification was quantified from the N concentration depletion ($[N]_{initial} - [N]_{final}$) of the incubation solution. The xylem sap flux was measured according to Shurr (1998) in plants incubated with nutritive solution containing 10 mM NO_3^- or NH_4^+, as a sole N source, by 24 h. The stems were cut at 5 cm above root and the xylem sap was harvested for 60 min and cooled down for the

quantification of nitrate and ammonium. The influx of N was expressed in μmol NO_3^- or NH_4^+ g^{-1} DW h^{-1} and the N xylem flux was expressed in mmol NO_3^- or NH_4^+ L^{-1} xylem sap h^{-1}.

Nitrate, ammonium and amino acids concentration

The concentration of nitrate in the nutritive solution and xylem sap was quantified using the perchloric acid method, according to Cawse (1967) at A_{210}. For the quantification of nitrate, ammonium and amino acid content in plant tissue, lyophilized leaves and roots samples were incubated with distilled water at 100 ºC for 1 h and filtered to obtain the crude extract. Subsequently, the nitrate concentration was measured by salicylic acid method, according to Cataldo et al. (1975). All the ammonium determinations (in the nutritive solution, xylem sap and plant tissue) were performed by using the phenol-hypochlorite-ammonia method (Felker, 1977) and the total free amino acids were measured according to Yemm and Cocking (1955). The concentration of NO_3^-, NH_4^+ and amino acids were expressed in μmol g^{-1} DW.

Glutamine synthetase and NADH-Glutamate synthase activities

Fresh leaves and roots were ground until obtaining a fine powder in presence of liquid N_2, ice-cold 200 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 1 mM $MgCl_2$. 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 ºC. Total and cytosolic GS (EC 6.3.1.2) activity were determined by the hydroxamate biosynthetic method as described by Hirel and Gadal (1980). For the 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. For the GS_1 activity, 5 mM glucosamine 6-phospahte was added in the assay buffer to inhibits GS_2 activity. 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 solution. 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}. The GS_2 activity was calculated as follow: $GS_2 = GS_{Total} - GS_1$.

NADH-GOGAT (EC 1.4.1.14) activity was determined according to Matoh and Takahashi (1981). The assay buffer consisted of 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM glutamine, 5 mM α-ketoglutarate and 0.25 mM NADH in a final volume of 2 mL. The reaction was initiated by the addition of 0.2 mL of extract and carried out at 30 °C. After mixing, a linear decrease in absorbance at 340 nm over 3 min was observed and the enzyme activity was measured. A control was performed by omitting glutamine. The blank consisted of the assay buffer in the absence of enzymatic extract. NADH-GOGAT activity was calculated from the NADH molar extinction coefficient (ε_{340} = 6.22 mM^{-1} cm^{-1}) and the activity was expressed as μmol NADH g^{-1} FW h^{-1}.

Statistical analysis and experimental design

The experiments were arranged in a completely randomized block, with four replicates per treatment. In the leaf segment experiment, one replicate was represented by one Petri dishes containing 40 leaf segments of 3 cm length, and in the whole plant experiments, one replicate was represented by two plants per pot. The data were subjected to the analysis of variance (ANOVA) and the averages were compared by Tukey or *t*-test at a confidence level of 5% ($p < 0.05$), as referred in figure captions.

RESULTS

Ammonium-treated rice plants exhibit low NH_4^+ flux from roots to leaves

In order to investigate rice mechanisms of ammonium allocation in different plant organs, the content of ammonium (NH_4^+) and nitrate (NO_3^-) were estimated in leaves and roots, in parallel to N influx and N xylem flux determinations. Rice plants exposed for 10mM NO_3^- during 24 h after N starvation (72 h), exhibited nitrate influx and nitrate xylem flux equalled to 54.31 (μmol g^{-1} DW h^{-1}) and 4.17 (mmol L^{-1}Xylem Sap h^{-1}), respectively (**Table 1**). Nitrate content in roots did not differ from NO_3^- content in leaves (**Table 1**). In other hand, ammonium influx and ammonium xylem flux reached 79.7 (μmol g^{-1} DW h^{-1}) and 0.55 (mmol L^{-1}Xylem Sap h^{-1}), respectively in ammonium treated plants (**Table 1**). Ammonium content in roots from NH_4^+-treated rice was 5-fold higher than ammonium content in leaves (**Table 1**).

Rice exposure to ammonium does not induce significant changes in GS-GOGAT enzymatic activities and photosynthesis, but increases the aminoacid content in leaves and roots

The long-term experiments were designed to evaluate the possibility of rice long-standing periods of ammonium exposure and accumulation activating contrasting enzymatic responses related to N metabolism and consequently, ammonium tolerance. After 21 days of different N treatments, rice plants exposed to NH_4^+ exhibited 50% increase in shoot fresh weight but no significant differences in root fresh weight, in comparison to NO_3^--exposed plants (**Fig. S1**). In parallel, ammonium- and nitrate-treated rice plants presented no significant differences in glutamine synthetase (GS) and glutamine oxoglutarate aminotransferase (GOGAT) activities, in both leaves and roots (**Table 2**). Differently, NH_4^+-treated plants exhibited approximately 20% and 70% increase in aminoacid contents of leaves and roots, respectively, in comparison to nitrate-treated plants (**Table 2**).

The absence of ammonium toxicity in rice plants was corroborated by leaf cell membrane integrity (electrolyte leakage), which did not display significant differences among 10 mM NO_3^-, 10 mM NH_4^+ and even 20 mM NH_4^+ treated plants (**Table 3**).

These results were associated to photosynthesis, since ammonium-treated rice also did not exhibit any significant difference in important photosynthetic parameters, such as CO_2 assimilation, internal CO_2 concentration, stomatal conductance and transpiration, as compared to nitrate-treated plants (**Table 3**). In opposition, PSII quantum efficiency (ΦPSII) of rice plants exposed to from 10 mM NH_4^+ exhibited a slight increase (from 0.18 to 0.26), in comparison to nitrate treated plants (0 mM of NH_4^+). However, 20 mM NH_4^+ treated rice did not exhibit significant differences in ΦPSII as regarding 10 mM ammonium or 10 mM nitrate treatments (**Table 3**).

High ammonium generates delays in rice PSII quantum efficiency recovery

Aiming to investigate the high ammonium toxic effects on rice photochemical activity, plants were grown for 7 days with 10 mM NO_3^- or 10 mM NH_4^+ as exclusive N source, in combination with 8 hours of moderate light (ML, 400 µmol m^{-2} s^{-1}) or high light (HL, 2000 µmol m^{-2} s^{-1}), at the 7th day of treatment. Rice plants treated with 10 mM NH_4^+ did not exhibit significant differences in maximum quantum efficiency of PSII in dark- and illuminate-acclimated leaves (Fv/Fm and Fv'/Fm', respectively) as compared to nitrate-treated leaves, in both light regimes ML and HL (**Fig. 1 a, b**). Accordingly, the actual quantum efficiency of PSII (ΦPSII) induction kinetics was similar in both ammonium- and nitrate-treated leaves, even under high light conditions (**Fig. 1 c, d**). In contrast, the ΦPSII relaxing kinetics was delayed approximately 20% in NH_4^+ treated leaves. A similar delay induced by ammonium treatment was observed in both light regimes (**Fig. 1 e, f**).

In parallel, non-photochemical quenching (NPQ) induction kinetics of rice leaves treated with high ammonium concentration displayed a similar pattern as compared with nitrate-treated plants, in both light regimes (**Fig. 1 e, f**). This response was contrasting to NPQ relaxation kinetics. During relaxation kinetics, the ML-ammonium-treated leaves exhibited proportional delay in NPQ dissipation (20%), but no differences were noted for HL-ammonium treated leaves in NPQ relaxation kinetics, as compared to HL-NO_3^--treated rice (**Fig. 1 e, f**). Taken together these results suggest that PSII activity induction in rice leaves is not affected by excess ammonium treatment, which is consistent with effective rice ammonium tolerance. However, the

fact that PSII relaxation process is affected by ammonium indicates a potential susceptibility under specific conditions.

PSI quantum efficiency (ΦPSI) in both ammonium- and nitrate-treated rice did not exhibit significant differences regarding induction kinetics, in both HL and ML light conditions (**Fig. 2 a, b**). These results are contrasting with ΦPSI relaxation kinetics, which evidenced faster PSI relaxation in ammonium-treated plants as compared to NO3- treatment under ML conditions, and this difference between N source treatments was approximately 20% greater in HL conditions (**Fig. 2 a, b**). In addition, the acceptor and donor side limitations of PSI, ΦNA and ΦND, respectively, evidenced that ammonium induced changes to rice PSI were present only under HL, and these differences were related to decreased ΦNA during post-illumination relaxing kinetics (**Fig. 2 c-f**).

Rice leaf segments directly exposed to ammonium exhibit differences in cytosolic GS isoforms dependent on light regime and similar cellular membrane integrity

In order to investigate deeply the process related to a potential ammonium toxicity regarding NH_4^+ metabolism, rice leaf segments were exposed to 10 mM NO_3^- or 10 mM NH_4^+ during 8 hours in presence of ML or HL conditions. These experiments evidenced that cellular membrane integrity was similar in both ammonium- and nitrate-treated segments, regardless light conditions (**Fig. 3 A**). This response in ammonium-treated segments occurred in parallel to intense accumulation of NH_4^+, especially in HL conditions (approximately 120 %), as compared to nitrate treatment (**Fig. 3 B**). In addition, the nitrate content decreased 25% and 50% in ammonium-treated segments in comparison to nitrate-treated leaves, under ML and HL, respectively (**Fig. 3 C**).

Ammonium-treated rice segments exhibited similar activity of total GS, cytosolic GS_1 and chloroplastic GS_2 in comparison to NO_3^--treated segments, in ML conditions (**Fig. 11**). Oppositely, HL induced increase in total GS activity and GS_1 only in ammonium-treated segments, reaching activities 20% and 200% higher than activities of nitrate-treated segments, at similar light conditions (**Fig. 4**).

Leaf segment experiments reveal that direct exposure to NH_4^+ generates photoinhibition and increase the leaf susceptibility to light stress

The photochemical activity was deeply investigated in rice leaf segments exposed to excess ammonium or nitrate, to better comprehend the results found in intact plants experiments. In contrary to intact plants experiments, ammonium-incubated leaf segments exhibited 19% decrease in Fv/Fm (**Fig. 5 a**). PSII-light curves evidenced that direct ammonium treatment to rice leaves is able to induce strong impairment in ΦPSII, especially at moderate light intensities, which generates deficiency in electron transport rate from PSII (ETRII), more prominent at higher light intensities (**Fig. 5 b, c**). These responses were similar to ΦPSI and ETRI curves in function of light intensity, where ETRI from ammonium treated segments reaching approximately 70% of this parameter measured in nitrate-exposed leaves (**Fig. 5 d, e**). These differences in ETRII and ETRI culminated in approximately 20% increase in cyclic electron flux (CEF) in NH_4^+ segments, compared to nitrate treatment, especially at lower light intensities (**Fig. 5 f**).

The PSII- and PSI-induction/relaxation kinetics in rice leaf segments treated with different N sources corroborated the results from photochemical light curves. The ΦPSII induction kinetics in ammonium-treated segments revealed significant decrease in comparison to nitrate-treated ΦPSII kinetics (20%), only in HL exposed segments (**Fig. 6 a, b**). These responses were associated to ammonium-induced impediment in ΦPSII relaxing kinetics, which was delayed approximately 25% and 50% in ML- and HL-exposed segments, as compared to nitrate-treated leaves under similar conditions (**Fig. 6 a, b**). This impairment on PSII activity was associated to photoinhibition and not to PSII down-regulations, as suggested by Fv/Fm*Fo parameter, which was decreased in similar proportion in ammonium treated segments in comparison to Fv/Fm (**Fig. S2**). Oppositely, excess ammonium induced no differences in ΦPSI induction kinetics in both light regime treatments, but a more efficient ΦPSI relaxing (20%) kinetics in ammonium-treated segments exposed to ML (**Fig. 6 c, d**). No significant differences in re-induction kinetics were observed between ammonium- and nitrate-treated leaf segments, regardless light regime, ML or HL (**Fig. 6**).

In addition, ammonium induced significant differences in PSI limitation processes in comparison to nitrate-treated segments, but exclusively in ML conditions (**Fig. 7**). During photochemical induction ammonium-treated segments showed higher

donor side limitation and lower acceptor side limitation, as compared to NO_3^-, under ML conditions (**Fig. 7 a**). These differences in ΦNA were followed by lower acceptor side limitation of PSI during post-illumination kinetics in ML-ammonium treated segments (**Fig. 7 c**). Oppositely, no significant differences in ΦND and ΦNA kinetics during induction, relaxation and re-induction phases of leaf segments incubated with ammonium in presence of HL, as compared to nitrate-treated leaves under similar conditions (**Fig. 7 b, d**).

DISCUSSION

Nitrate (NO_3^-) and ammonium (NH_4^+) are the two major forms that land plants utilize for N assimilation. However, NH_4^+ is a potentially toxic molecule, which could generate disturbances in energetic metabolism, restrict plant growth and, at extreme conditions, cause plant death (Britto and Kronzucker, 2013). Therefore, plants that preferably utilize NH_4^+ as N source, needed to develop differential adaptations and mechanisms to become able to grow at high ammonium concentrations. Among such adapted species, rice (*Oryza sativa*) have been referred in literature as very tolerant to ammonium (Harada et al., 1968; Sasakawa and Yamamoto, 1978; Wang et al., 1993). Despite the rice tolerance to ammonium be commonly reported, the mechanism of toxicity and tolerance to high ammonium concentration is still not fully comprehended (Balkos et al., 2010; Esteban et al., 2016a).

Our results clearly show that rice is capable to accumulate high ammonium content in leaves. However, the fact that rice plants exhibited lower NH_4^+ xylem transport in comparison to NO_3^- transport, could indicate a possible avoidance strategy in this species. Indeed, rice plants accumulated ammonium preferably in roots tissues, which certainly contributed for this plant tolerance (Loqué and Von Wirén, 2004; Yuan et al., 1993). Important protective mechanisms against ammonium toxicity in root cells have been proposed in literature (Balkos et al., 2010; Kronzucker et al., 1999; Sonoda et al., 2003). Among such mechanisms, the regulation of ammonium influx trough plasma membrane, ammonium efflux throughout the cytoplasm, NH_4^+ compartmentalization inside vacuoles and effective ammonium metabolization are expected to play an important role in rice root cells (Loqué and Von Wirén, 2004).

The effective metabolization of ammonium in rice roots evokes the induction of important enzymes related to this metabolic process (Ishiyama et al., 2004). Indeed,

rice root tips, where NH_4^+ uptake is maximum, coincide with high expression of NADH-GOGAT and OsAMT, important proteins related to ammonium assimilation and uptake transporters respectively (Loqué and Von Wirén, 2004; Sonoda et al., 2003). In the current study, the total GS and NADH-GOGAT did not presented significant changes in nitrate- or ammonium-treated plants when measured in roots and leaf tissues. However, the great increase of amino acids detected in rice roots after NH_4^+ exposure could indicate that this molecule was intense metabolized, despite absence of changes in GS-GOGAT activities. This response could have implicated other important enzymes not measured here, such as glutamate dehydrogenase - GDH (Miflin, 2002), and should have occurred in parallel to high ammonium compartmentalization in vacuoles, as indicated by high ammonium contents in rice roots.

Higher ammonium content in root cells in comparison to leaves was an important mechanism in rice plants exposed to 10 mM NH_4^+. This avoidance mechanism certainly contributed to protection of photosynthetic mechanism in these plants. Stomatal conductance and CO_2 assimilation of rice plants was not impaired even after 21 days of exposure to ammonium as exclusive N source. Several studies have reported the photosynthetic responses in plants exposed to ammonium (Foyer et al., 1994; S. Guo et al., 2007; Shiwei Guo et al., 2007; Konnerup and Brix, 2010). Tolerant species as rice, are expected to not exhibit negative effects in photosynthetic activity in presence of high ammonium concentrations (Shiwei Guo et al., 2007). Indeed, rice plants exposed to osmotic stress in combination with ammonium as exclusive N source exhibited increased Rubisco content and photosynthetic activity (Shiwei Guo et al., 2007).

Ammonium toxicity mechanism in photosynthetic metabolism is usually discussed as a consequence of two important chemical features related to this molecule: 1) the potential activity of ammonium as ΔpH uncoupler (García-Mendoza and Colombo-Pallotta, 2007) and its consequence for impairment of important processes such as NPQ and ATP synthesis (Zhu et al., 2000); 2) the potential capacity of ammonia to bind PSII oxygen evolving complex in two sites and consequently limiting water split efficiency (Askerka et al., 2015; Beck et al., 1986; Drath et al., 2008; Mohanty et al., 1991). However, the argument for potential effects of ammonium as ΔpH uncoupler during photochemical activity have lost power, especially due to several reports of plants grown with NH_4^+ as exclusive N source do not present changes in NPQ formation

(Bendixen et al., 2001; Schinner et al., 2001; Zhu et al., 2000). The thermal component of NPQ (qE) is essentially formed by trans-thylakoidal ΔpH, and therefore should be decreased in such conditions (Ruban, 2016).

Our results evidenced that rice plants do not display any limitation in NPQ formation capacity even after seven days of exposure to 10 mM NH_4^+. For instance, this found is in accordance with literature for *Phaseolus vulgaris*, but the ammonium concentrations used in the current study are considerably higher (Bendixen et al., 2001; Zhu et al., 2000). Moreover, ammonium-treated rice plants did not exhibit any difference in FV/Fm and PSII and PSI activity induction, even after 8 hours of combined exposition to HL. Based on these data, it is reasonable to argue that high ammonium accumulation in rice roots prevents chloroplasts of direct exposure to excessive NH_4^+ concentrations, which protected rice leave against toxicity symptoms at photochemical activity level (Kronzucker et al., 1999; Sonoda et al., 2003). However, ammonium-treated plants exhibited indicators of impairment in PSII activity by a delay in dark-recovery. This event suggests an early sign of NH_4^+ toxicity in rice leaves.

Aiming to better understand the mechanisms of a possible toxic effect for ammonium in rice leaves, leaf segment experiments involving NO_3^- and NH_4^+ direct exposure in presence of ML and HL were performed. These experiments allowed high NH_4^+ accumulation in leaf tissues, especially at HL, which was associated with higher induction of cytosolic GS1. The induction of this protein probably was related to its importance for ammonium metabolization, especially in cytosol were this molecule present high toxicity potential (Britto et al., 2002; Wang et al., 1993). Ishiyama et al., 2004 reported that in Arabidopsis root cells, the expression of GS1 is highly induced by ammonium. Indeed, a region located within a 1.3-kb of the promoter of GS1 and GS2 in rice leaves is responsive to ammonia, probably by cis-acting regulatory elements (Kozaki et al., 1992).

Despite up-regulation in GS1 activity, the data strongly suggest that high ammonium concentrations provided directly to rice leaves is capable to generate toxicity and photoinhibition. Our data shows that Fv/Fm was strongly impaired in ammonium-treated leaf segments, and this effect was more prominent under HL conditions, were ammonium reached highest concentrations. Moreover, the aggravation of ammonium-induced impairment in PSII recovery in combination with higher PSI limitation at donor side (ΦND), indicates that rice leaf segments exhibited

limitations at PSII repair mechanisms. Interestingly, silenced Arabidopsis plants for GS_2 expression presented disrupted protection against high light, higher photoinhibition and slightly higher PSI donor side limitation, indicating lower electron supply from PSII (Brestic et al., 2014). In the current study, the increment of donor side of PSI linked to ammonium accumulation in leaves was much more prominent than reported for *gs2* Arabidopsis. We propose that ammonium toxicity in rice leaf segments is associated with disturbances in PSII repair process, which induces delay in PSII activity recovery and increases the limitation at PSI donor side.

The PSII repair mechanism is a complex event that involves several proteins and fine regulation, including D1 protein *de novo* synthesis (Bhuiyan et al., 2015; Tikkanen et al., 2008). Several environmental conditions are believed to generate impairment in this mechanism, most markedly accumulation of reactive oxygen species (Nishiyama and Murata, 2014). The levels of ROS in the current study were not properly assessed. However, evidences of ammonium-induce oxidative stress were reported previously in the literature (Podgórska et al., 2017, 2013). Arabidopsis plants grown for 8 weeks at 5 mM ammonium exhibited strong impaired growth and increase production of ROS in mitochondria (Podgórska et al., 2013). Despite the increased generation of ROS in mitochondria, this study revealed that chloroplasts were not affected by oxidative stress in such conditions, which was associated to unchanged photochemical activity (Podgórska et al., 2013).

Taken together, our results suggest that rice plants are tolerant to very high concentration of ammonium probably due to high accumulation of NH_4^+ in root cells and low xylem efflux to leaves. In rice roots ammonium is effectively metabolized to aminoacids, which prevents its toxic effects. When ammonium is supplied in high concentration to rice leaves, this molecule can reach the photosynthetic apparatus, generating photoinhibition and higher susceptibility to high light. Ammonium toxicity in rice is related to delay in PSII dark recovery, but the underlying mechanisms of toxicity are not completely understood to date. Delays in PSII recovery could be caused by higher ROS generation in rice leaf segments and subsequent impairment in D1 recovery process (Nishiyama et al., 2011). However, the possibility of impaired mitochondrial activity by ammonium toxicity (Podgórska et al., 2013) and subsequent decrease in reducing power sink (dark respiration) should not be ruled out. Further

studies are important to clarify the importance of these mechanisms to explain ammonium toxicity to rice leaves.

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4 CONCLUSÃO

Plantas de arroz (*Oryza sativa* ssp *Japonica* cv. Nipponbare) mais uma vez mostraram ser resistentes ao amônio, pelo menos quando expostas à 20 mM desse íon por 21 dias. Essa forte tolerância a altas concentrações de amônio se dá pela capacidade dessas plantas poderem concentrar todo o seu excesso nas raízes, evitando assim que esse íon chegue à parte aérea e protegendo todo o metabolismo foliar, principalmente a fotossíntese. Entretanto, os resultados dessa dissertação mostram que parte desse amônio é translocado para a parte aérea via xilema e que, apesar desse pequeno acúmulo na folha, os parâmetros fotossintéticos de trocas gasosas não são afetados em planta intacta. Já quando a concentração de amônio se acumula em grandes proporções na folha, quando aplicado diretamente em segmento foliares, esse íon prejudica as reações fotoquímicas da fotossíntese, principalmente a recuperação do fotossistema II. Esse fenômeno pode estar relacionado principalmente ao processo de ressíntese da proteína D1, o qual pode ser afetado pelo excesso de amônio. Portanto, concluímos neste trabalho que plantas de arroz apresentam tolerância elevada a altas concentrações de NH_4^+ por reduzir o seu transporte para a parte aérea e acumular todo o seu excesso na raiz, mas que em situações de desbalanço energético (alta luz) o aparato fotoquímico sofre fotoinibição.

Tabela 1 Nitrogen acquisition of rice plants exposed to 10 mM NO_3^- or 10 mM NH_4^+ by 24 hours under greenhouse conditions. Each measurement is represented by the average of four replicates (±SE).

Measurements	**10 mM NO_3^-**	**10 mM NH_4^+**
	[NO_3^-]	**[NH_4^+]**
N influx ($\mu mol\ g^{-1}\ DW\ h^{-1}$)	54.31 (± 6.70)	79.70 (± 7.72)
N xylem flux ($mmol\ L^{-1}$ Xylem Sap h^{-1})	4.17 (± 0.23)	0.55 (± 0.03)
N leaf content ($\mu mol\ g^{-1}$ DW)	26.10 (± 2.5)	19.64 (± 0.62)
N root content ($\mu mol\ g^{-1}$ DW)	25.71 (±2.63)	101.47 (± 6.92)

Tabela 2 Activities of glutamine synthetase and NADH-glutamate synthase and amino acids content in leaves and roots of rice plants exposed to 10 mM NO_3^- or NH_4^+ by 21 days under greenhouse conditions. Each measurement is represented by the average of four replicates (±SE), different letters represent significant differences at 5% between treatments according to the t-test.

Measurements	**10 mM NO_3^-**		**10 mM NH_4^+**	
	Leaf	**Root**	**Leaf**	**Root**
GS activity ($\mu mol\ g^{-1}\ FW\ h^{-1}$)	14.63 (± 1.00) A	1.61 (± 0.16) A	13.92 (± 0.51) A	1.20 (± 0.10) A
NADH-GOGAT activity ($\mu mol\ g^{-1}\ FW\ h^{-1}$)	9.22 (± 0.02) A	7.21 (± 0.17) A	9.20 (± 0.15) A	7.52 (± 0.21) A
Amino acids content ($\mu mol\ g^{-1}\ DW$)	374.03 (± 18.49) B	597.87 (± 90.90) B	434.11 (± 10.79) A	1000.49 (± 92.77) A

Tabela 3 Cell integrity, net CO_2 assimilation (P_N), internal CO_2 partial pressure (*Ci*), stomatal conductance (g_S), transpiration rate (E) and actual quantum efficiency of PSII (Φ PSII) in leaves of rice plants exposed to 0, 10 or 20 mM NH_4^+ by 21 days under greenhouse conditions. Control plants (0 mM NH_4^+) were exposed to 10 mM NO_3^- as N source. Each measurement is represented the average of four replicates (±SE), different letters represent significant differences at 5% between treatments according to the *t*-test.

Measurements	**0 mM NH_4^+**	**10 mM NH_4^+**	**20 mM NH_4^+**
Cell Integrity (%)	10.91 (± 1.59) A	13.66 (± 1.07) A	10.11 (± 0.74) A
P_N (μmol m^{-2} s^{-1})	21.08 (± 0.40) A	24.10 (± 1.10) A	24.35 (± 1.67) A
Ci (Pa)	29.02 (± 0.49) A	27.30 (± 0.74) A	27.07 (± 0.69) A
g_S (mol m^{-2} s^{-1})	0.46 (± 0.04) A	0.44 (± 0.06) A	0.43 (± 0.01) A
E (mmol m^{-2} s^{-1})	9.97 (± 0.39) A	9.45 (± 0.55) A	9.57 (± 0.10) A
Φ PSII	0.18 (± 0.01) B	0.26 (± 0.01) A	0.23 (± 0.02) AB

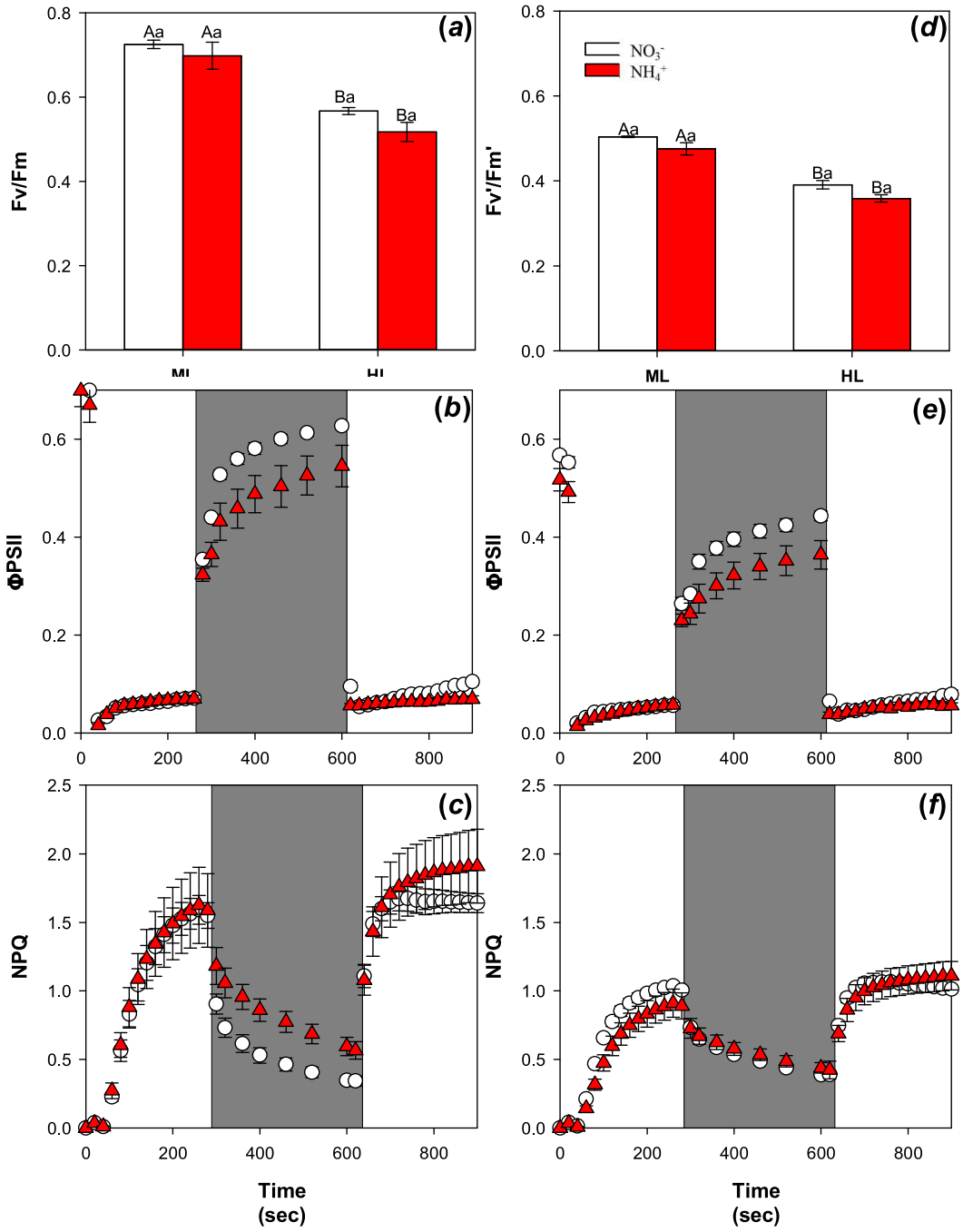

Figure 1 Photochemical kinetics of rice leaves (intact plants) grown with 10 mM NO_3^- and 10 mM NH_4^+ as N source by 7 days. After 7 days plants were exposed to 8 hours of moderate light (ML – 400 µmol m^{-2} s^{-1}) or high light (HL – 2000 µmol m^{-2} s^{-1}). (a) Maximum quantum efficiency of PSII in 30 min dark-acclimated leaves – Fv/Fm. (b) Maximum quantum efficiency of PSII in light-acclimated leaves – Fv'/Fm'. (c,d) Actual quantum efficiency of PSII – ΦPSII, (e,f) Actual quantum efficiency of PSI – ΦPSI. Photochemical parameters were noted in response to time (900 s), with 5 min of light induction (0-300 s), 5 min of dark relaxation (300-600 s) and 5 min of light re-induction (600-900 s). Values represented indicate average of three independent replicates (n=3) and bars represent S.E.M. Different letters mean significant difference at 5%, according to Tukey tests.

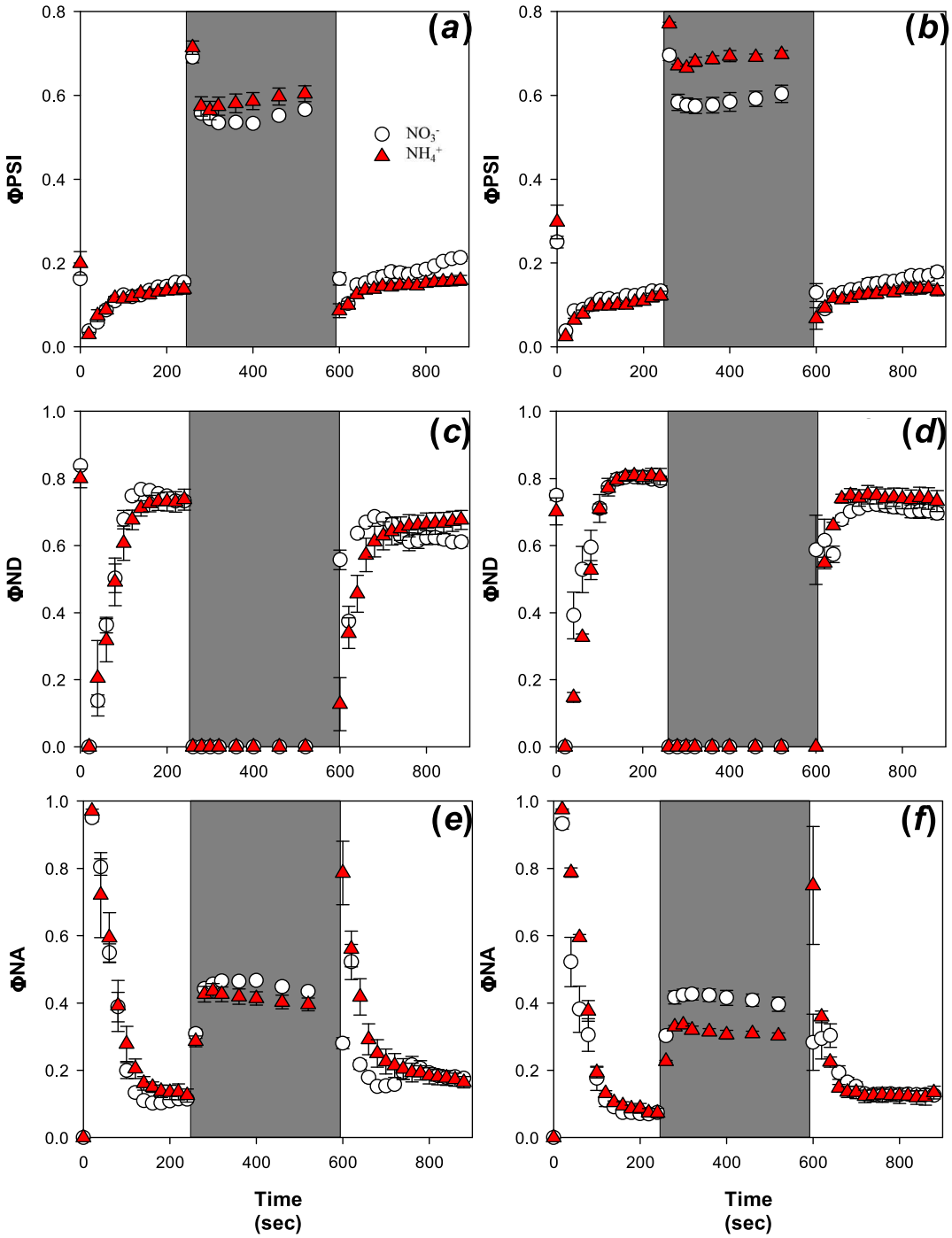

Figure 2 Photochemical kinetics of rice leaves (intact plants) grown with 10 mM NO_3^- and 10 mM NH_4^+ as N source by 7 days. After 7 days plants were exposed to 8 hours of moderate light (ML – 400 µmol m^{-2} s^{-1}) or high light (HL – 2000 µmol m^{-2} s^{-1}). (a,b) Actual quantum efficiency of PSI – ΦPSI, (c,d) PSI donor side limitation – ΦND and (e,f) PSI acceptor side limitation – ΦNA. Photochemical parameters were noted in response to time (900 s), with 5 min of light induction (0-300 s), 5 min of dark. relaxation (300-600 s) and 5 min of light re-induction (600-900 s). Values represented indicate average of three independent replicates (n=3) and bars represent S.E.M.

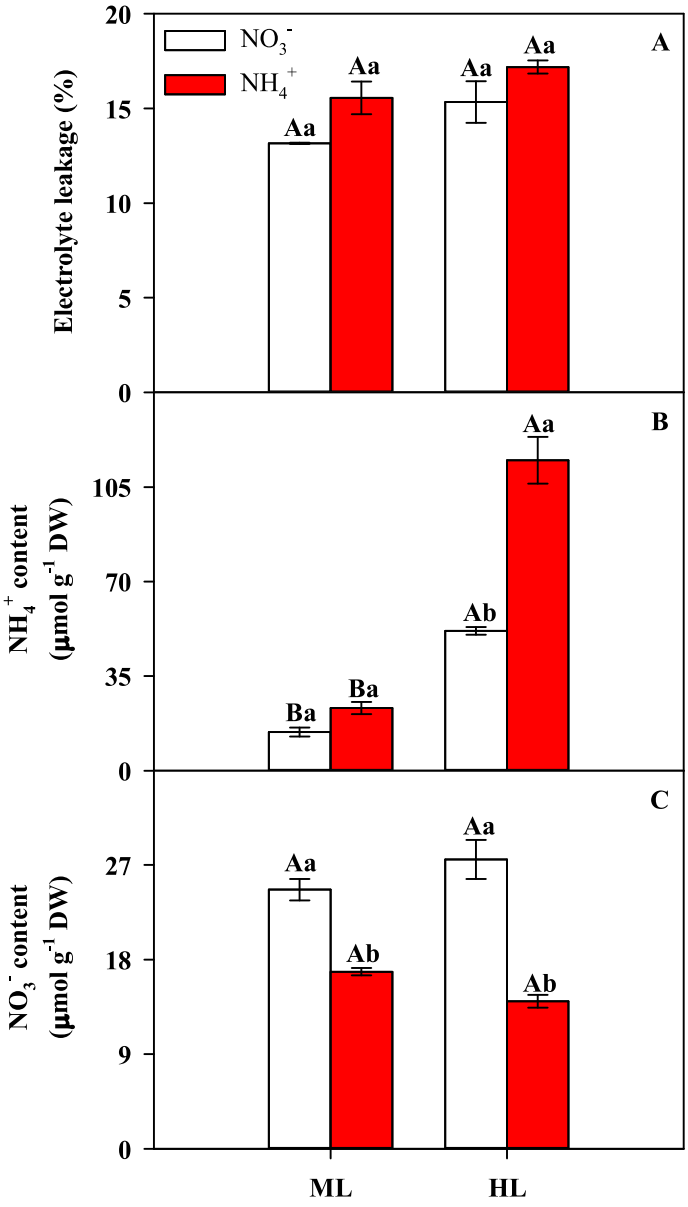

Figure 3 Electrolyte leakage (A), NH_4^+ (B) and NO_3^- (C) content in leaf segments of rice plants exposed to 10 mM NO_3^- or $NH_4^+$by 8 hours under moderate (400 mmol m^{-2} s^{-1}) or high (2000 mmol m^{-2} s^{-1}) light conditions. Each bar represents the average of four replicates (±SE), different capital and lowercase letters represent significant differences between light and N treatments, respectively, at 5% according to the t-test.

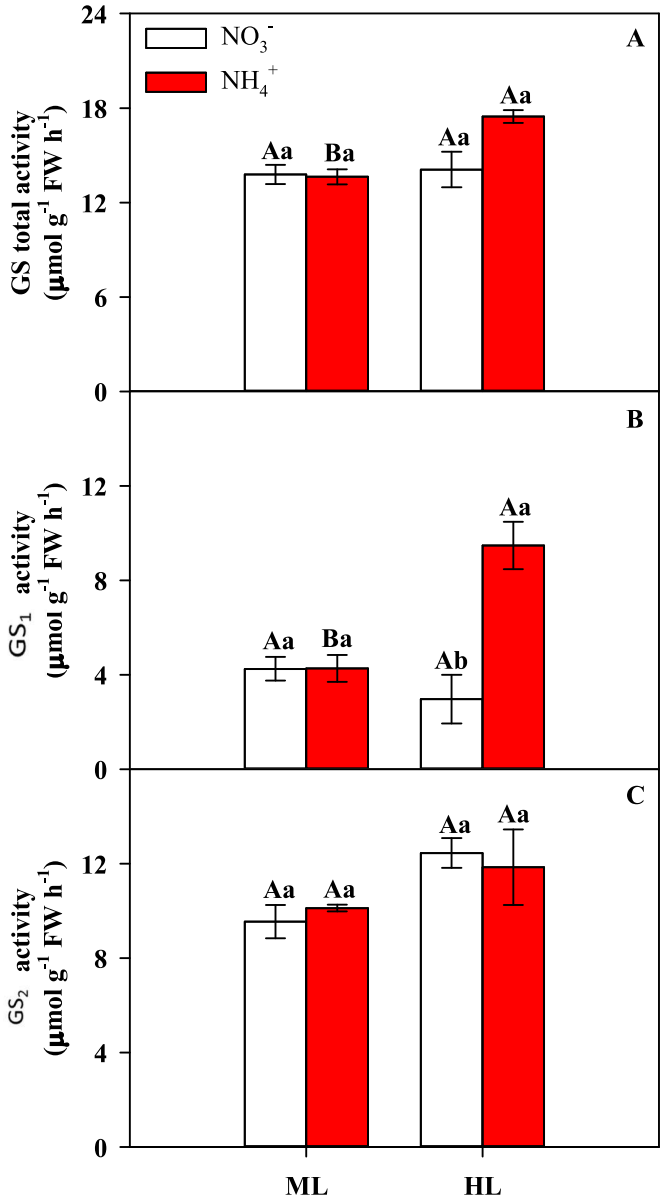

Figure 4 Activity of glutamine synthetase total (A), cytosolic glutamine synthetase isoform (B) and plastid glutamine synthetase isoform (C) in leaf segments of rice plants exposed to 10 mM NO_3^- or NH_4^+ by 8 hours under moderate (400 mmol m^{-2} s^{-1}) or high (2000 mmol m^{-2} s^{-1}) light conditions. Each bar represents the average of four replicates (±SE), different capital and lowercase letters represent significant differences between light and N treatments, respectively, at 5% according to the t-test.

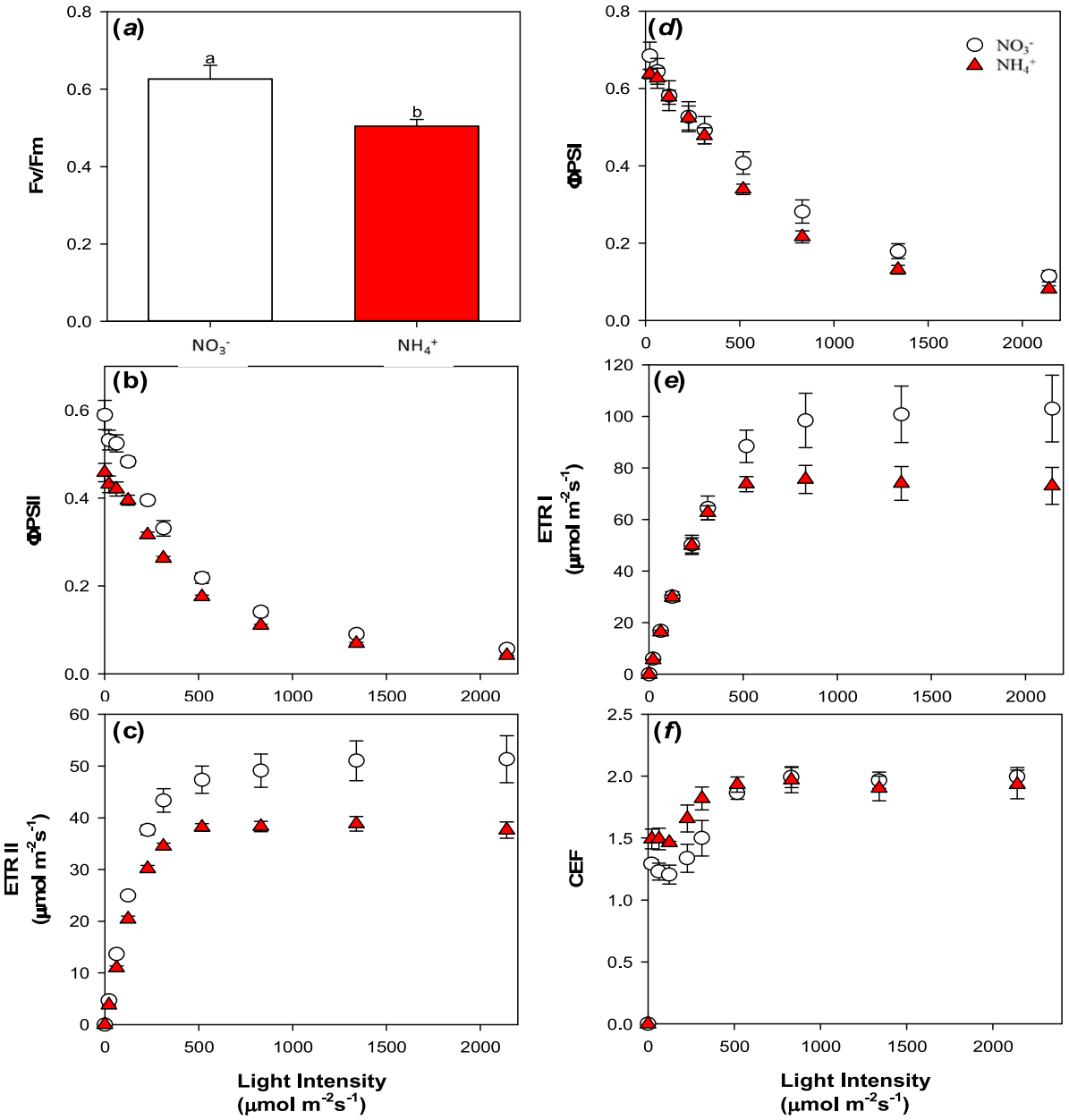

Figure 5 Photochemical activity of rice leaf segments exposed to 10 mM NO_3^- and 10 mM NH_4^+ by 8 hours. (a) Maximum quantum efficiency of PSII in 30 min dark-acclimated leaves – Fv/Fm. (b) Actual quantum efficiency of PSII – ΦPSII, (c) electron transport rate from PSII – ETRII, (d) Actual quantum efficiency of PSI – ΦPSI, (e) electron transport rate from PSI – ETRI and (f) and apparent cyclic electron flux – CEF. Photochemical parameters were noted in response to increased light intensities (2 min at each light intensity). Values represented indicate average of three independent replicates (n=3) and bars represent S.E.M. Different letters mean significant difference at 5%, according to Tukey tests.

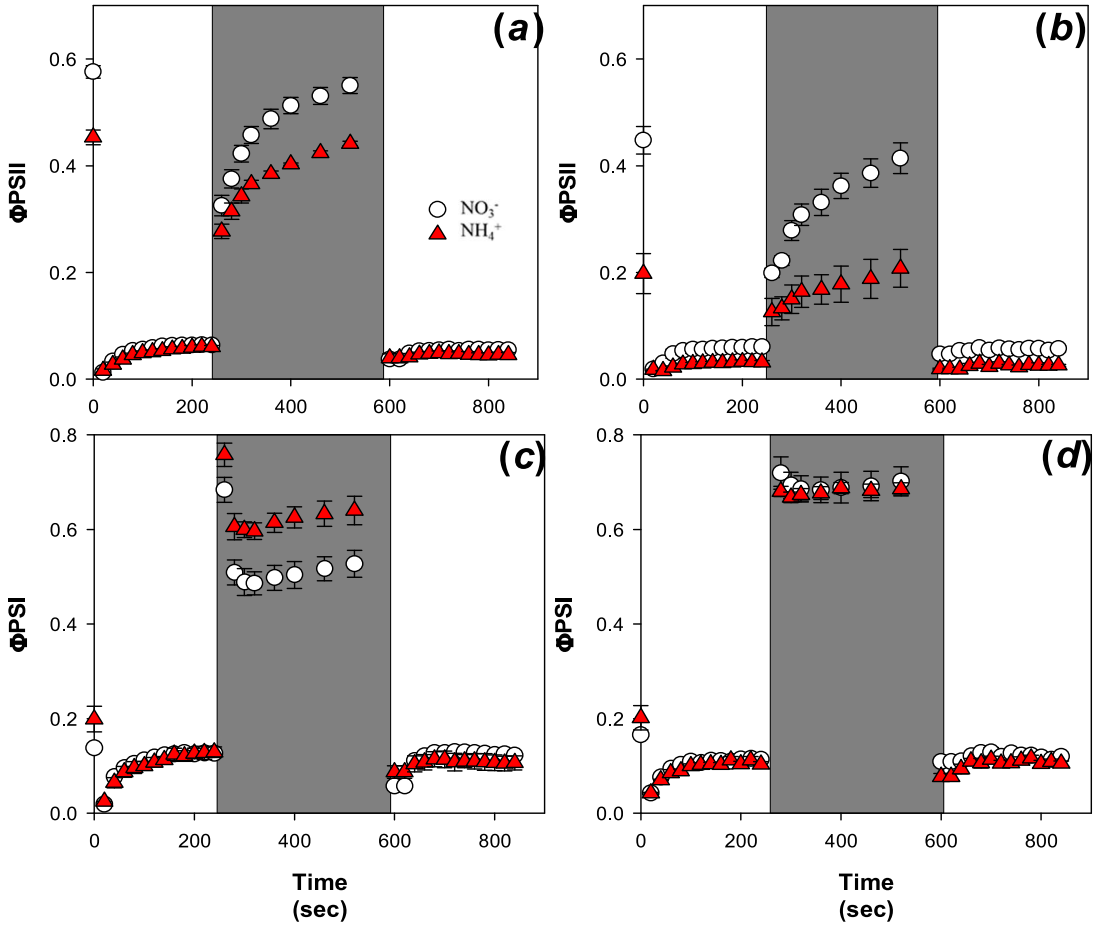

Figure 6 Photochemical kinetics of rice leaf segments exposed to 10 mM NO_3^- and 10 mM NH_4^+ by 8 hours at moderate light (ML – 400 µmol m^{-2} s^{-1}) or high light (HL – 2000 µmol m^{-2} s^{-1}). (a,b) Actual quantum efficiency of PSII – ΦPSI, (c,d) Actual quantum efficiency of PSI – ΦPSI. Photochemical parameters were noted in response to time (900 s), with 5 min of light induction (0-300 s), 5 min of dark relaxation (300-600 s) and 5 min of light re-induction (600-900 s). Values represented indicate average of three independent replicates (n=3) and bars represent S.E.M.

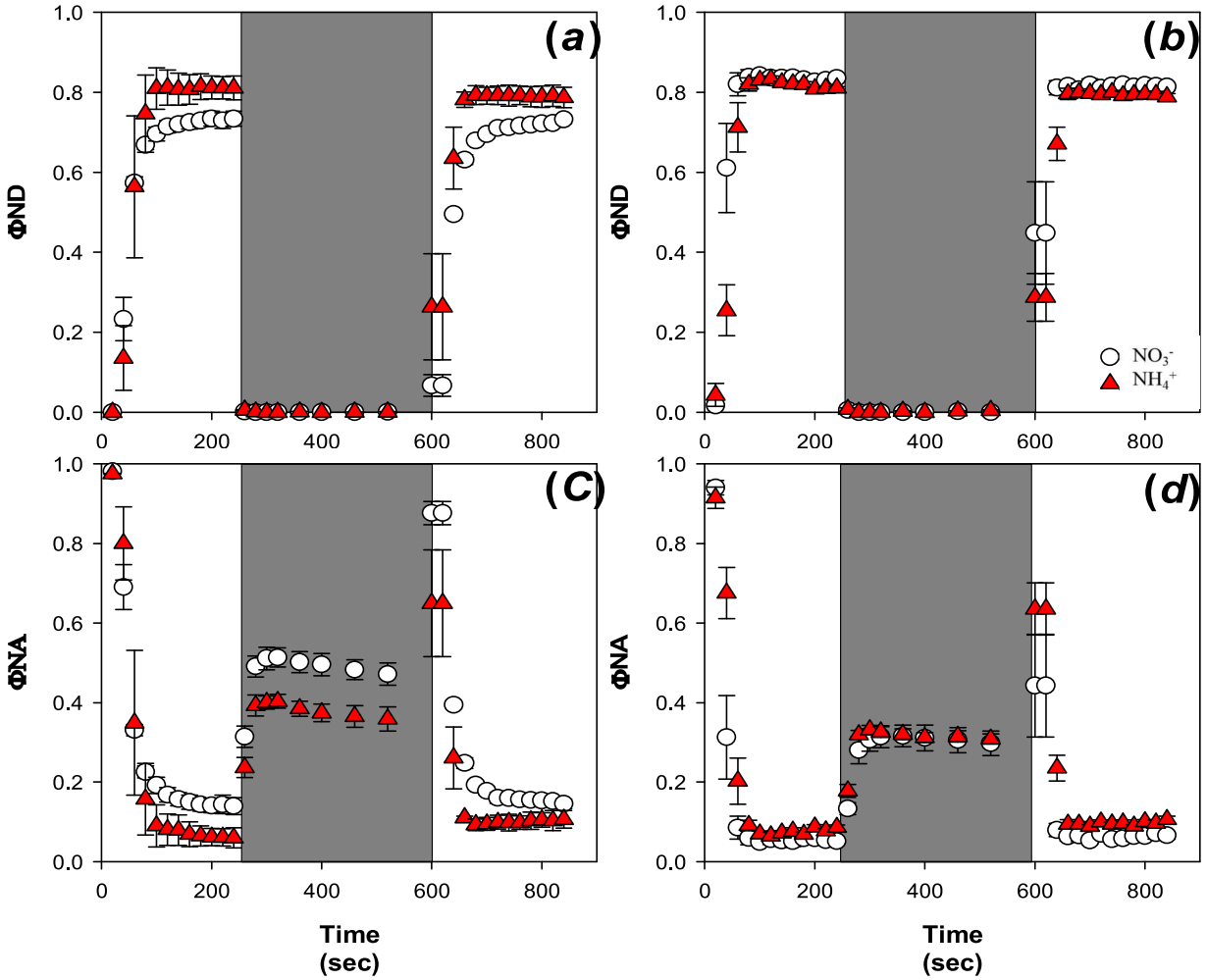

Figure 7 Photochemical kinetics of rice leaf segments exposed to 10 mM NO_3^- and 10 mM NH_4^+ by 8 hours at moderate light (ML – 400 µmol m^{-2} s^{-1}) or high light (HL – 2000 µmol m^{-2} s^{-1}). (a, b) PSI donor side limitation – ΦND and (c,d) PSI acceptor side limitation – ΦNA. Photochemical parameters were noted in response to time (900 s), with 5 min of light induction (0-300 s), 5 min of dark relaxation (300-600 s) and 5 min of light re-induction (600-900 s). Values represented indicate average of three independent replicates (n=3) and bars represent S.E.M.

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APÊNDICE A

Figura 1: Crescimento e desenvolvimento de plantas de arroz (*Orysa sativa* sp japonaica cv nipponbare) em casa de vegetação.

Figura 2: Experimento fluxo de [NH_4^+] na seiva xilemática em plantas de arroz (*Oryza sativa* sp Japonaica cv Nipponbare).

Figura 3: Experimento dose dependente de amônio em cultivares de arroz de sequeiro (*Oryza sativa* sp Indica BRS Sertaneja, Pepita e Primavera). Na primeira imagem semente da cultivar de arroz de sequeiro BRS Sertaneja.

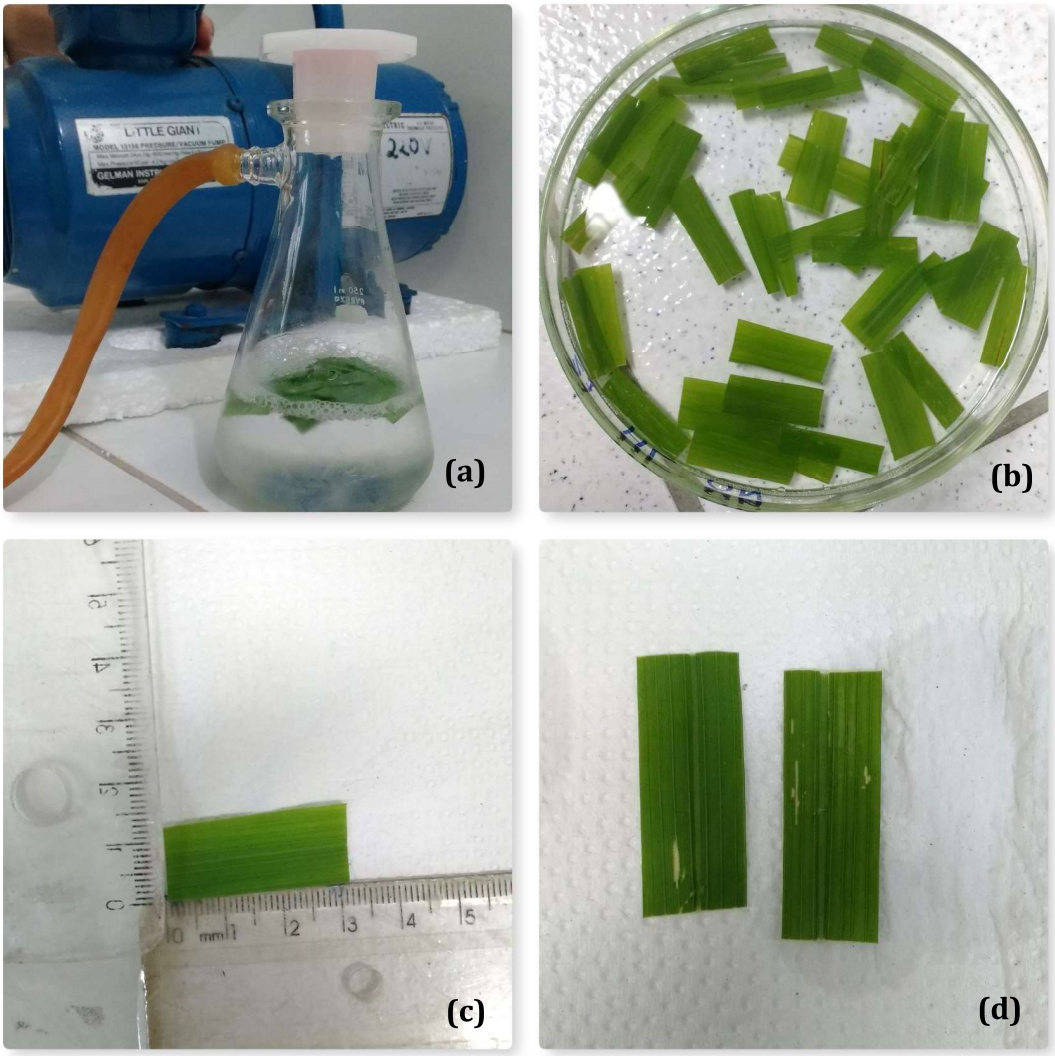

Figura 4: Experimento com segmentos de folhas de arroz (Oryza sativa sp Japonica cv Nipponbare). (a) Segmentos de folhas de arroz em kitasato com 5 ml de solução com 10 mM de NO_3^- ou NH_4^+ sendo filtrados com bomba a vácuo (b) após incubação com solução de NO_3^- ou NH_4^+ em placa de petri (c) medindo 3 cm cada (d) após todo processo de filtração e incubação.

Figura 5: Experimento em seguimento de folha de arroz e planta inteira em câmera de crescimento.

APÊNDICE B

How tolerant is rice plants to ammonium excess? New insights from photochemical responses under two contrasting light regimes

SUPPLEMENTARY MATERIAL

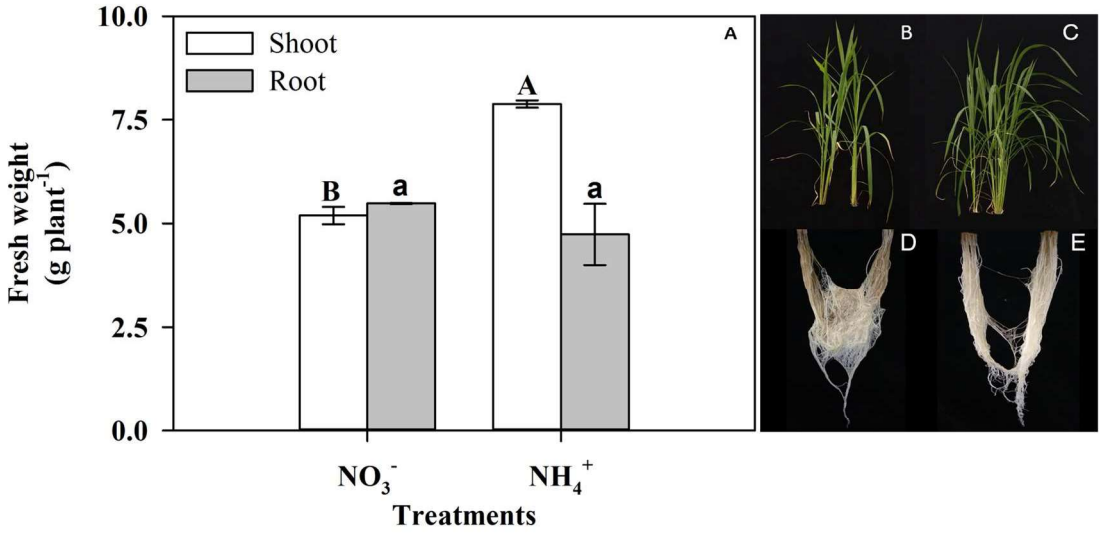

Figure S1. Fresh weight of shoot and root (A) and morphological aspects of representative shoot (B, C) and root (D, E) of rice plants exposed to 10 mM NO_3^- or NH_4^+ by 21 days under greenhouse conditions. Each bar represents the average of four replicates (±SE), different letters represent significant differences at 5% between treatments according to the t-test.

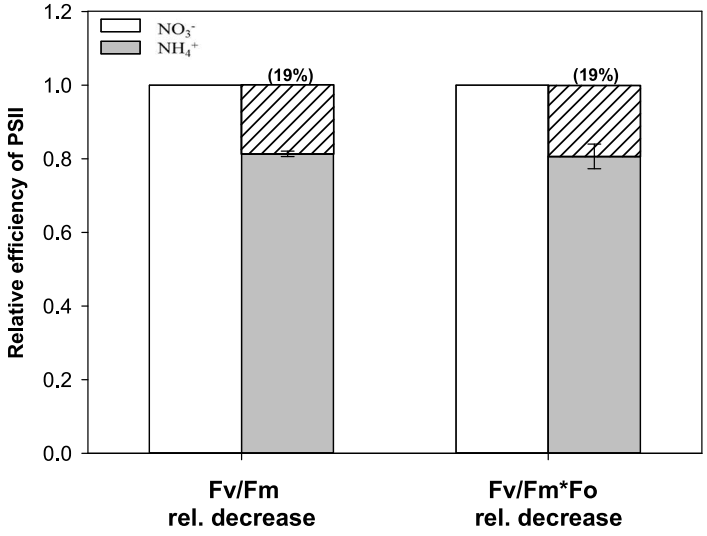

Figure S2. Photoinhibition (Fv/Fm relative decrease) and PSII down-regulation (Fv/Fm*Fo) in rice leaf segments exposed to 10 mM NH_4^+ by 8 hours at moderate light (ML – 400 µmol m^{-2} s^{-1}). Values are relative to Fv/Fm and Fv/Fm*Fo noted in leaf segments exposed to 10 mM NO_3^- at similar environmental conditions. Values are average of three independent replicates (n=3) and bars represent S.E.M. Inside brackets are represented the percentage of each relative decrease.