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FRANCISCO WILLIAM VIANA MARTINS

PHOTOSYNTHETIC EFFICIENCY MECHANISMS INVOLVED WITH TOLERANCE TO EXCESS AMMONIUM AND LIGHT IN RICE PLANTS

FORTALEZA
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OPPONENTS

_____
Prof. Dr. Joaquim Albenísio Gomes da Silveira (Advisor)
Federal University of Ceará (UFC)

_____
Prof. Dr. Fabrício Eulálio Leite Carvalho
Federal University of Ceará (UFC)

_____
Prof. Dra. Rosilene Oliveira Mesquita
Federal University of Ceará (UFC)

_____
Prof. Dra. Ana Luiza Sobral Paiva
Federal University of Ceará (UFC)

My mother and father, who, even in the difficulties of life, strove to make my dreams come true, supporting me and helping me at all stages of my life, I dedicate this work.

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Chico Xavier

RESUMO

Plantas podem obter nitrogênio do solo em duas formas principais: nitrato e amônio. Em particular, essa última é predominate em solos alagados onde a baixa oxigenação possibilita sua acumulação. Todavia, diversos efeitos negativos associados ao acúmulo de amônio em plantas podem ocorrer, incluindo desde modificações morfológicas até modificações metabólicas. Para investigar como as plantas tolerantes ao amônio podem proteger seu sistema fotoquímico, o arroz, uma espécie conhecidamente amônio-tolerante, foi exposto ao suprimento exclusivo de amônio (10 mM) ou nitrato (10 mM) como fonte N por até oito dias e exposto à luz alta (2000 µE) e luz moderada (400 µE). As plantas fornecidas com amônio não apresentaram alterações significativas na atividade do PSII sob luz moderada, em comparação com as plantas nutridas com nitrato. Sob alto suprimento de NH_4^+ e a luz alta, as plantas de arroz apresentaram um atraso significativo na recuperação do PSII escuro (50%) somente após o sexto dia de exposição. Em contrapartida, quaisquer alterações significativas relacionadas ao acúmulo de proteínas fotoquímicas importantes, como PsbA, PsbE, PsbS, CP43 e LCA1, foram observadas nas plantas de amônio e nitrato. Paralelamente, nenhuma evidência significativa de estresse oxidativo nas proteínas localizadas na fração tilacoidal foi observada nas duas fontes-N. Curiosamente, as plantas fornecidas com amônio apresentaram maior indução da luz dependente do tempo no NPQ associada à diminuição do relaxamento no escuro após o sexto dia, o que coincide com o atraso na recuperação do PSII no escuro. Posteriormente, a análise BN-PAGE em tilacóides também revelou que as plantas de amônio acumularam significativamente maiores quantidades de trímeros de LHCII-CP24-CP29 e LHC livres sob alta luz. Tomados em conjunto, nossos resultados sugerem que o alto teor de amônio é capaz de induzir retardo na recuperação do PSII, o que provavelmente não depende exclusivamente do acúmulo de proteínas fotoquímicas ou danos oxidativos nas proteínas dos tilacóides. Caso contrário, o alto teor de amônio combinado com a alta luz é capaz de desencadear alterações no estado de agregação dos subcomplexos da antena PSII, que podem afetar diretamente a atividade de NPQ e PSII. Esses processos podem representar mecanismos fotoprotetores incomuns que podem ser essenciais para preservar a fotossíntese em plantas de arroz cultivadas em ambientes extremos de alto teor de amônio e excesso de luz, onde o fechamento estomático é frequente, o que pode contribuir para agravar o balanço energético prejudicial nos cloroplastos.

Palavras-chaves: *Oriza sativa*. Alta luz. Luz moderada. Amônio. PSII.

ABSTRACT

Plants can obtain nitrogen from the soil in two main forms: nitrate and ammonium. In particular, the latter is predominant in flooded soils where low oxygenation allows its accumulation. However, several negative effects associated with the accumulation of ammonium in plants can occur, including from morphological changes to metabolic changes. To investigate how ammonium tolerant plants can protect its photochemical system, rice was exposed to sole ammonium (10 mM) or nitrate (10mM) supply as N-sources for up to eight days and exposed to high light (2000 µE) and moderate light (400 µE). Ammonium supplied plants did not exhibit any significant changes in PSII activity under moderate light, as compared to nitrate plants. Under both high NH_4^+ and light, rice plants exhibited a significant delay in dark PSII recovery (50%) only after the sixth day of exposure. In opposition, any significant change related to the accumulation of important photochemical proteins, such as PsbA, PsbE, PsbS, CP43 and LCA1 were observed in both ammonium and nitrate plants. In parallel, no significant evidence of oxidative stress in proteins localized in the thylakoidal fraction was observed in both N-sources. Interestingly, ammonium supplied plants displayed higher time-dependent light induction in NPQ associated with decrease in dark relaxation after the sixth day, which coincides with delay in dark PSII recovery. Subsequently, the BN-PAGE analysis in thylakoids also revealed that ammonium-plants significantly accumulated higher amounts of free LHCII-CP24-CP29 and free LHC trimmers in high light. Taken together, our results suggest that high ammonium is able to induce delaying in PSII recovery, which is probably not dependent exclusively on photochemical proteins accumulation or oxidative damage on thylakoidal proteins. Otherwise, high ammonium combined with high light is able to trigger changes on the aggregation state of PSII antenna sub-complexes, which can affect directly NPQ and PSII activity. These processes may represent unusual photoprotective mechanisms that might be essential to preserve photosynthesis in rice plants grown under extreme environments of high ammonium and excess light where stomatal closure is frequent, which can contribute to aggravating the harmful energy balance in chloroplasts.

Keywords: *Oriza sativa*. High light. Moderate light. Ammonium. PSII.

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ABBREVIATION LIST

$(NH_4)_2CO_3$	Ammonium carbonate
$(NH_4)_2SO_4$	Ammonium sulfate
AMT	Ammonium transporter
ATP	Adenosine triphosphate
CO_2	Carbon dioxide
ROS	Oxigen-reactive species
Fv/Fm	Maximum quantum efficiency of photosystem II
Fv'/Fm'	Maximum quantum efficiency of photosystem II leaves acclimated to light
H^+ - ATPase	Proton bomb
HL	High light
ML	Moderate light
mM	Millimolar
N	Nitrogen
NAD	Nicotinamide and adenine dinucleotide
NH_3	Ammonia
NH_4	Ammonium
pH	Hydrogen potential
NPQ	Non-photochemical quenching
Pi	Inorganic Phosphate
qE	Thermal component of NPQ
RCII	PSII reaction center
P_N	Liquid CO_2 assimilation
pKa	Ionization constant potential
PSI	Photosystem I
PSII	Photosystem II
RN	Nitrate reductase
RNi	Nitrite reductase
ΔpH	H^+ variation
Y(NA)	Acceptance side limitation
Y(ND)	Donor side limitation
ΦPSI	Real quantum efficiency of photosystem I

ΦPSII	Real quantum efficiency of photosystem II
NH_4Cl	Ammonium chloride
NH_4NO_3	Ammonium nitrate
N_2	Liquid nitrogen
K_m	Michaelis-Menten constant

SUMMARY

1 GENERAL INTRODUCTION

Among the essential elements present in the soil, nitrogen (N) is the mineral element most required by plants, as it is the primary constituent of nucleotides, proteins and chlorophylls, thus being, commonly, the main restrictive factor for their growth and development (LEA; AZEVEDO , 2006). For this reason, there will be a need to carry out high applications of nitrogen fertilizers in the soil, since their availability will directly define the quality and the productivity rate of the crops (MOTA et al., 2015). Therefore, nitrogen fertilizers are often applied to the soil, which have high concentrations of ammonium and nitrate in order to meet crop requirements. The main inorganic forms of N used in fertilizers are ammonium nitrate (NH_4NO_3), ammonium sulfate ($(NH4)_2SO_4$) and ammonium phosphate ($NH_4H_2PO_4$). In addition to these salts, urea ($CO(NH_2)_2$) and / or anhydrous ammonia (NH_3) can also be applied to the soil surface (BITTSÁNSZKY et al., 2015).

The vast majority of plants absorb predominantly the ammonium (NH_4^+) and nitrate (NO_3^-) ions as sources of N (SARASKETA et al., 2016). In the soil the proportion of each in the soil will depend on the environmental factors, the chemical and physical characteristics of the soil (pH and organic matter), the rates of nitrification and ammonification in the soil and its use (MARINO et al., 2016). In most agricultural soils, NO_3^- is the main source of nitrogen for plants, being preferentially absorbed by most cereals, with the exception of rice, where the NH_4^+ form is considered ideal (KANT et al., 2011; WANG et al., 1993). In well-aerated soils, the concentration of NO_3^- is generally higher than that of NH_4^+, whereas in the cultivation of wetlands, the opposite occurs. This modification of the availability of mineral N forms in the soil promotes a series of different responses in the metabolism of plants (MARSCHNER, 2011). These changes are still highlighted due to the fact that the cultivation conditions in flooded soils commonly involve the concomitant occurrence of high light, a recurrent situation in rice growing systems in tropical regions (ALENCAR et al., 2019).

Regardless of how plants acquire nitrogen, this nutrient is only assimilated, that is, structurally incorporated into biomolecules, starting from NH_4^+ and, therefore, its direct absorption implies lower energy costs than NO_3^- (MEHRER; MOHR, 1989). After being absorbed, nitrate is first reduced to nitrite, which in turn must be quickly reduced to ammonium. This process uses high energy, a consumption equivalent to 12 ATP (BLOOM et al., 1992). However, ammonium becomes a paradox, since high concentrations of this ion inside cells cause phytotoxicity (LI et al., 2014). In addition, this phytotoxic potential should

be further aggravated in the presence of excess light, given that the energy input in these conditions largely exceeds the metabolic demand.

The susceptibility of ammonium toxicity has been described in *Arabidopsis thaliana* and plants of the Brassicaceae family, which generally present a reduction in biomass and a decrease in the growth of the aerial part, leaf chlorosis and atrophied root when exposed to high concentrations of this ion (SARASKETA et al., 2014; LI et al., 2014). However, other species are considered tolerant to high concentrations of NH_4^+, and are often described as NH_4^+ -specialists, such as rice (*Oryza sativa*. L). In fact, this species is generally grown in flooded environments, which only contain ammonium as a primary source of N (WANG et al., 1993a, b; BRITTO; KRONZUCKER, 2002; CRUZ et al., 2006; ESTEBAN et al., 2016). So, because it grows under conditions of high concentrations of N-ammoniacal, flooded rice is considered one of the most tolerant species to NH_4^+ (WANG et al., 1993a, b).

One of the most common symptoms of ammonium toxicity in plants is their ability to decouple the H^+ gradient, decrease photosynthesis and consequently induce oxidative stress (BRITTO et al., 2002; BITTSÀNSZKY et al., 2015). In fact, several classic studies have reported that one of the main toxic effects of ammonium in plants is the depolarization of membranes, which promotes a reduction in ATP synthesis and several metabolic routes, including photosynthesis (BITTSÁNSZKY et al., 2015). It is believed that the deleterious effects of NH_4^+ on the photosynthetic apparatus are related to the reduction of the activity of the oxygen evolution complex and the proton gradient in the thylakoid membrane (NAVARRO et al., 2013; OYALA et al., 2015). However, currently several studies have shown that increased ammonium concentration does not cause major changes in cytoplasmic electrochemical potential and, therefore, this effect would not be the primary cause of inhibition of photosynthesis and other metabolic processes (BITTSÁNSZKY et al., 2015; ESTEBAN et al., 2016). Therefore, the molecular mechanisms related to the occurrence of ammonium-induced toxicity, as well as the defense processes triggered in resistant plants, are still poorly understood.

Several studies have been applied in order to understand the effects of both toxicity and tolerance. To explain the effects that lead to NH_4^+ toxicity in plants, some hypotheses have been proposed, such as the reduction of root carbon induced by the assimilation of NH_4^+ (FINNEMANN; SCHJOERRING, 1999), mineral deficiencies (SIDDIQI et al., 2002) and in Protein N-glycosylation (BARTH et al., 2009) and mainly the NH_4^+ futile cycle (BRITTO et al., 2001; KRONZUCKER et al., 2001; BRITTO; KRONZUCKER, 2002; SZCZERBA et al., 2008). Some studies indicate that tolerance to

high ammonium concentrations is related to the greater activity of glutamine synthetase (GS) and less accumulation of free NH_4^+ in plant tissues (MAGALHÃES; HUBER, 1991; BALKOS; BRITTO; KRONZUCKER, 2010). The increase in the 0vacuole has also been reported as a mechanism that some plants perform to prevent the damage caused by the excessive accumulation of NH_4^+ (BRITTO et al., 2001; SZCZERBA et al., 2008). Although the understanding of NH_4^+ toxicity and / or sensitivity has improved in recent decades, the mechanisms involved in this process have not yet been fully clarified (ESTEBAN et al., 2016).

2 CHAPTER ONE: LITERATURE REVIEW

2.1 INTRODUCTION

Nitrogen (N) has attracted attention in recent years because of its importance in plant development, because of its presence as a constituent of various plant molecules and because it is required at many stages of plant metabolism (KANT et al., 2018). For this reason, plants have a preference for specific forms of N, such as nitrate (NO_3^-) and ammonium (NH_4^+) (TANG et al., 2020). Ammonium-nourished plants often exhibit symptoms of toxicity, such as morphological and metabolic changes. Regarding these modifications, some authors also address the effects caused by the combined action between ammonium and high light in plants and photosystems I and II were the most affected (ALENCAR et al., 2019; BRITTO et al., 2002). Although the understanding of the causes of NH_4^+ sensitivity/tolerance has greatly improved over the past decades, the characteristics of the plants responsible for the sensor tolerance or NH_4^+ sensitivity of the plant remain uncertain. This review highlights the major responses to NH_4^+ toxicity compared to NO_3^-, showing its effects at the structural and metabolic levels.

2.2 NO_3^- / NH_4^+ absorption and transportation

Among all the mineral nutrients present in plants, nitrogen is the most required, as it is the primary constituent of nucleotides and proteins, being therefore the main limiting factor for their growth (LEA; AZEVEDO, 2006; KANT, 2018). Given its importance, plants preferentially absorb N as ammonium (NH_4^+) and nitrate (NO_3^-) ions (SARASKETA et al., 2016). External factors such as pH can lead to one of these two nitrogenous forms. But this absorption is subject to strict regulation according to total plant demand (CRAWFORD; GLASS, 1998). The pKa value for NH_4^+ ion deprotonation to change to NH_3^- is 9.24. Therefore, pH values greater than 9.25 are predominantly the neutral nitrogen form (HOWITT; UDVARDI, 2000; BITTSÁNSZKY ET AL., 2015). Both forms of N can be absorbed by plants, but which form predominates in plant uptake is not yet fully understood. But some experiments using tea plants show that preferential uptake of NH_4^+ occurs. (KRONZUCKER; SIDDIQI; GLASS, 1996; YUAN et al., 2007; COSKUN et al., 2013; TANG et al., 2020).

NH_4^+ absorbance by plants has a lower energy cost than NO_3^- (MEHRER; MOHR, 1989). After being absorbed, nitrate by the action of the enzyme nitrate reductase is reduced to

nitrite which in turn is reduced to ammonium by the action of the enzyme nitrite reductase. This process uses high energy, a consumption equivalent to 12 ATP as shown in **Figure 1** (BLOOM et al., 1992; HAGEMAN et al., 1971; LOSADA; PANEQUE, 1971). Regardless of how plants uptake nitrogen, it is either assimilated or incorporated into biomolecules from NH_4^+. However, ammonium becomes a paradox since it is elevated from within cells causing phytotoxicity (LI et al., 2014).

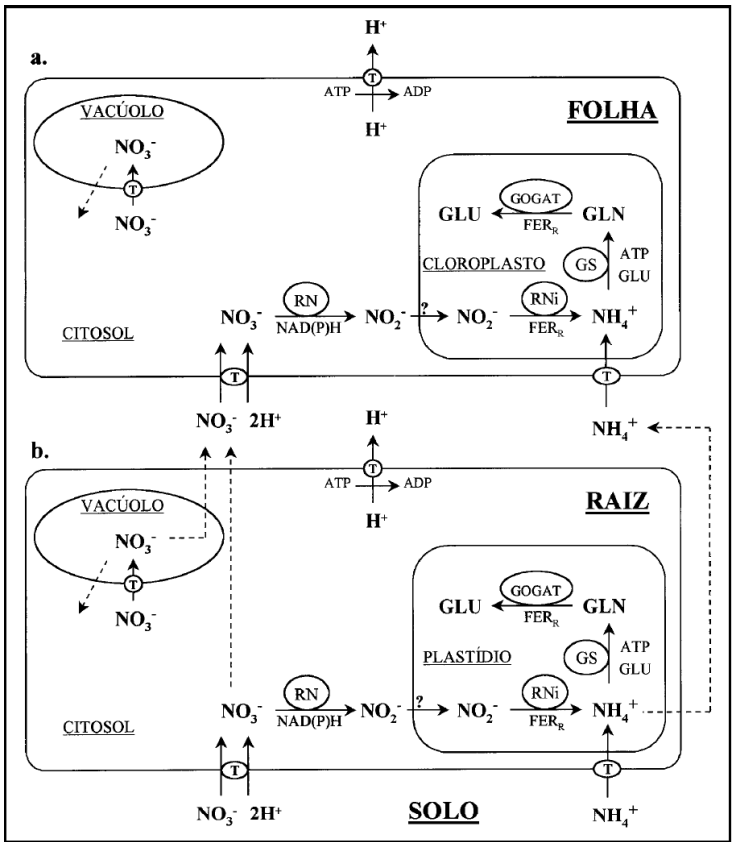

Figure 1 Schematic representation of the nitrogen assimilation pathway in the roots and leaves of plants. (NO_3^- nitrate; NO_2^- nitrite; NH_4^+ ammonium; GLN: glutamine; GLU: glutamate; RN: nitrate reductase; Rni: nitrite reductase; GS: glutamine synthetase; GOGAT: glutamate synthetase; T: transporter). Source: Bredemeier and Mundstock (2000).

Ammonium uptake in plant roots is accomplished through membrane-integrated carrier proteins that are classified into Ammonium Transporter (AMT) families, mainly AMT1 and AMT2. The amount of ammonium absorbed depends on the studied species. (LI et al., 2016; LOQUÉ; VON WIRÉN, 2014). In order to respond to the plant's external N availability and internal N concentration, AMT gene expression is highly controlled in the roots at various regulatory levels. At the transcriptional level, ammonium supply can regulate

or decrease the expression of AMT genes, which is likely triggered by downstream ammonium metabolites, i.e. glutamine (GAZZARRINI et al., 1999; RAWAT et al., 1999; VON WIRÉN et al., 2000; SONODA et al., 2003a, b; WU et al., 2016). In experiments aimed at investigating whether root AtAMT1;3 transporter activity responds differently to ammonium or nitrate replenishment in Arabidopsis mutants. Wu et al., 2019 have observed post-translational regulation, as abundances of AtAMT1;3 transcribed proteins did not change accordingly.

Nitrate, in turn, can be absorbed into the root system through membrane carriers classified as NTR and NPF genes, can be temporarily stored in the vacuole or reduced to NH_4^+, which is mainly used for glutamine and asparagine synthesis. These two amino acids can be carried to the leaves by the transpiratory flow in the xylem vessels. In several species when the capacity for nitrate assimilation by the roots is saturated, the NO_3^- released from the roots to the xylem vessels is carried by the transpiratory flow to the leaves. Large amounts of nitrate can be stored in leaf vacuoles (HELDT, 1997). Different evolutionary pressures led each plant species to develop a preference for a particular source among N forms, namely ammonium (NH_4^+) and nitrate (NO_3^-) (BOUDSOCQ et al., 2012). However, deleterious responses can be triggered by ammonium in many plant species as mentioned above.

To understand the relationship between membrane transporter gene expression and NH_4^+ influx, Kumar et al (2003) performed experiments using rice plants under high irradiance and high NH_4^+ supply. The results showed that gene expression occurs according to the concentration of NH_4^+ present outside the roots, thus causing a negative regulation when rice plants are exposed to 10 mM NH_4^+. Regarding the effect of irradiance it was observed that in day time the gene expression was higher than in night time.

2.3 Physiological, biochemical and biophysical mechanisms of NH_4^+ toxicity

Toxicity caused by NH_4^+ is not a new subject. This phenomenon was noted by Chales Darwin in 1882 who demonstrated that *Euphorbia peplus* plants had inhibition in their growth after being cultivated with ammonium carbonate salt ($(NH_4)_2CO_3$). Since then, several studies have been conducted to clarify the damage done by this ion. As reported in a critical review of ammonium toxicity, BRITTO AND KRONZUCKER (2002) listed susceptible and tolerant plants.

Among the notable symptoms attributed to ammonium phytotoxicity, these biochemical and physiological changes, such as changes in intracellular pH, intracellular

alkalinization and extracellular acidification, osmotic imbalance, induction of nutritional deficiencies caused by inhibition of cation absorption (K^+, Mg^{2+} or Ca^{2+}), root respiration inhibition and photorespiration stimulation, interference with photosynthetic activity (lower CO_2 assimilation rate, stomatal conductance and transpiration), increased H_2O_2 content causing oxidative stress and decreased chlorophyll a, b and carotenoids, change in enzyme activities related to NH_4^+ uptake and high energy cost to maintain low ammonium levels in cytosol (GERENDÁS et al., 1997; CRUZ et al., 2003; DOMÍNGUEZ-VALDIVIA et al., 2008; ARIZ et al., 2010; CRUZ et al., 2001; SHENGQI et al., 2012; BORGOGNONE et al., 2013; BITTSÁNSZKY et al., 2015).

The main visible symptoms of ammonium toxicity are stunted roots and leaf chlorosis (BRITTO; KRONZUCKER, 2002; LI et al., 2014). Ammonium toxicity is typically observed when plants are exposed to high exogenous concentrations or when plant cells are under stressful environmental conditions. Under such conditions high ammonium production occurs through increased proteolytic activities and photorespiration (SKOPELITIS et al., 2006). In a study with $^{13}NH4^+$, Britto et al (2001) found that in susceptible species the efflux rate show that these plants have little assimilation of this nitrogen source. With the increase of efflux, the extracellular and intracellular environment is modified. Normally this happens by absorbing ammonium thus rhizosphere becomes acidic while the cytosol becomes alkaline (MARSCHNER, 2011).

In tolerant plants such as rice, it was observed that from 1 mM NH_4^+ a trans-membrane potential is generated, allowing the high flow of ammonium (BRITTO et al., 2001). Ammonium concentrations in healthy plant tissues are always believed to remain low and under these conditions it is suggested that NH_4^+ absorbed or formed in the roots is rapidly assimilated and not translocated to the shoots (TOBIN; YAMAYA, 2001).

2.4 Ammonium toxicity and its negative effects on photosynthesis

Susceptibility to ammonium toxicity has been described for *Arabidopsis thaliana* and plants from Brassicaceae family, which generally show a decrease in biomass, in shoot growth, leaf chlorosis and stunted roots when exposed to high concentrations of this ion (SARASKETA et al., 2014; LI et al., 2014). However, other species are described as tolerant to high concentrations of NH_4^+, such as rice plant (*Oryza sativa*. L) which is usually grown in flooded environments, where ammonium is in higher concentrations (WANG et al., 1993 a, b; BRITTO; KRONZUCKER, 2002; CRUZ et al., 2006; ESTEBAN et al., 2016). Rice plants

grown in flooded conditions, where high ammonium concentrations are found, are considered to be NH_4^+ tolerant species (WANG at al., 1993 a, b).

Some researchers show that one of the main toxic effect of ammonium on plants is membrane depolarization, which promotes reduced synthesis of ATP in various metabolic pathways, including photosynthesis as shown in table 1 (BITTSÁNSZKY et al., 2015). The deleterious effects of NH_4^+ on the photosynthetic apparatus are believed to be related to reduced activity of the oxygen evolution complex and proton gradient in the thylakoid membrane as shown in table 1 (PEREZ-NAVARRO et al., 2013; OYALA et al., 2015). However, some studies have shown that increasing ammonium concentration does not cause major changes in cytoplasmic electrochemical potential and therefore this effect would not be the primary cause of inhibition of photosynthesis and other metabolic processes (BITTSÁNSZKY et al., 2015; ESTEBAN et al., 2016).

Many studies have been conducted to understand the effects of both ammonium toxicity and tolerance. To explain the effects that lead to NH_4^+ toxicity in plants, some hypotheses have been proposed, such as the reduction of root carbon induced by NH_4^+ assimilation (FINNEMANN; SCHJOERRING, 1999), mineral deficiency (SIDDIQI et al., 2002) and disturbances. N-glycosylation of proteins increasing sensitivity to this ion as can be seen from table 1 (BARTH et al., 2009) and especially the futile cycle of NH_4^+ (BRITTO et al., 2001; KRONZUCKER et al., 2001; BRITTO; KRONZUCKER, 2002; SZCZERBA et al., 2008). Some studies indicate that tolerance and high ammonium concentrations are related to higher glutamine synthetase (GS) activity and lower free NH_4^+ accumulation in plant tissues (MAGALHÃES; HUBER, 1991; BALKOS; BRITTO; KRONZUCKER, 2010). Increased NH_4^+ uptake and transport of this ion to the apoplast (efflux) and / or to the vacuole has also been reported as a mechanism that some plants possess to prevent damage caused by excessive $NH4^+$ accumulation (BRITTO et al., 2001; SZCZERBA et al., 2008). In experiments involving relaxation kinetics, it is observed that ammonium-treated plants have a delay in PSII recovery compared to nitrate-treated plants. On the other hand, ammonium-treated plants have a faster PSI recovery than those undergoing nitrate treatments (ALENCAR et al., 2019). Although understanding of NH_4^+ toxicity and / or sensitivity has improved in recent decades, the mechanisms involved in these processes have not yet been fully clarified (ESTEBAN et al., 2016).

Table 1. Photosynthetic responses related to ammonium supply in plants.

Effects	Authors
Delayed PSII recovery, Severe NPQ induction; Delay in protein D1 turnover process;	Alencar et al., 2019
Reduction of shoot growth;	Sarasketa et al., 2014
Stunted roots;	Li et al., 2014
Leaf Chlorosis;	Britto et al., 2002
Reduction of oxygen evolution complex activity;	Perez-Navarro et al., 2013
Reduction of proton gradient activity in the thylakoid membrane;	Oyala et al., 2015
N-glycosylation disorders of proteins;	Barth et al., 2010
Affects photosynthesis through links with OEC	Drath et al., 2008

2.5 NH_3/NH_4^+ -induced photochemical disorders

Among the forms of mineral N used by plants, nitrate (NO_3^-) and ammonium (NH_4^+), from different evolutionary pressures each specie independently developed a preference for a particular source conventionally called β values (BOUDSOCQ et al., 2012). However, ammonium can trigger deleterious responses in many plant species as mentioned above. Therefore, plants with high β values, which have a high affinity for NH_4^+ compared to NO_3^- need to develop different protection mechanisms over the course of evolution and thus can guarantee their growth (BRITTO; KRONZUCKER, 2002).

Photosynthesis is one of the main metabolic processes that determine plant growth (KRAUSE et al., 2013). By means of photochemical reactions carried out via the electron transport chains of the thylakoid membrane (PETC) and the reducing reactions concerning the CO_2 assimilation cycle, the Calvin-Benson cycle, the plant produces all its reducing power and molecules with high energy (HILL; BENDALL, 1960; CALVIN, 1962). These metabolic pathways are crucial for providing the energy needed to meet the metabolic demand for plant growth. The role of NH_4^+ as a membrane potential decouple (GARCÍA-MENDOZA; COLOMBO-PALLOTTA, 2007) has been related to an important deleterious effect on ATP production from PETC, since the ATP synthesis complex is energy dependent proton motive force created from electron transport in PETC (ZHU et al., 2000).

According to this hypothesis, the decrease in ATP production could affect the other reducing metabolic processes essential for plant growth. However, this hypothetical mechanism of toxicity caused by NH_4^+ has met with great opposition. Plants subjected to high light irradiance and high NH_4^+ concentration exhibit visual symptoms of toxicity but have no decrease in the thermal component of NPQ (qE), which is fundamentally dependent on ΔpH (ZHU et al., 2000; RUBAN, 2016). On the other hand, it was observed that plants subjected to NH_4^+ and high light showed a marked induction of NPQ compared to plants supplied with nitrate as can be seen in **table 1** (ALENCAR et al., 2019). This result apparently contradicts with the classic chemical effect of NH_4^+, commonly reported in assays involving microalgae and chloroplasts isolated in suspension (GARCÍA-MENDOZA; COLOMBO-PALLOTTA, 2007). In fact, intact plants consist of a much more complex model than single-celled organisms, or chloroplasts in suspension. Compartmentalization processes (BRITTO et al., 2002), futile flow of NH_4+ through the plasma membrane, energetic cross-talking involving chloroplasts and mitochondria, and control of the root-leaf xylem transport could at least in part justify responses to NH_4^+ contrast between these models.

During the 1980s, American researchers using low-temperature electronic paramagnetic resonance (EPR) spectroscopy techniques found that membranes of 100 mM NH_4Cl-treated spinach isolated thylakoids exhibited signal changes. These changes were compatible with NH_3 binding to the S2 stage of the photosystem II (PSII) oxygen evolution complex (OEC). Consequently, NH_4^+ could be associated with a deleterious effect on PSII activity via direct interaction with the PSII reaction center (RCII), thus causing decreases in effective quantum yield (ΦPSII) and energy deficit. This hypothesis is corroborated by the increased sensitivity to NH_4^+ presented by cyanobacteria deficient in ftSH2 protein, a key protein involved in D1 protein turnover and RCII repair (DRATH et al., 2008). Another fact that helps explain this process is the difference in D1 protein pool in NH_4^+ and lincomycin-treated rice plants that did not recover from the D1 synthesis process when compared to NO_3^- and lincomycin-treated plants (ALENCAR et al., 2019).

Although the evidence for the direct action of photoinhibition generated by excess NH_4^+ on microorganisms is quite plausible, the mechanism inherent in its toxicity in intact plants exposed to 5 mM ammonium does not show evidence of photoinhibition (THEIS; SCHRODA, 2016). As previously argued, avoidance processes could be activated in such a way as not to allow direct toxic action of ammonium on chloroplasts of intact plants. Alternatively, processes such as pH gradient decoupling and melting of the RCII might not be the main factors in the process inherent to NH_4^+ toxicity in plants.

The RCII repair cycle in higher plants is crucial and finely regulated process. This process involves the dismantling of the damaged PSII super-complex, D1 protein phosphorylation and referral for its degradation (MELIS, 1999), de novo D1 protein synthesis and photosynthetic apparatus reassembly. This is often related to the inhibitors of de novo synthesis process of D1 protein through the inhibitory action of factor G elongation protein (NISHIYAMA; ALLAKHVERDIEV; MURATA, 2011). Arabidopsis thaliana plants grown for eight weeks with NH_4^+ as their sole source of N exhibited strongly restricted growth, increased NAD(P)H / $NAD(P)^+$ ratio, ROS accumulation and oxidized protein accumulation (PODGÓRSKA et al., 2013).The use of plants capable of tolerating high concentrations of ammonium, in particular, is a promising study model. Despite this fact, in-depth studies on photochemical details and CO_2 assimilation in response to excess ammonium in this tolerant species, such as rice, are practically not available.

2.6 NH_3/NH_4^+ -induced Calvin-Benson cycle reactions disturbance

Photosynthetic and N-absorption and assimilation reactions should be finely arranged so as not to cause any disturbance to plants, given that N metabolism is considered essential for a strong carbon skeleton drain and reducing power (BLOOM, 2015). The consequences of using ammonium as the sole source of N in CO_2 uptake depends on ion concentration, exposure time, plant species and stage of development. Claussen and Lenz (1995) found that leaf chlorosis and reduction of photosynthesis disappeared when aubergine plants were exposed to 10 mM NH_4^+ for 10 days in the reproductive stage compared to the vegetative stage. Blueberry plants are known to have higher photosynthetic rate and more dry mass production in the presence of NH_4^+ (6 mM) for 6 weeks when compared to plants fed only with NO_3^- (CLAUSSEN; LENZ, 1999). In contrast, strawberry and raspberry plants under the same conditions exhibit chlorosis, reduced photosynthesis and biomass.

In *Phaseolus vulgaris* under low NH_4^+ concentration (5 mM), CO_2 uptake remained unchanged, however, the apparent CO_2 offset point (Γ*app) decreased and mitochondrial respiration increased compared to plants provided with NO_3^- (GUO, et al., 2005). The largest application Γ*app in NO_3^- treated plants demonstrates that non-photorespiratory CO_2 uptake depends on high light, while high ammonium concentration facilitates high respiratory rate in order to support the uptake of this ion in amino acids. In an experiment with rice plants under 3 mM NH_4^+ concentration, GUO et al. (2007) showed that gas exchange parameters were not modified and that the efficiency of RuBisCO carboxylation

increased by 23% when compared to plants provided with NO_3^- (3 mM).

Although few studies address the influence of NH_3/NH_4^+ on plant photosynthesis, generally when in excess in leaves, this ion directly modifies mitochondrial reactions. This ultimately affects photochemical reactions of photosynthesis thus having little influence on the Calvin- Benson cycle directly. (GUO et al., 2007). Some researchers also claim that reserves of carbohydrates in root and high respiratory rate support increased uptake of NH_4^+ in plants supplied with this as a chief source of N. As a result of this protein synthesis is increased, especially the synthesis of Rubisco, which promote the production of biomass as well as stimulate photosynthesis (BRUCK; GUO, 2006; GUO et al., 2007).

Rubisco has the largest foliar N drain, as this enzyme represents about 50% of total plant protein (PARRY et al., 2012). Thus, under high concentration of NH_4^+ amino acid synthesis is stimulated. This in turn favors the synthesis of Rubisco and other photosynthetic proteins in order to fulfill the demand of C required for the assimilation of N (TERCÉ-LAFORGUE et al., 2004; GUO et al., 2007). However, further studies are needed to evaluate in detail the role of high ammonium concentration on photosynthetic reactions in higher plants.

2.7 Mitochondrial and chloroplast interaction associated with NH_3/NH_4^+ toxicity

Plants during their development in an environment without biotic and / or abiotic stress have interaction between chloroplast and mitochondria for the exchange of energy, metabolites, carbon skeleton and mainly reducers such as ATP and NAD(P)H (HOEFNAGEL; ATKIN; WISKICH, 1998). Many of these reducing species are used in various metabolic reactions occurring in cytosol that reduces NAD^+ to NADH, such as glycolysis and nitrate reduction (PODGÓRSKA et al., 2013). For nitrogen absorption plants need more than 25% of these reducing species from photosynthesis to be available to reduce nitrate from the soil (NOCTOR; FOYER, 1998). Therefore, when a plant is supplied only with nitrate as a nitrogen source, the reduction of this ion competes with the Mehler reaction for electrons (e^-) from photosystem I (PSI) (GERENDÁS et al., 1997). On the other hand, in plants supplied only with ammonium as a nitrogen source this phenomenon occurs without competing with other reactions.

Mehler reaction takes place when there is a decline in the number of reducing species due to other competitors as well as a decrease in the nitrate concentration inside cytosol. As a result of this, there is a transport of electrons to the thylakoid membrane during

photosynthesis leading to photo-reduction of oxygen which produces the superoxide radical (O^{2-}). Then, O^{2-} dismutation occurs through the enzyme superoxide dismutase (SOD). The hydrogen peroxide (H_2O_2) is the product of SOD and then the reaction of H2O2 with O^{2-} results in the formation of the highly reactive hydroxyl radical (HO^-) (GERENDÁS et al., 1997). Therefore to avoid this leaf stress, mitochondria may be involved in regulating the cellular redox state (NOCTOR; PAEPE; FOYER, 2007). This is perceived in ammonium-grown plants as the only nitrogen source where increased respiration has been observed (ESCOBAR; GEISLER; RASMUSSON, 2006).

With steady state photosynthesis, more than 50% of NADH can be exported to cytosol (KROMER; HELDT, 1991; KROMER, 1995). In mitochondria there is a “malate shuttle” which during photorespiration increases the NADH/NAD rate in cytosol further raising the level of reducers that served to reduce cytosol nitrate (RACHMILEVITCH; COUSINS; BLOOM, 2004; BLOOM et al., 2010). Therefore, if nitrogen is absorbed as nitrate being the only source of N, a low regulation of a type II NAD (P)H dehydrogenase goes back to the matrix and several AOXs can effectively decrease the matrix's respiratory reoxidation during NDSH 2-oxoglutarate synthesis (ESTEBAN et al., 2016).

However, a plant supplied with only ammonium as a source of N will have alternative respiratory chain routes that will be over-regulated. And in the absence of reduced cytosolic NADH, nitrate may accumulate allowing activation of NAD(P)H dehydrogenase type II and AOX induced by this ion (ESTEBAN et al., 2016). Thus, redox equivalents may be released from the transport chain by stabilizing the cytoplasmic redox level (GARDESTROM; IGAMBERDIEV; RAGHAVENDRA, 2002; KROMER, 1995). Primary NH_4^+ assimilation occurs mainly in the root, thus avoiding the increase of alternative respiratory capacity pathways in the mitochondria may also result in increased matrix respiratory oxidation and little export of redox equivalents to cytosol (ESTEBAN et al., 2016). Thus, nitrogen metabolism is interconnected with carbon metabolism through anaplerotic metabolites from chloroplast, photosynthesis and tricarboxylic acid cycle metabolism and mitochondrial glycolysis (YANG et al., 2016).

N-metabolism in mitochondria is known in the context of carbon skeleton supplementation to assimilate ammonium (SZAL; PODGÓRSKA, 2012). In the chloroplast, the GS/GOGAT cycle, responsible for ammonium assimilation, requires 2-oxoglutarate (carbon skeleton) that comes from mitochondria (TCHERKEZ et al., 2009; GAUTHIER et al., 2010). Thus, the interaction of these two organelles under toxic conditions caused by ammonium is very important for plants.

3 OBJECTIVES

The general objective is to elucidate the molecular mechanisms related to the photosystem with ammonium toxicity in rice plants exposed to high light.

In order to achieve the general objective, some specific goals were proposed:

1. Identify alterations in the protein profile related to the membranes of the thylakoids.
2. To investigate processes of damage mediated by reactive oxygen species in proteins of the thylakoid membranes.
3. Quantify photosystem II core-related proteins under high light and ammonium conditions as well as during the photosynthetic relaxation step.
4. To verify the organizational regulation of the relationship between antennas and reaction center in native supercomplexes of photosystem I and II under ammonium and high light conditions, as well as during the photosynthetic relaxation step.
5. To determine the amount of total chlorophylls, carotenoids as well as the chlorophyll *a/b* ratio present in high light and ammonium rice plants.

4 HYPOTHESIS

High ammonium supply combined with excessive light induce specific photochemical protective mechanisms in rice, an ammonium-tolerant species. These mechanisms might involve NPQ dynamics, PSII down-regulation and oxidative stress protection in the thylakoids.

5 HIGH AMMONIUM COMBINED WITH EXCESS LIGHT INDUCES TIME-DEPENDENT CONTRASTING NPQ DYNAMICS AND ANTENNA CHANGES AS A PROTECTIVE MECHANISM IN RICE

ABSTRACT – Effects of high ammonium concentration combined with excessive light on the photochemical dynamics of NH_4^+-tolerant plants are still poorly understood. To investigate how these plants can protect its photochemical system, rice was supplied with sole ammonium (10 mM) or nitrate (10 mM) for up to eight days under high light (HL) or moderate light (ML). Ammonium-supplied plants did not exhibit significant changes in PSII activity under ML, as compared to nitrate plants. Under NH_4^+ and HL, plants exhibited delays in dark PSII recovery only after the sixth day of exposure. Changes related to accumulation of important photochemical proteins or evidence of oxidative stress in these proteins, were not observed. Ammonium-supplied plants displayed higher time-dependent light induction in NPQ associated with decrease in dark relaxation after the sixth day, which coincides with delay in dark PSII recovery. BN-PAGE analysis revealed that ammonium-plants accumulated more LHCII-CP24-CP29 and LHC trimmers under HL. Therefore, data suggest that high ammonium is able to induce delaying in PSII recovery and trigger changes on the aggregation state of PSII antennas, which can affect NPQ and PSII activity. These processes may represent unusual photoprotective mechanisms essential to preserve photosynthesis in rice plants grown under high ammonium and light.

Keywords: excess light, NH_4^+ toxicity, *Oryza sativa*, PSII activity.

INTRODUCTION

Rice is an important crop that is utilized as the main source of food for billions of people worldwide (WU et al. 2016). Its cultivation is commonly performed in paddy soils, where NH_4^+ is the main N-source and frequently reaches high concentrations that are toxic for the most of the cultivated species (TABUCHI et al. 2007). High ammonium supply to non-adapted plants causes several metabolical disturbances, including respiration, ionic unbalance and hormonal changes, among others (BRITTO AND KRONZUCKER 2002). These adverse physiological responses are aggravated by the presence of additional stressful environmental factors such as high light radiation (MURCHIE et al. 2005). Rice is considered as an ammonium specialized or tolerant species (BRITTO AND KRONZUCKER 2013) but the involved tolerance mechanisms are poorly known. Among the physiological processes affected by excess ammonium, decreases in photosynthesis and PSII activity and CO_2 assimilation have been reported (ESTEBAN et al. 2016).

Despite some works have revealed that high ammonium supply affects photosynthesis, in general these studies are incipient and specific reactions and action mechanisms are virtually unknown. It has been known for many years that NH_3 in very high concentrations can compete with H_2O and establishing bind with oxygen evolving complex at PSII level (BECK et al. 1986b; VINYARD et al. 2016), affecting activity and recovery of this system (DRATH et al. 2008). Nevertheless, the majority of these studies were performed employing microorganisms and isolated chloroplasts in presence of excessively high NH_4^+/NH_3 and non-physiological concentrations compared to those commonly employed to plant growth (BECK et al. 1986a; DAI et al. 2014). Indeed, in these conditions some studies have evidenced that high ammonium supply can induce disturbances in photochemical activity, decreasing PSII recovery via decreased D1 protein synthesis; inducing photoinhibition and impairment in electron transport at PSII level (DRATH et al. 2008).

Some evidences have suggested that high light can stimulate the ammonium transport from roots towards leaves (DING et al. 2015) and this impact could exacerbate the harmful ammonium effects on photosynthesis (ALENCAR et al. 2019). Actually, is widely known that single excess light causes several dangerous consequences on the photochemical reactions, inducing photoinhibition and decreasing drastically efficiency of both PSII and PSI and decreasing CO_2 assimilation (RUBAN 2015). Assuming that photosynthesis represents a central process involved in plant growth and productivity (KROMDIJK et al. 2016), the elucidation of weak and strong points within of the photosynthetic network under excess ammonium could contribute to comprehension and production of resistant plants in response

to that harmful N-form. This contribution should be more important to crops cultivated in poorly aerated soils and, especially, in tropical regions where NH_4^+ can reach high concentrations and frequently associated with other adverse factors such as high sunlight radiation (MURCHIE et al. 2005).

Rice cultivars adapted to paddy soils in tropical regions should be good model for ammonium tolerance mechanisms elucidation since these plants display high photosynthetic rates and are considered as a NH_4^+ tolerant species, besides being cultivated in these regions worldwide (TABUCHI et al. 2007) and exhibiting sensitivity to high light (MURCHIE et al. 2015). Paradoxically, the majority of the reported works employing ammonium responses in different plants species have applied relatively low NH_4^+ concentrations in the root medium associated with mild or low light and the photosynthesis studies are incipient and fragmented (BRÜCK AND GUO 2006; DING et al. 2015; GUO et al. 2007). Indeed, most of these studies are concentrated in comparing NH_4^+ and NO_3^- nutrition, in general aiming to compare these two N-sources in terms of performance to growth or to evaluate the effects of ammonium toxicity on sensitive or non-tolerant species (PODGÓRSKA et al. 2013; ZHU et al. 2000). Unfortunately, these comparative and descriptive works are not enough to uncover key reactions, genes or metabolites directly involved in plant resistance/sensitivity to high ammonium supply.

Recently, our group add new insights on some questions involving photosynthetic processes that are more affected by high ammonium supply in presence of excess light utilizing rice as a model (ALENCAR et al. 2019). We evidenced that in these circumstances excess ammonium triggers several effects on photosynthetic reactions. Firstly, that condition induces a significant stomatal closure, affecting CO_2 assimilation by stomatal and carboxylation restrictions. In addition, PSII dark recovery is delayed, also affecting PSI activities in parallel to strong induction of NPQ, possibly associated with excess energy and ATP synthase inhibition. We have concluded that high ammonium combined with light induces many disturbances on photosynthesis, but the cause-effect relationships remain poorly understood. In these adverse photosynthetic situations, plants could avoid excess energy restricting light capture by antenna, increasing heat dissipation via NPQ and regulating the photochemical activity, as a protective photoinhibition (FOYER et al. 2017).

Our group have demonstrated that cashew plants, a species adapted to extreme environmental conditions, such as drought and high light, are able to employ NPQ induction, PSII down regulation and decrease in accumulation of photosynthetic pigments as an effective photoprotective mechanism (LIMA et al. 2018). Despite is relatively well established that

regulation of antenna components synthesis/degradation and NPQ kinetics are vital processes to photoprotection, the detailed biochemical mechanisms involving the relationships between them are still controversial (PINNOLA AND BASSI 2018; RUBAN 2016; RUBAN et al. 2012). As high ammonium supply combined with excessive light can trigger strongly the NPQ induction in rice, what could occur in parallel with antenna proteins? In these circumstances, how these two stressful factors should regulate the photochemical system in a time-dependent way? Thus, in this work we tried elucidating how specific photochemical mechanisms are regulated by high ammonium supply combined with excessive light in a simultaneously NH_4^+ tolerant and high-light sensitive species.

Our results reinforce previous finds and evidence that rice plants display high plasticity in terms of PSII activity in presence of high ammonium and excessive light. This photochemical homeostasis involves delayed capacity to recovery NPQ induction and PSII activity in the dark after HL exposure associated with important changes at level of some antenna sub-complexes in order to minimize energy utilization and preserving thylakoid proteins against oxidative stress by avoiding reactive oxygen species (ROS) accumulation caused by energy excess in chloroplast. These finds are discussed in terms of photosynthetic tolerance potential to understand underlying mechanisms and generating plants resistant to high ammonium supply.

MATERIAL AND METHODS

Plant growth conditions and treatments

Rice plants (*Oryza sativa* L. Cv. Nipponbare) were cultivated in 3 L plastic pots filled with Hoagland-Arnon's nutritive solution (Hoagland and Arnon 1950) as described by Alencar et al. (2019) in a greenhouse under natural conditions: average day/night temperature of 32/25 °C, average relative humidity of 65%, maximum photosynthetic photon flux density (PPFD) around 600 µmol m^{-2} s^{-1} at noon and a 12 h photoperiod. To deeply investigate the dynamic of photochemical activity in intact plants exposed to high NH_4^+ concentration combined with excess light, an experiment (Experiment I) was performed. First, these plants with 35-day-old, grown in the conditions described above, were transferred to an N-free solution for 72 h aiming to induce a transient N-deprivation and the expression of N transporters. Subsequently, plants were exposed to a complete nutrient solution containing 10 mM NO_3^- or 10 mM NH_4^+, as a sole N source, for 8 days in a controlled growth chamber

(28/24 °C day/night temperature, 60% relative humidity, and 12 h photoperiod) exposed to different light regimes: moderate light – ML (400 µmol m^{-2} s^{-1}) or high light – HL (1,700 ± 100 µmol m^{-2} s^{-1}). Photochemical measurements were taken periodically over the experimental period. Later, a second experiment (Experiment II) was performed to study the effect of the combination of high NH_4^+ concentration and a short period of excess light in photosynthesis and thylakoid membrane protein profile. Plants, cultivated as described in experiment I, were transferred to a complete nutrient solution containing 10 mM NO_3^- or 10 mM NH_4^+, as a sole N source, for 8 days in a controlled growth chamber conditions (28/24 °C day/night temperature, 60% relative humidity, 400 µmol m^{-2} s^{-1} PPFD and 12 h photoperiod). After this acclimation period, in the last day, plants were exposed to moderate light – ML (400 µmol m^{-2} s^{-1}) or high light – HL (2,000 µmol m^{-2} s^{-1}) for 8 hours. Subsequently, were performed analysis of photosynthesis and harvest of leaf samples in the presence of liquid nitrogen followed by storage at -80 °C until the analysis.

Total chlorophyll, membrane damage, lipid peroxidation

Leaf total chlorophyll content and the membrane damage were quantified according to Porra et al. (1989) and Alencar et al. (2019), respectively. The quantification of lipid peroxidation was performed according to Cakmak and Horst (1991) by measuring the formation of thiobarbituric acid-reactive substances (TBARS). Fresh leaf samples were ground in presence of liquid N_2 and 5% (w/v) TCA, followed by centrifugation at 12,000 g (4 °C) for 15 min, the supernatant was immediately used for TBARS determination. The TBARS content was calculated using its absorption coefficient (155 mM^{-1} cm^{-1}), and the results were expressed as µmol MDA g^{-1} FM.

Gas exchange and photochemical measurements

The steady state gas exchange parameters (P_N – Net CO_2 assimilation and g_S – stomatal conductance) 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), in plants acclimated to growth light conditions. The conditions inside IRGA's chamber during the measurements were near to those of the growth chamber: PPFD of 1,500 mmol m^{-2} s^{-1}, 28 ºC, air vapor pressure deficit of 1.85 ± 0.14 kPa and air CO_2 partial pressure of 40 Pa. The amount of blue light was set to 10% of the PPFD to maximize stomatal aperture (FLEXAS et al. 2008).

Chlorophyll fluorescence and $P700^{+}$ absorbance were measured *in vivo* using a Dual-PAM 100 (Walz, Germany). The photochemical parameters were measured by the saturation pulse method (SCHREIBER et al. 1995) and the leaves were previously acclimated in the dark for 30 minutes. Induction and recovery kinetics were performed, which consisted of 6 minutes of light induction with 400 µmol m^{-2} s^{-1} or 1,700 µmol m^{-2} s^{-1} of actinic light for ML and HL plants, respectively, followed by 15 minutes of dark relaxation. Saturation pulses were employed throughout the kinetics with intensity of 8,000 µmol m^{-2} s^{-1} and 0.6 s duration. The following PSII parameters were evaluated: the effective quantum yield of PSII [YII = (Fm - Fs)/Fm '] and the non-photochemical quenching, which was calculated as [NPQ = (Fm/Fm ') - 1]. For NPQ calculation the Fm was estimated at day 0, before the beginning of the experiment. In parallel, following PSI parameters were evaluated: effective quantum yield of PSI [YI = 1 – (YND - YNA)], the PSI limitation on the donor side [YND = 1- P700red] and the acceptor side limitation of the PSI [YNA = (Pm-Pm')/Pm].

Thylakoid membrane proteins immunodetection

The abundance of proteins involved with photochemical activity was performed using fraction enriched of thylakoid membrane of leaf samples from experiment II. The thylakoid membrane was isolated as described by RUBAN et al. (2016) using dark-adapted leaves and the amount of total chlorophyll was determined according to PORRA et al. (1989). The proteins were first separated by SDS-PAGE (LAEMMLI 1970). Equal amounts of chlorophyll (2 □g) were electrophoretically transferred to a PVDF membrane (TOWBIN et al. 1979). Polypeptide detection was performed using specific polyclonal antibodies against PsbA, PsbE, PsbS, LCA1 and CP43 proteins (Agrisera©, Sweden). Membranes were blocked for 3 hours with 5% non-fat milk in saline Tris-HCl buffer (100 mM Tris-HCl, pH 7.6, 150 mM NaCl), incubated with primary antibody overnight and after with alkaline phosphatase-conjugated secondary antibody (A3812 - Sigma-Aldrich©, USA) by 6 hours. The protein detection was developed using NBT/BCIP (Sigma-Aldrich©, USA) by adding 1 tablet to 10 mL dH_2O, until bands were visualized. The bands abundance was calculated using the SmartView Pro 1200 Imager System Version 1.0.0.3 (Major Science).

Thylakoid membrane oxidized proteins immunodetection

The quantification of oxidized proteins from thylakoid membrane was measured in the same extract described above using the OxyBlot Protein Oxidation Detection Kit

(S7150, Merck millipore), including the molecular weight markers, following the manufacturer's instructions. The bands abundance was quantified using the software Image J (version 1.52p).

Blue Native PAGE electrophoresis

The photosystems protein complex structure was quantified by using the native blue gel electrophoresis (BN-PAGE) as described by SCHAGGER et al. (1994), with modifications. The same extract of thylakoid membrane described above (2 µg of chlorophyll) was solubilized in 100 µL of 50 mM BisTris-HCl, pH 7.0, 0.5 M ε-aminocaproic acid and 10% (w/v) glycerol containing 1% (w/v) n-dodecyl-D-maltoside and incubated for 10 min on ice. After, the samples were centrifuged at 15,000 g for 10 min at 4 ºC. The supernatant (8 □L) was mixed with 2 µL of coomassie dye stock solution (5% (w/v) Coomassie Serva Blue G, 50 mM BisTris-HCl, pH 7.0 and 0.5 M ε-aminocaproic acid) to give a detergent/Coomassie ratio of 8:1 (v/v). Equal amounts of samples (50 □L) were loaded in a 4-16% Bis-Tris gradient gel (Novex by life technologies, Invitrogen). The electrophoresis was performed at 100 V at room temperature. The cathode buffer initially had 0.02% Coomassie dye and was replaced by a dye-free buffer after half the electrophoretic run.

Statistical analysis and experimental design

The experiments were organized in a completely randomized design, with three replicates per treatment, each one represented by a pot containing two plants. The data were submitted to analysis of variance (ANOVA) and the averages were compared by Tukey's test or t-test with 5% of confidence level ($p < 0.05$), as indicated in the figure captions. All statistical analyses were conducted using SigmaPlot 12.0 (Systat Software, San Jose, USA).

RESULTS

Ammonium supplying affects the dynamics of PSII and PSI activity in rice plants

In order to clarify the effects of ammonium exclusive supplement in a NH_4^+ specialized species, rice plants were supplied with 10 mM NO_3^- or 10 mM NH_4^+ for up to 8 days and simultaneously exposed to contrasting light regimes of 400 or 1700 µmol photons m^{-2} s^{-1}, with photoperiod of 8 hours per day. Rice plants did not exhibit any remarkable visual

change as regarding ammonium or nitrate exclusive nutrition (**Fig. 9**). In contrast PSII activity was differently affected by these treatments in rice plants. Under moderate light (ML), ammonium-supplied plants exhibited a significant decrease in the steady state of effective quantum yield of PSII (YII), by 10% and by 20% at 2 and 4 days of experiment, respectively and in comparison to nitrate-treated plants (**Fig. 2A, Fig. 10**). No significant differences were observed in YII steady state in these plants exposed to ML after the day 4. Despite high light (HL) has induced a prominent decrease in steady state YII as compared to ML, any significant changes were observed as comparing ammonium and nitrate-supplied plants (**Fig. 2B**).

Regarding the ratio of YII dark recovery, which was calculated following the initial slope of exponential increase of YII in the dark (**Fig. 10**), rice plants did not exhibit any significant difference, comparing nitrate and ammonium exclusive nutrition, under ML (**Fig. 2C**). In contrast, the presence of HL induced remarkable changes in the ratios of PSII dark recovery, where ammonium supplied rice exhibited decrease of 50% in these ratios at 6 and 8 days after the beginning of the experiment, in comparison to nitrate supplied plants (**Fig 2D**). In parallel, rice plants supplied with high ammonium showed no significant differences in net CO_2 assimilation, stomatal conductance and total chlorophyll content, compared to plants supplied with nitrate (**Table 9**). However, corroborating the results previously obtained by Alencar et al. (2019), under HL, the NH_4^+-plants showed a decrease in all these parameters, in the order of 25%, 40% and 11% in P_N, g_S and CHL, respectively and in comparison with plants supplied only with nitrate (**Table 2**).

Regarding the steady state of PSI quantum yield (YI), the donor side limitation of PSI (YND) and acceptor side limitation of PSI (YNA), no significant difference between nitrate and ammonium-supplied plants was observed under moderate light (**Fig 3A, 3C, 3E, 11, 12 and 13**). In contrast, HL induced a slight decrease in YI steady state (by 20%) of ammonium treated rice at the 6^{th} and 8^{th} days of treatment, as compared to nitrate supplied plants under similar light conditions (**Fig 3B**). Under HL, ammonium supplied plants displayed a slight increase in the donor side limitation only at the 8^{th} day of the experiment, in comparison to nitrate-supplied plants (**Fig. 3D**). It was not observed any significant differences in YNA of nitrate and ammonium-grown rice under HL (**Fig. 3F**).

The ammonium effects on PSII dynamics are probably not related to oxidative stress or differences in the accumulation of photochemical proteins

Aiming to understand the molecular mechanisms behind the effects of ammonium supplying and the changes in PSII activity dynamics of rice plants, a biochemical analysis of

the accumulation of some important thylakoidal proteins (ImmunoBlot), as well as the protein-carbonylation analysis (OxyBlot) of the thylakoidal fraction were performed. The SDS-PAGE profile obtained with proteins of the thylakoidal enriched fraction did not display any evidence of differential accumulation of thylakoidal proteins between nitrate- and ammonium-supplied plants (**Fig. 4**). This result was also supported by the immunoblotting analysis related to the core proteins (CP43, PsbA, PsbE), the NPQ enhancing protein (PsbS) and the PSI antenna protein (LCA1). This data evidenced that no changes between nitrate and ammonium supplying to rice plants are related to a differential accumulation of these plants (**Fig. 5**). Finally, the possibility of functional damage of thylakoidal proteins due to the occurrence of oxidative stress as consequence of ammonium exclusive nutrition was also ruled out, as no differences were observed in the OxyBlot analysis of thylakoidal membranes enriched fraction (**Fig. 6**). In parallel, increases in TBARS levels (10%) and membrane damage (50%) from plants supplied with ammonium compared to nitrate-plants were observed only under high light conditions (**Table 9**). However, the levels of these parameters remained too low to represent evidence of oxidative stress.

Ammonium supplying combined with HL induces delay in the NPQ recovery and accumulation of free antenna sub-complexes

Aiming to investigate the involvement of NPQ as an important energy sink for PSII activity in rice plants supplied solely with ammonium or nitrate and exposed to HL, NPQ induction and relaxation kinetics were performed (**Fig. 14**). Under HL, rice plants supplied with both contrasting N forms exhibited a progressive and linear increase in maximum reached NPQ from the beginning of the experiment until the eighth day (**Fig. 7A**). However, ammonium supplied plants exhibited an increase trend of 12% more prominent than nitrate-supplied ones, throughout the time of HL exposure (**Fig. 7A**). In parallel, NPQ relaxation dynamics were similar in the beginning of the experiment and were maintained similar to both ammonium- and nitrate-provided plants until the 8th day under ML (**Fig. 7B-F**). Conversely, under HL ammonium-provided plants exhibited a slight delay in NPQ relaxation that was evident from the 4th day until the end of the experiment, as compared with nitrate-supplied plants under similar light regime (**Fig. 7B-F**).

In order to better comprehend the changes in NPQ dynamics induced by ammonium supplying to rice plants, a BN-PAGE was performed to assess the responses regarding complexes and sub-complexes of the photosystems. The BN-PAGE analysis in samples from pre-dawn plants did not reveal any prominent difference in profiles from nitrate

and ammonium-supplied rice (**Fig. 8**). However, samples obtained from plants after 8 hours of exposure to HL, revealed a strong accumulation of free trimmers of LHCII in plants from both N-treatments, which were more prominent in ammonium-supplied plants than in nitrate ones (**Fig. 8**). In addition, an intense accumulation of free LHCII-CP24-CP29 sub-complexes in HL was evident in ammonium supplied plants, but not in nitrate-provided rice **(Fig. 8)**. Interestingly, after 30 min of dark relaxation, the accumulation of these sub-complexes in both nitrate- and ammonium-supplied rice was completely similar, indicating full recovery from HL treatment (**Fig. 8**).

DISCUSSION

Rice plants are commonly cultivated in paddy soils, where low oxygenation levels allow that microorganisms promote ammonification, favouring the accumulation of high NH_4^+ concentrations over nitrate in the root medium (DAI et al. 2008). Despite these plants are commonly adapted to this environment, the combined presence of an additional stress factor, such as high light, could impose new challenges for plant surviving, and the responses triggered under such conditions are poorly studied. Here we have reinforced that high ammonium supplying in combination with a recurrent exposure to HL is able to trigger changes in the PSII activity, and these alterations are time-dependent in a long-term scale (days). These responses involve a significant delay of PSII dark recovery, which is clearly absent until the 6^{th} day or when ammonium is supplied under ML regime. Moreover, we provided here evidences that suggest a delay in PSII dark recovery which could be linked to biochemical alterations involving some PSII antenna components and consequently, affecting NPQ induction/relaxation dynamics.

In a recent previous study, our group evidenced that, in fact, despite rice is considered a tolerant species, when NH_4^+ is supplied in high concentration, followed by a single exposure to HL for 8 hours, these plants exhibit delays in PSII dark recovery and become more sensitive to inhibitors of chloroplast protein synthesis than nitrate-supplied rice (ALENCAR et al. 2019). Interestingly, these plants also suffered other non-specific alterations in photosynthesis-related processes such as restriction in stomatal conductance that can have exerted indirect effects on photochemical and Calvin-Benson cycle activity. Here, the immunoblotting analysis of some important proteins belonging to PSII core (PsbA, PsbE and CP43), as well as one protein of the PSI antenna system (LCA1) did not reveal any significant

difference in accumulation of these proteins, regardless the light condition (dark, HL and recovery). These results suggest that despite the greater sensitivity of ammonium-supplied rice to lincomycin, a deficiency in the synthesis of PSII proteins by itself may not be the main reason underlying the delay in PSII dark recovery.

Indeed, studies involving the cyanobacteria *Synechocystis* sp. with knockout in a protein from the PSII repair mechanism (FtsH2) also revealed a greater sensitivity to ammonium (DRATH et al. 2008). However, further studies with this same species concluded that ammonium directly accelerated photodamage of PSII rather than affecting the repair of photodamaged system (DAI et al. 2014). These authors also suggested that ammonium-induced photodamage on PSII primarily occurred at the oxygen evolution complex, which has a known binding site for ammonium at very high concentrations (DAI et al. 2014). Thus, we suggest that in the presence of high doses of ammonium, PSII repair mechanisms are crucial for the protection of this photosystem (ALENCAR et al. 2019), but it is probable that chloroplast protein synthesis *per se* may not be the main target of ammonium-induced toxicity in plants, as currently speculated in literature. However, further studies involving other tools such as proteomics and transcriptomics associated with genetic reverse for photosynthetic genes are required.

NPQ an important mechanism involved in the protection of PSII and avoidance of photoinhibition under adverse environmental conditions (RUBAN 2015). In fact, NPQ needs to be seen as a set of integrated and complex biological processes involving from photochemical components related to loss of absorbed energy in the form of heat by the PSII antenna (qE) via disassembling of antenna complexes until direct changes in the reaction center (qI, qT and others). In terms of photoprotection qE is considered the most important NPQ component (CARVALHO et al. 2015), but the specific site of its occurrence in the PSII is still controversial (RUBAN 2015). Actually, two models are proposed as the most relevant to explain qE generation mechanism: 1) The Bassi and colleagues model, involving zeaxanthin and a quenching association encompassing CP29, CP26 and CP24 (CAZZANIGA et al. 2013) and 2) the qE switch model based upon the major LHCII trimmers, proposing a small conformational alteration within the monomeric unit of the complex comprising neoxanthin and lutein pigments (JOHNSON et al. 2011).

In the current study, high ammonium supplying to rice plants induced not only higher NPQ induction in response to HL treatment but also was related to a progressive delay in NPQ dark relaxation, which could be directly linked to the previous discussed delay in PSII dark recovery, since these two processes are competitive (BAKER 2008). Interestingly, the

fact that under HL, ammonium supplied plants accumulate more free antenna components, including both LHCII trimmers and free LHCII-CP24-CP29 sub-complexes, than nitrate supplied ones, suggest a possible biochemical basis for the differences observed in NPQ dynamics. Indeed, under several adverse environmental conditions, plants can regulate the disassembling of LHC components and sustain a quenched state from minutes to days, which was former known as the NPQ qI parameter, and currently is considered as a photoprotective PSII down-regulation mechanism (ADAMS et al. 2013; FOYER et al. 2017). Thus, our obtained data strongly suggest that an earlier PSII down-regulation mechanism in rice plants supplied with high ammonium and, concomitantly, exposed to excess light, might have occurred as a photoprotective mechanism.

Excessive light is an environmental condition established when the total amount of light energy absorbed by the harvesting antenna overcome the photochemical and biochemical demand from the plant cell metabolism associated with the reductive reactions, especially related to C, N and S assimilatory pathways (FOYER et al. 2012; LI et al. 2009). Therefore, any environmental conditions capable to induce stomatal closure and, consequently inducing decrease in the reductive phase of photosynthesis, might potentially generate a condition of excess energy in the thylakoids. Previous studies have reported that rice plants grown with ~5 mM NO_3^- or 5 mM NH_4^+ did not exhibit any significant alteration in the stomatal conductance (GUO et al. 2007) but we have clearly demonstrated previously (ALENCAR et al. 2019) and also here, that 10 mM ammonium is able to restrict stomatal conductance, especially when in presence of high light. In fact, despite rice plants have been largely reported in the literature as an ammonium specialized species (BRITTO AND KRONZUCKER 2013), their acclimation to high ammonium in presence of excess energy – a problem commonly recurrent in tropical regions, has been neglected and still need further studies for a complete comprehension.

Thus, a crucial question discussed here is if these ammonium-HL induced changes induced on photochemical system are stress or damage indicators or if they are simply acclimation responses to avoid excess energy in thylakoids. Recently our group have demonstrated that after approximately one week of high ammonium exclusive supplying to rice plants followed by a single 12h-photoperiod under HL, these plants display significant reduction in stomatal aperture in comparison with nitrate-supplied plants (ALENCAR et al. 2019). Thus, under a scenario of great stomatal restriction, the induction of high levels of NPQ, in parallel with down-regulation of PSII activity, might represent an effective strategy to rice plants cope with the excess energy induced by ammonium supplement and HL. In

absence of such features, rice plants could suffer an unbalance between the oxidative and reductive phases of photosynthesis, leading to accumulation of reducing power and generation of ROS over accumulation and subsequent PSII photoinhibition, which under extreme conditions can lead to irreversible cellular damages and leaf senescence (ESTEBAN et al. 2016).

Working with *Arabidopsis thaliana*, a relatively sensitive species to ammonium toxicity, Podgóska et al. (2013) have demonstrated that after a long-term exposure to NH_4^+ these plants displayed a higher ratio NDPH/$NADP^+$ and several physiological evidences of occurrence of oxidative stress, especially in the mitochondria. In our previous and study with rice plants the absence of no evidence of membrane damage and ROS accumulation suggested that rice plants are capable to avoid oxidative stress, even under prominent stomatal closure induced by ammonium supplying and HL (ALENCAR et al. 2019). The data obtained here considering the profile of protein oxidation in the thylakoids enriched fraction corroborate the previous finds (ALENCAR et al. 2019) and allow us to conclude that rice plants are capable to avoid ROS over-accumulation even in presence of a high NH_4^+/HL for long-term exposure. This mechanism must represent an important achievement since this species is able to survive under paddy soils in presence of high ammonium levels.

More importantly, these obtained results could contribute to understand, at least in part, some photosynthetic mechanisms that confer tolerance/resistance to these common environments constituted by high ammonium supplying combined with excess solar radiation. In other terms, which genetic and physiological traits are crucial to induce ammonium resistance in non-adapted or sensitive species in terms of improvements on their photosynthetic apparatus? Indeed, this matter is practically unknown as for single ammonium exposure or its supplying combined with other environmental factors like high light. Actually, despite several physiological mechanisms have been commonly reported as related to ammonium toxicity and resistance, those encompassing photosynthesis are virtually absent (ALENCAR et al. 2019). As photosynthesis and other physiological processes act in an integrated and systemic way, the elucidation of individual resistance traits is a hard task that must involve many approaches, such as system biology employing integration between omics and physiological traits.

Taken together, the data obtained in the present investigation suggest that rice plants are able to trigger the NPQ photoprotection system more effectively in the presence of ammonium as compared to nitrate, especially associated with the simultaneous presence of subsequent cycles of high light for long-term. This ability may be related to the capability of

rice plants to regulate the protein aggregation state associated with photosystem II antenna, although further research in this area is still needed. The effective activation of energy dissipation at PSII level certainly might contribute significantly to the avoidance of ROS over accumulation. Moreover, the presence of sustained NPQ in the dark relaxation phase also suggests that under these conditions rice plants could have exhibited evidences for early PSII down regulation associated with later dark NPQ relaxation. We suggest that under those extreme environmental conditions rice plants are able to trigger an efficient photoprotective mechanism, employing a quenched state to avoid excess energy and allowing surviving under high ammonium concentrations.

5 CONCLUSION AND REMARKS

The effects caused by high ammonium supply in plants house morphological and metabolic changes in plants. The data obtained in the present work suggest that rice plants are able to activate the NPQ photoprotection system more effectively in the presence of ammonium when compared to nitrate, especially associated with the simultaneous presence of subsequent cycles of high light for long term. This ability may be related to the ability of rice plants to regulate the state of protein aggregation associated with the antenna of photosystem II, although further research in this area is still needed. The effective activation of energy dissipation at the PSII level can certainly contribute significantly to avoid ROS on accumulation. In addition, the presence of sustained NPQ in the dark relaxation phase also suggests that, under these conditions, rice plants could have evidenced evidence of early regulation of PSII associated with subsequent relaxation of dark NPQ. We suggest that, in these extreme environmental conditions, rice plants are able to activate an efficient photoprotective mechanism, employing an extinct state to avoid excess energy and allowing them to survive under high concentrations of ammonium.

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APÊNDICE A - EXPERIMENTAL RESULTS

High ammonium combined with excess light induces contrasting NPQ dynamics in a time-dependent way and antenna changes as a protective mechanism in rice

SUPPLEMENTARY MATERIAL

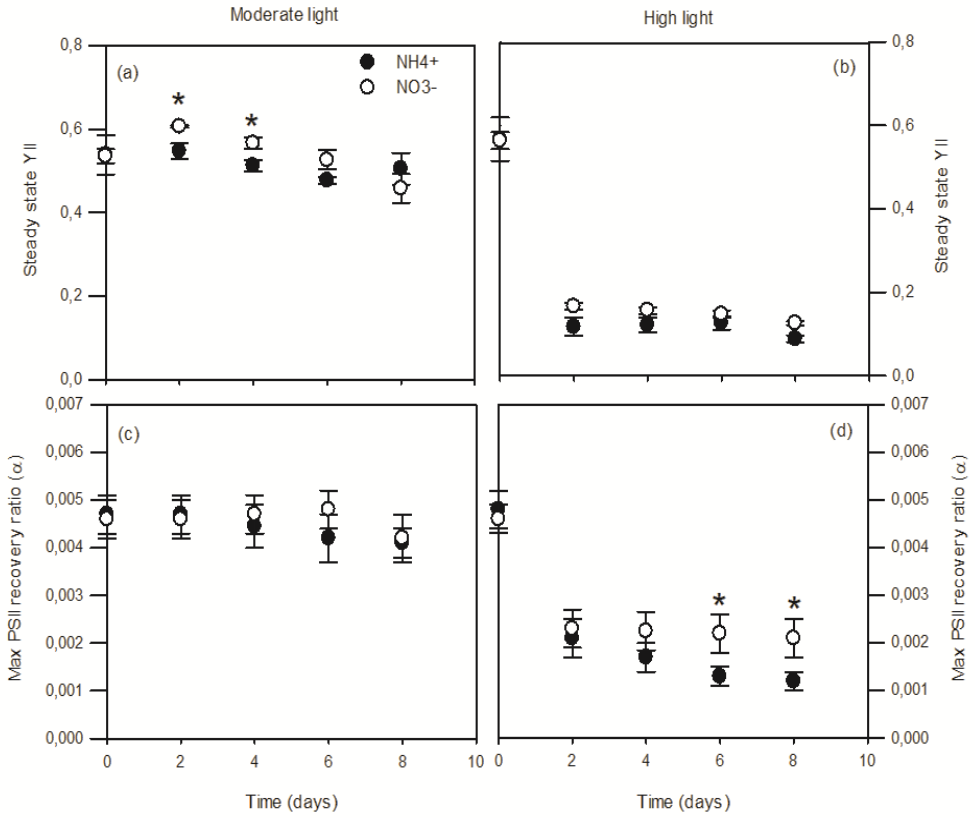


Figure 2. PSII effective quantum efficiency at steady state (A, B) and maximum PSII recovery rate in the dark (C, D) measured in rice plants previously acclimated for up to 8 days in moderate light – 400 µmol m^{-2} s^{-1} (left panel) or high light - 1700 µmol m^{-2} s^{-1} (right panel) conditions and simultaneously supplied with 10 mM nitrate (open circles) or 10 mM ammonium (closed circles). To calculate the YII of the stationary state, the measurement obtained after 6 minutes of photochemical induction was used (Fig. S2). To calculate the maximum PSII recovery rate in the dark, the inclination angle (α) of the relaxation phase in the dark was used, obtained from the function $Y = a(1\text{-}e^{-bx})$ that was estimated using the SigmaPlot 12.0 software. Asterisks represent a significant difference (ANOVA, Tukey Test at 0.05) between the treatment of nitrate and ammonium within the same day of treatment and light condition. Black bars represent ± standard error.

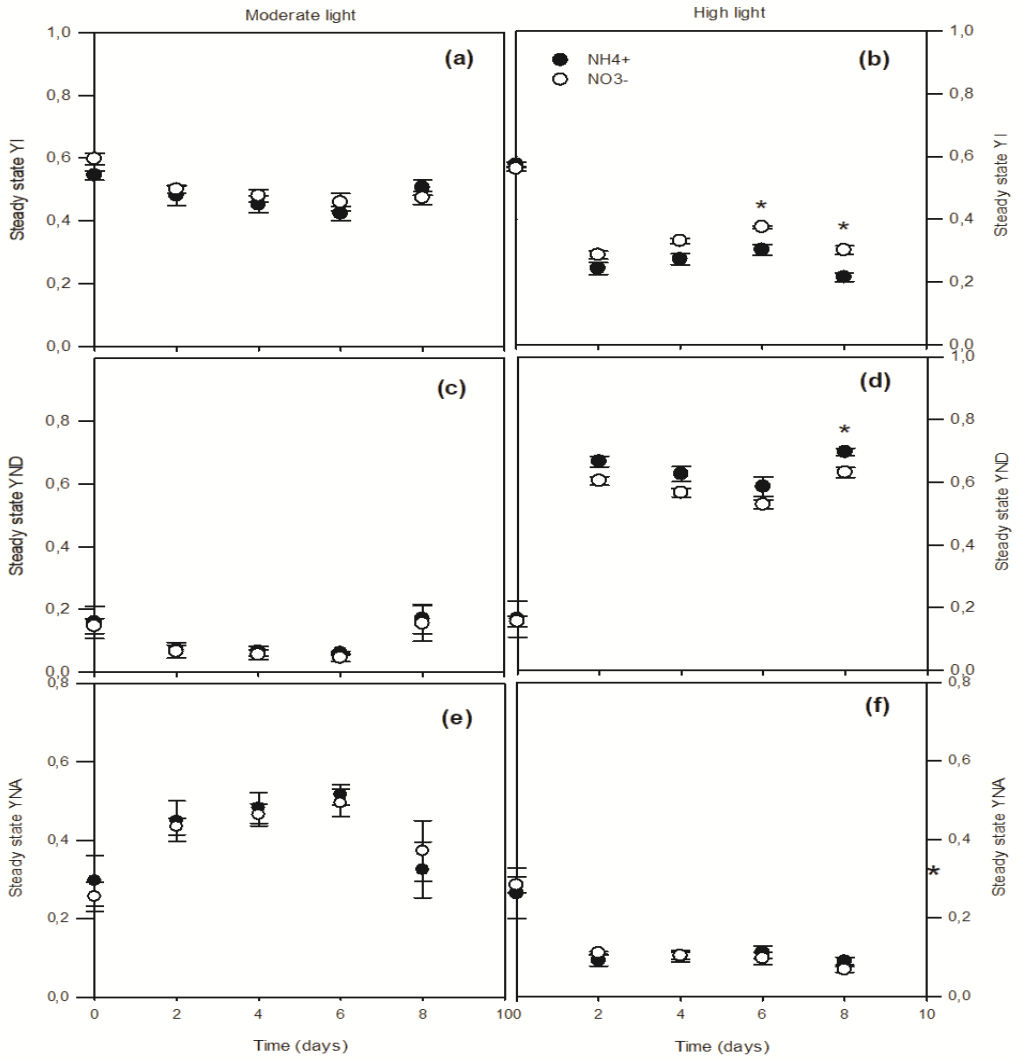


Figure 3. PSI effective quantum efficiency at steady state (A, B), PSI donor side limitation at steady state (C, D), PSI acceptor side limitation at steady state (E, F) measured in rice plants previously acclimated for up to 8 days in moderate light – 400 µmol m^{-2} s^{-1} (left panel) or high light - 1700 µmol m^{-2} s^{-1} (right panel) conditions and simultaneously supplied with 10 mM nitrate (open circles) or 10 mM ammonium (closed circles). To calculate the PSI parameters at the steady state, the measurement obtained after 6 minutes of photochemical induction was used (Fig. S3). Asterisks represent a significant difference (ANOVA, Tukey Test at 0.05) between the treatment of nitrate and ammonium within the same day of treatment. Black bars represent ± standard error.

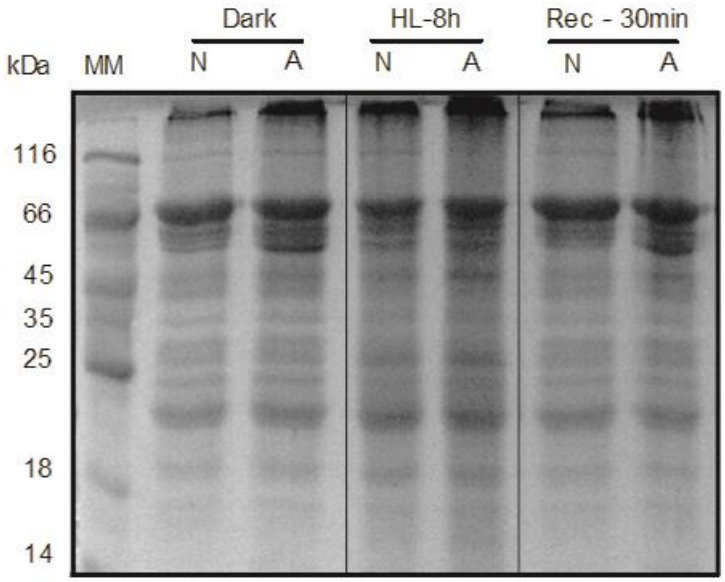


Figure 4. SDS-PAGE profile of proteins obtained from thylakoids enriched extracts of rice plants previously supplied with 10 mM nitrate or 10 mM ammonium for 8 days and subsequently exposed to moderate light – 400 µmol m^{-2} s^{-1} (left panel) or high light - 2000 µmol m^{-2} s^{-1} conditions for 8 hours. For thylakoids extraction, leaf samples were collected in the dark (pre-dawn), at the end of the high light treatment (8 hours) and after 30 minutes of recovery in the dark after high light treatment (recovery). A similar amount of 2 µg of total chlorophyll was applied to each well. The image is representative of three independent biological replicates.

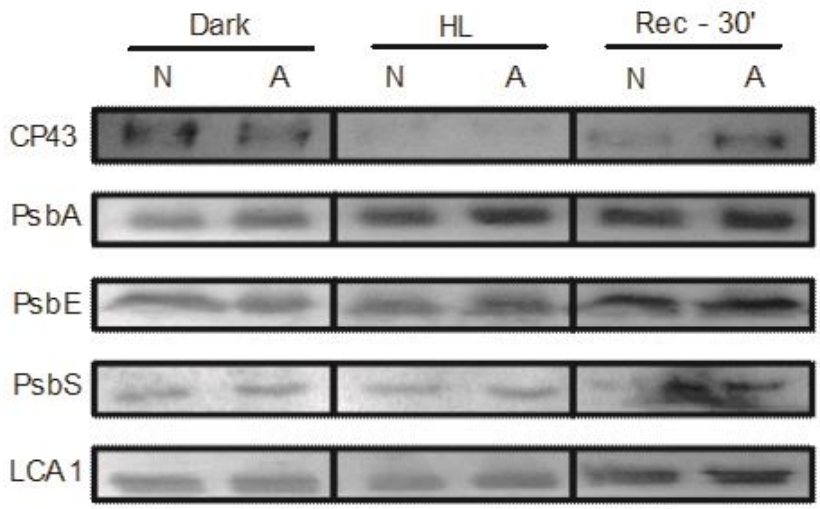


Figure 5. Western blot analysis of the photochemical proteins CP43, PsbA, PsbE, PsbS and LCA1. For immunodetection of target proteins, SDS-PAGE gels containing the protein profile of thylakoid extracts (Fig. 3) were first transferred electrophoretically to PVDF membranes and then incubated with the specific antibodies. For thylakoids extraction, leaf samples were collected in the dark (pre-dawn), at the end of the high light treatment (8 hours) and after 30 minutes of recovery in the dark after high light treatment (recovery). A similar amount of 2 µg of total chlorophyll was applied to each well. The image is representative of three independent biological replicates.

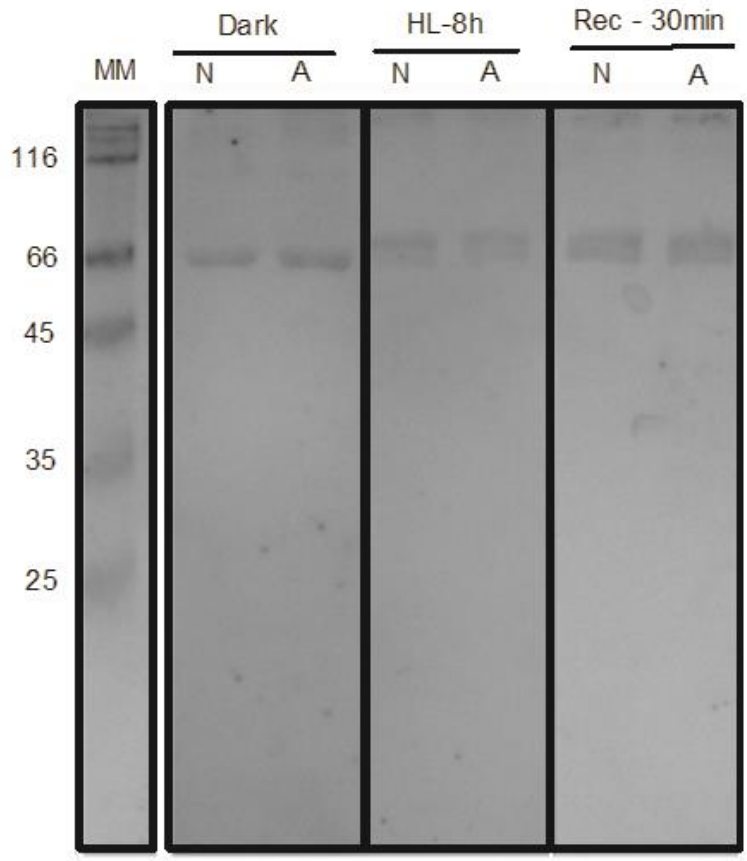


Figure 6. Oxyblot analysis of thylakoidal proteins. For oxyblot assay, thylakoid extracts were first subjected to derivatization with 2,4-dinitrophenylhydrazine and then separated by SDS-PAGE, as described in figure 3. The thylakoidal proteins in SDS-PAGE were subsequently transferred to PVDF membranes and then incubated with the specific antibodies. For thylakoids extraction, leaf samples were collected in the dark (pre- dawn), at the end of the high light treatment (8 hours) and after 30 minutes of recovery in the dark after high light treatment (recovery). The image is representative of three independent biological replicates.

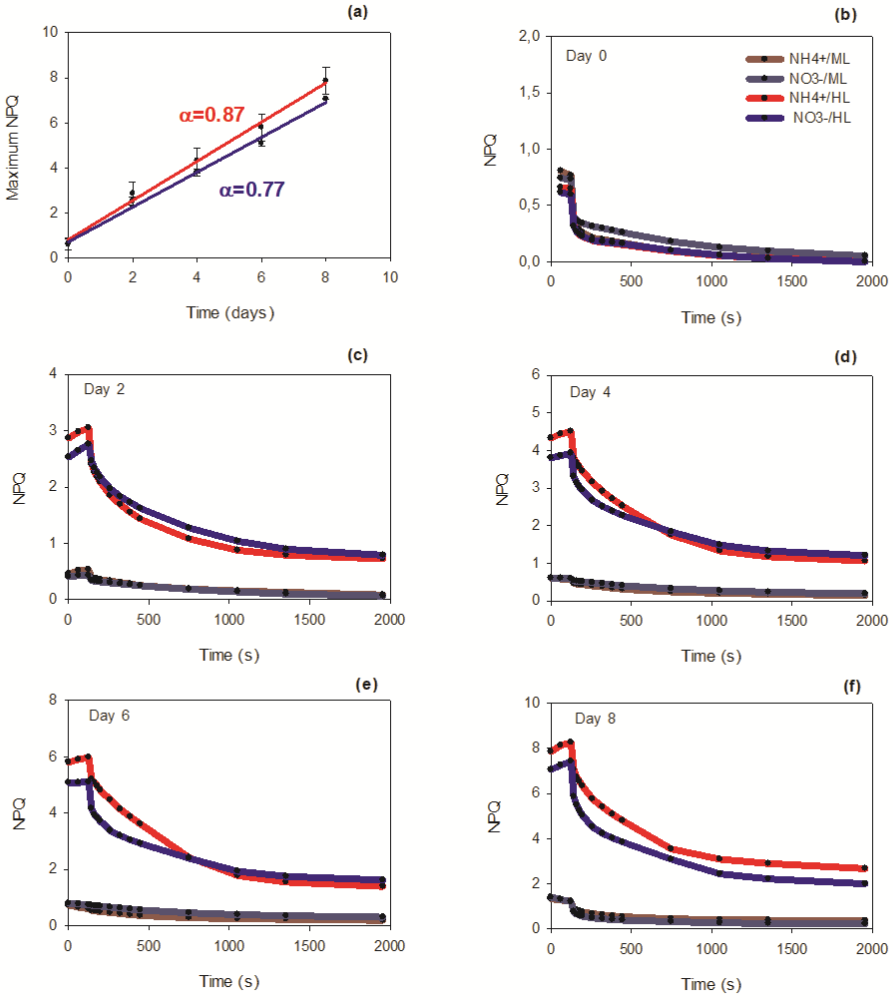
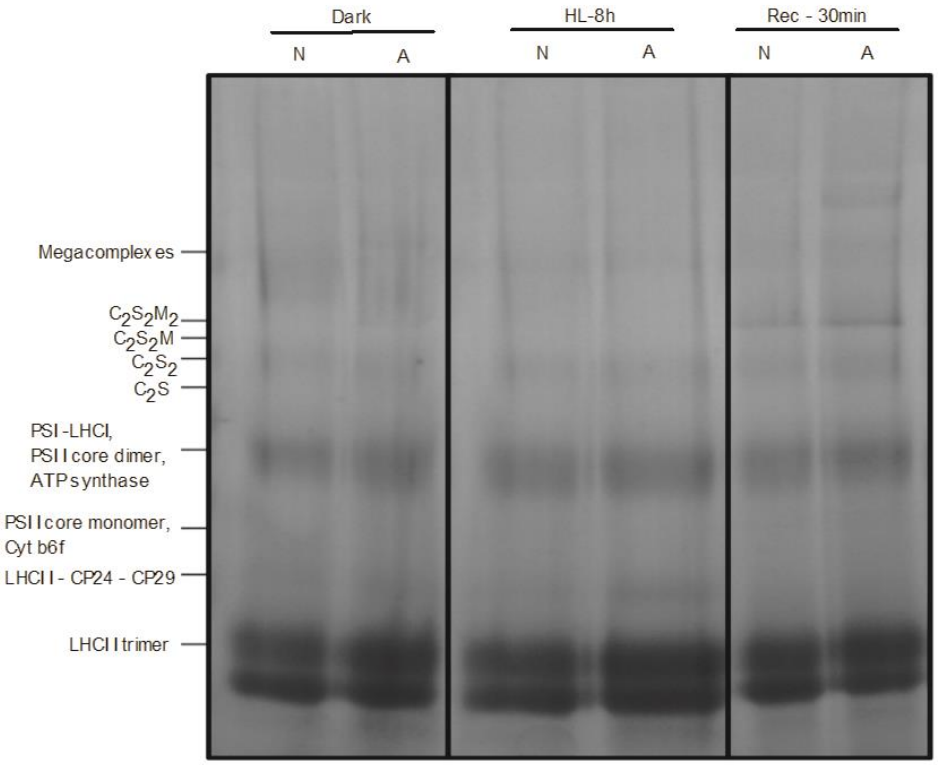


Figure 7. Maximum NPQ induction at steady state induced by high light (A) and NPQ dark recovery kinetics (B-E) measured in rice plants previously acclimated for up to 8 days in ML or HL. In (A), the slope angle (α) was calculated using the linear regression with function y = y0 + ax, obtained with the program SigmaPlot 12.0. Blue lines indicate the NPQ dynamics of rice plants supplied with 10 mM nitrate and red lines indicate the NPQ dynamics of rice plants supplied with 10 mM ammonium for up to 8 days. Black bars represent ± standard error.



Sub-Complex	Dark		HL		Rec	
	N	A	N	A	N	A
LHCII	1,0	1,2	1,6	1,9	0,9	1,4
LHCII-CP24-CP29	1,0	2,8	2,7	15,2	0,0	0,0
PSI-LHC1, ATPs, RC	1,0	1,3	1,6	2,1	1,4	1,1
C2S2	1,0	0,9	0,9	1,2	1,0	1,4
C2S2M2	0,0	0,0	0,0	0,0	1,0	1,6

Figure 8. Blue Native Page profile of proteins obtained from thylakoids enriched extracts of rice plants previously supplied with 10 mM nitrate or 10 mM ammonium for 8 days and subsequently exposed to moderate light – 400 µmol m^{-2} s^{-1} (left panel) or high light - 2000 µmol m^{-2} s^{-1} conditions for 8 hours. For thylakoids extraction, leaf samples were collected in the dark (pre-dawn), at the end of the high light treatment (8 hours) and after 30 minutes of recovery in the dark after high light treatment (recovery). A similar amount of 2 µg of total chlorophyll was applied to each well and thylakoidal extracts were previously treated with 1% (w/v) of α-DM. The image is representative of three independent biological replicates.

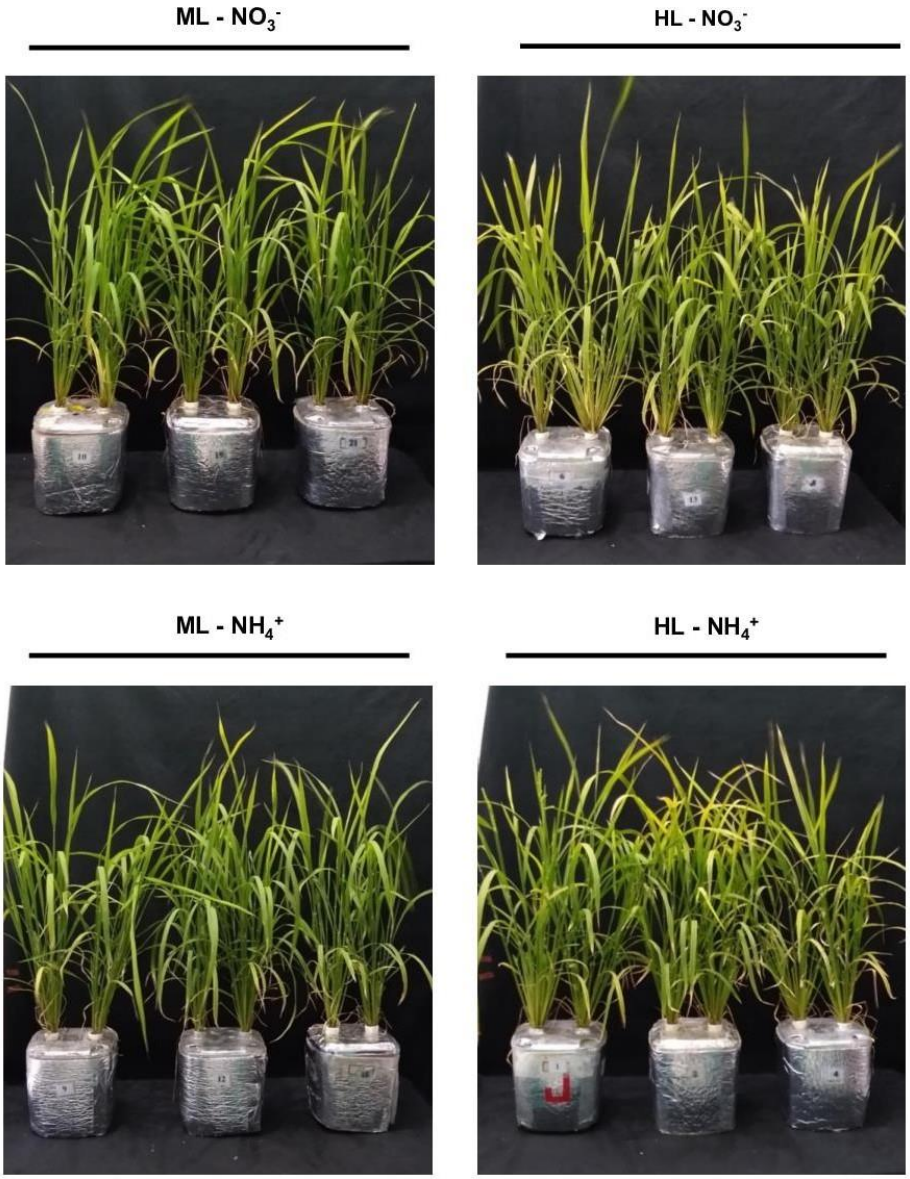


Figure 9. Morphological aspect of rice plants supplied for up to 8 days with nitrate (10 mM) or ammonium (10 mM) as the only form of N in the root medium and simultaneously exposed to contrasting light regimes, consisting of moderate light (400 µmol m^{-2} s^{-1}) and high light (1700 µmol m^{-2} s^{-1}), with photoperiod equivalent to 12 hours. The pictures were recorded at the end of the light treatment of the eighth day.

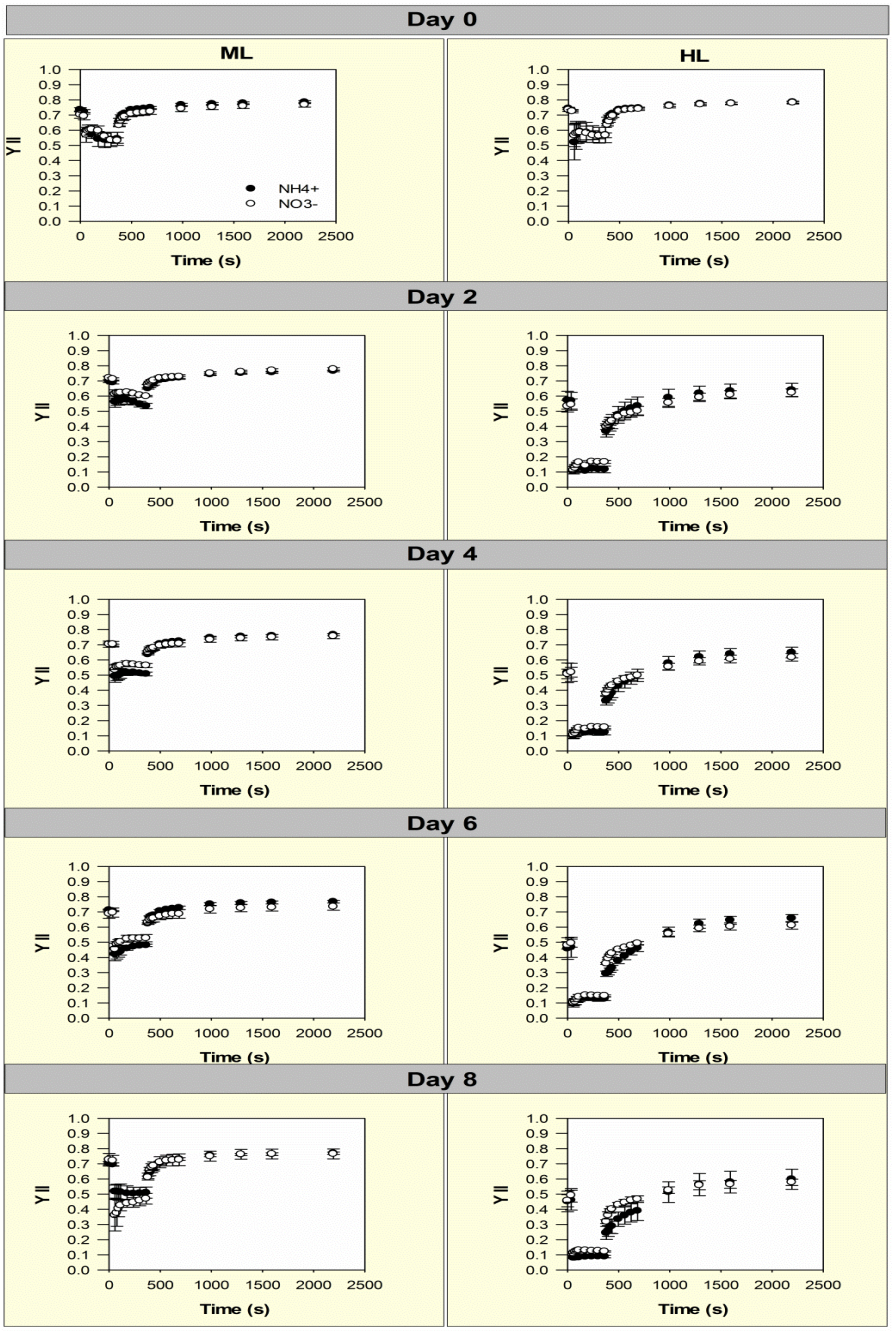


Figure 10. PSII effective quantum efficiency (YII) kinetics measured in rice plants previously acclimated for 0, 2, 4, 6 and 8 days under moderate light conditions – 400 µmol m^{-2} s^{-1} (left panel) or high light - 1700 µmol m^{-2} s^{-1} (right panel) and simultaneously supplied with 10 mM nitrate (open circles) or 10 mM ammonium (closed circles). The actinic light used in the induction phase was similar to the actinic acclimatization light of each plant in the growth chamber (ML and HL). In the graphs, gray squares represent the beginning of the photochemical recovery phase in the dark. Black bars represent ± standard error.

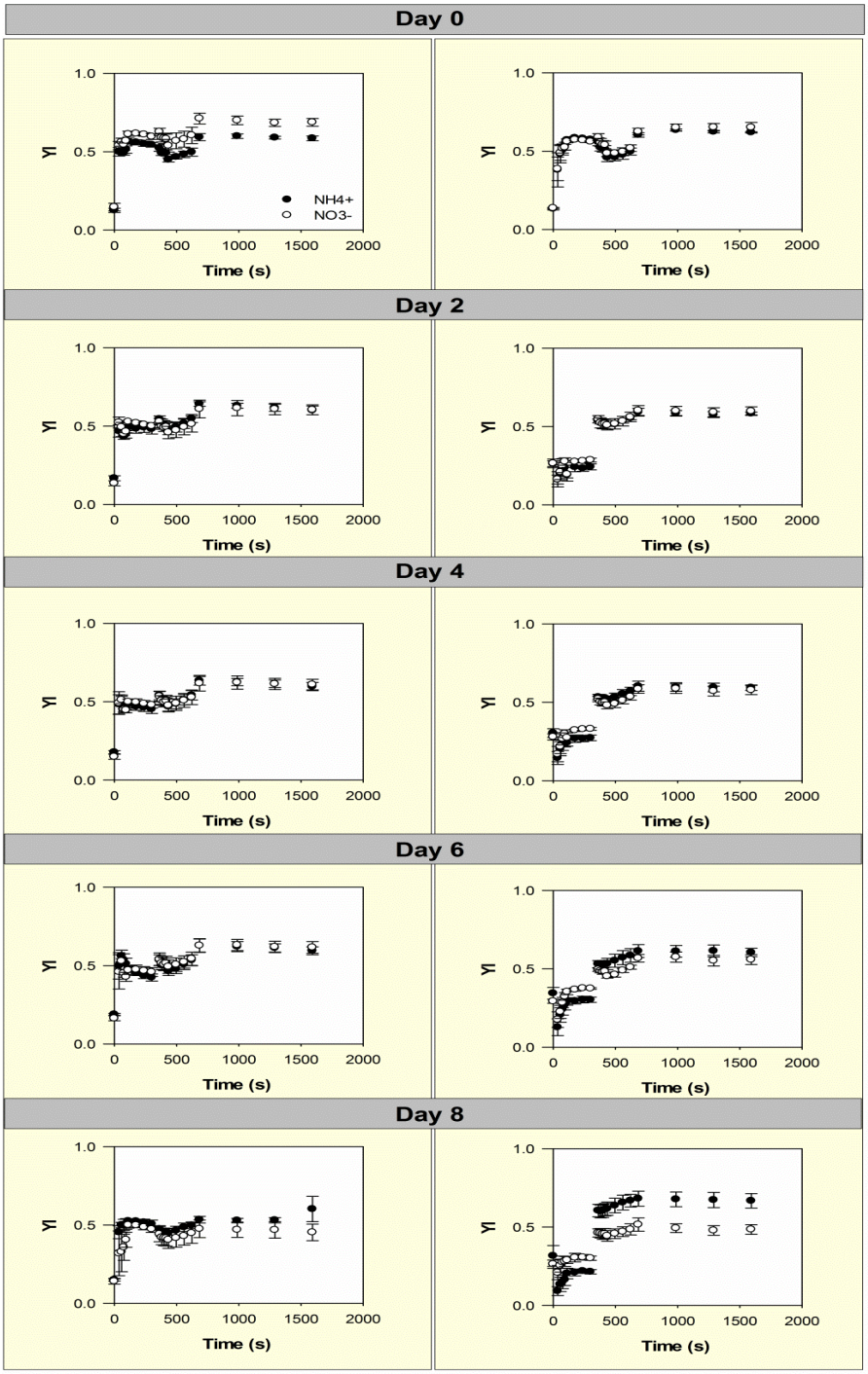


Figure 11. PSI effective quantum efficiency (YI) kinetics measured in rice plants previously acclimated for 0, 2, 4, 6 and 8 days under moderate light conditions – 400 µmol m^{-2} s^{-1} (left panel) or high light - 1700 µmol m^{-2} s^{-1} (right panel) and simultaneously supplied with 10 mM nitrate (open circles) or 10 mM ammonium (closed circles). The actinic light used in the induction phase was similar to the actinic acclimatization light of each plant in the growth chamber (ML and HL). In the graphs, gray squares represent the beginning of the photochemical recovery phase in the dark. Black bars represent ± standard error.

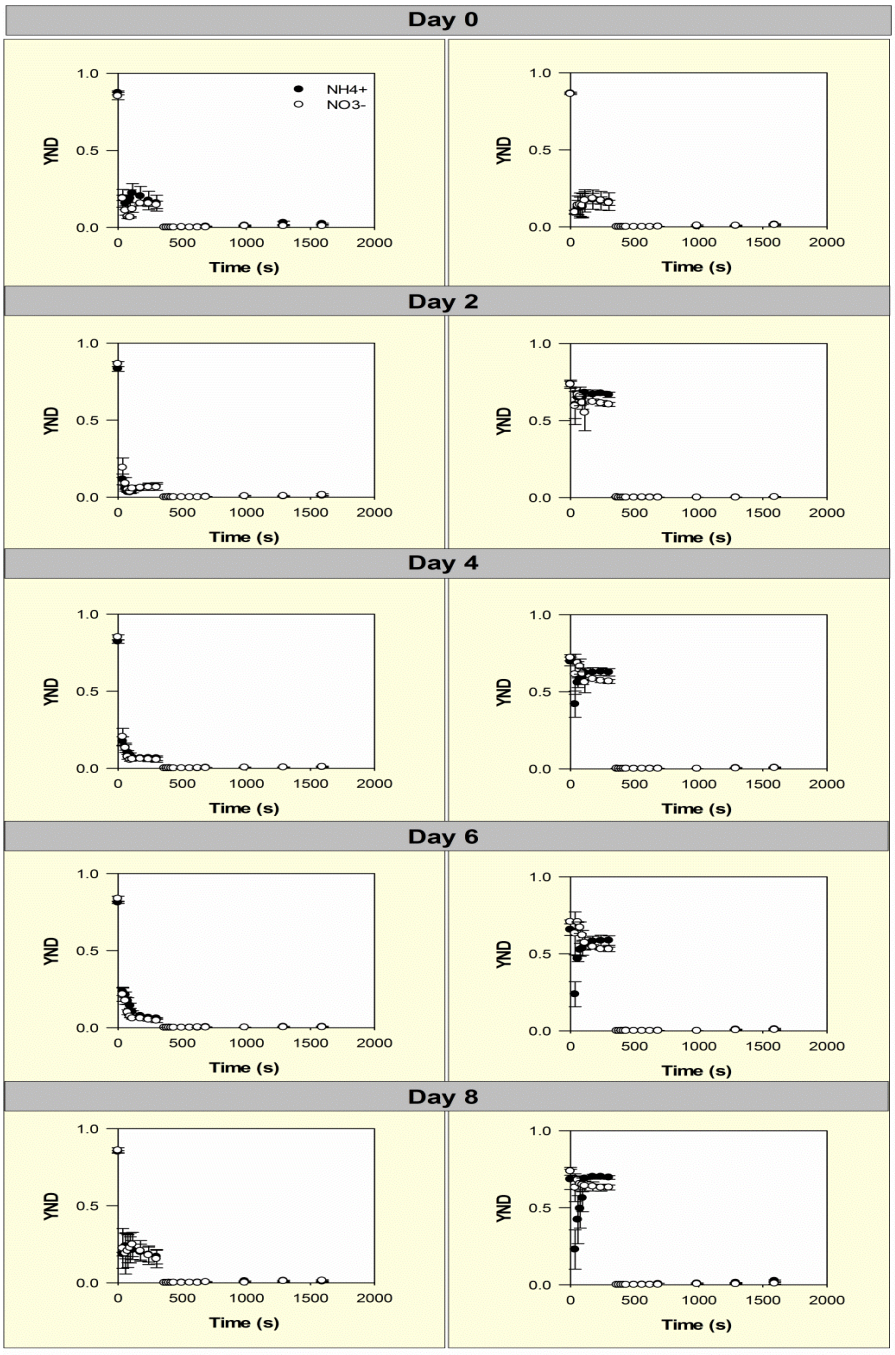


Figure 12. PSI donor side limitation (YND) kinetics measured in rice plants previously acclimated for 0, 2, 4, 6 and 8 days under moderate light conditions – 400 µmol m^{-2} s^{-1} (left panel) or high light - 1700 µmol m^{-2} s^{-1} (right panel) and simultaneously supplied with 10 mM nitrate (open circles) or 10 mM ammonium (closed circles). The actinic light used in the induction phase was similar to the actinic acclimatization light of each plant in the growth chamber (ML and HL). In the graphs, gray squares represent the beginning of the photochemical recovery phase in the dark. Black bars represent ± standard error.

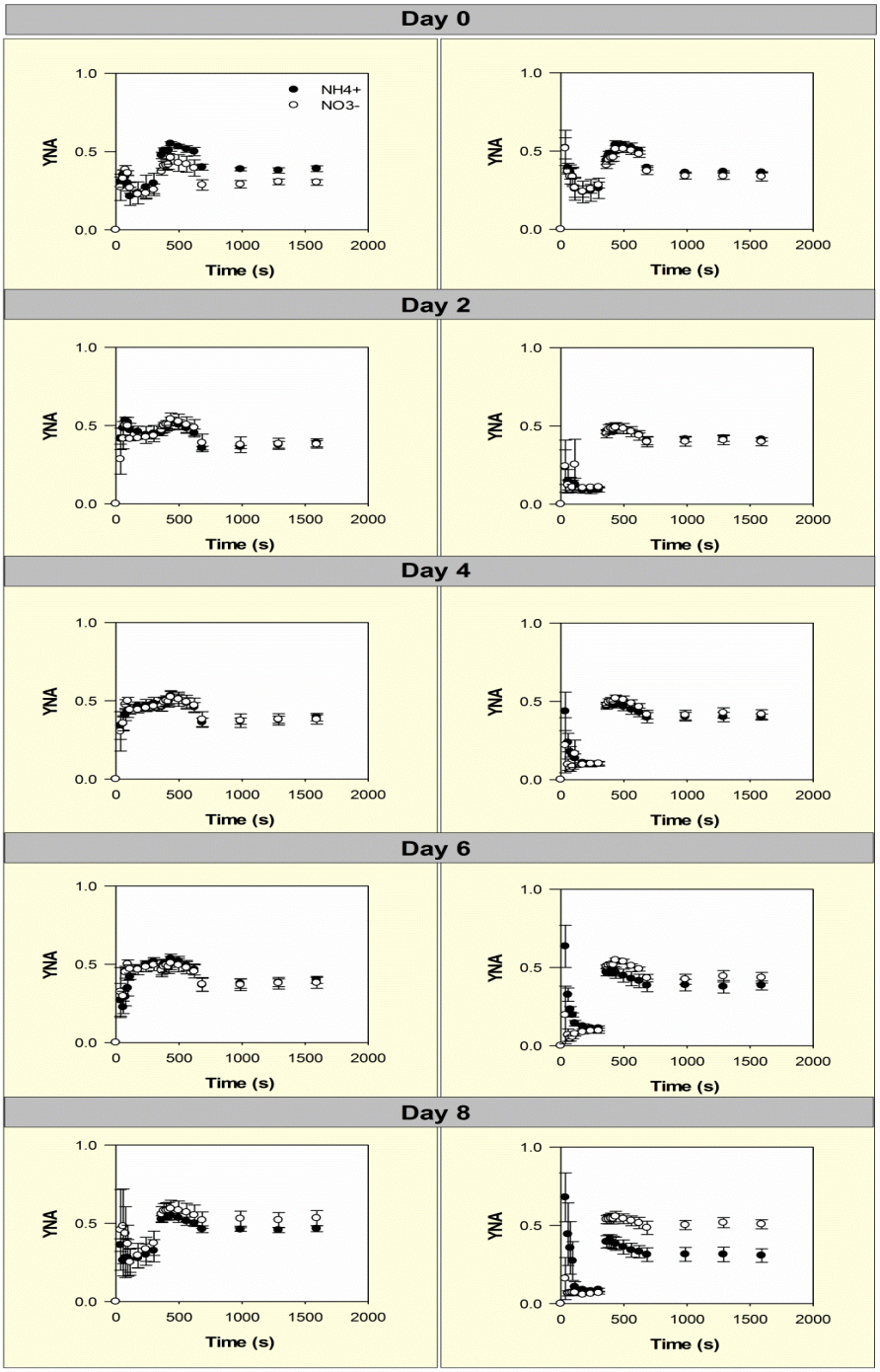


Figure 13. PSI acceptor side limitation (YNA) kinetics measured in rice plants previously acclimated for 0, 2, 4, 6 and 8 days under moderate light conditions – 400 µmol m^{-2} s^{-1} (left panel) or high light - 1700 µmol m^{-2} s^{-1} (right panel) and simultaneously supplied with 10 mM nitrate (open circles) or 10 mM ammonium (closed circles). The actinic light used in the induction phase was similar to the actinic acclimatization light of each plant in the growth chamber (ML and HL). In the graphs, gray squares represent the beginning of the photochemical recovery phase in the dark. Black bars represent ± standard error.

Parameter	ML		HL	
	N	A	N	A
gS (mol m^{-2} s^{-1})	0.61±0.044 aA	0.39±0.12 aA	0.53±0.02 aA	0.32±0.04 aB
P_N (µmol m^{-2} s^{-1})	24.53±1.54 aA	17.77±3.52 aA	19.20±3.18 aA	14.60±1.21 aB
Chlorophyll (mg g^{-1} DM)	5.33±0.35 aA	5.33±0.17 aA	5.43±0.20 aA	4.83±0.23aB
TBARS (ηmol g^{-1} FM)	20.84±1.08 aA	20.90±0.96 aA	25.72±3.10 aA	28.04±1.68 aB
Membrane Damage (%)	8.18±1.54 aA	11.40±2.20 aA	8.74±0.77 aA	13.71±2.50aB

Table 2. Net CO_2 assimilation (P_N), stomatal conductance (g_S), total chlorophyll content (CHL), content of thiobarbituric reactive species (TBARS) and electrolytes leakage (MD) of rice plants previously supplied with 10 mM nitrate or 10 mM ammonium for 8 days and subsequently exposed to moderate light conditions – 400 µmol m^{-2} s^{-1} or high light - 2000 µmol m^{-2} s^{-1} for 8 hours. Values indicate the average ± standard error (n=3). Different low case letters represent significant difference between light regimes and different capital letters mean significant differences between contrasting N treatments (Tukey at 0.05).

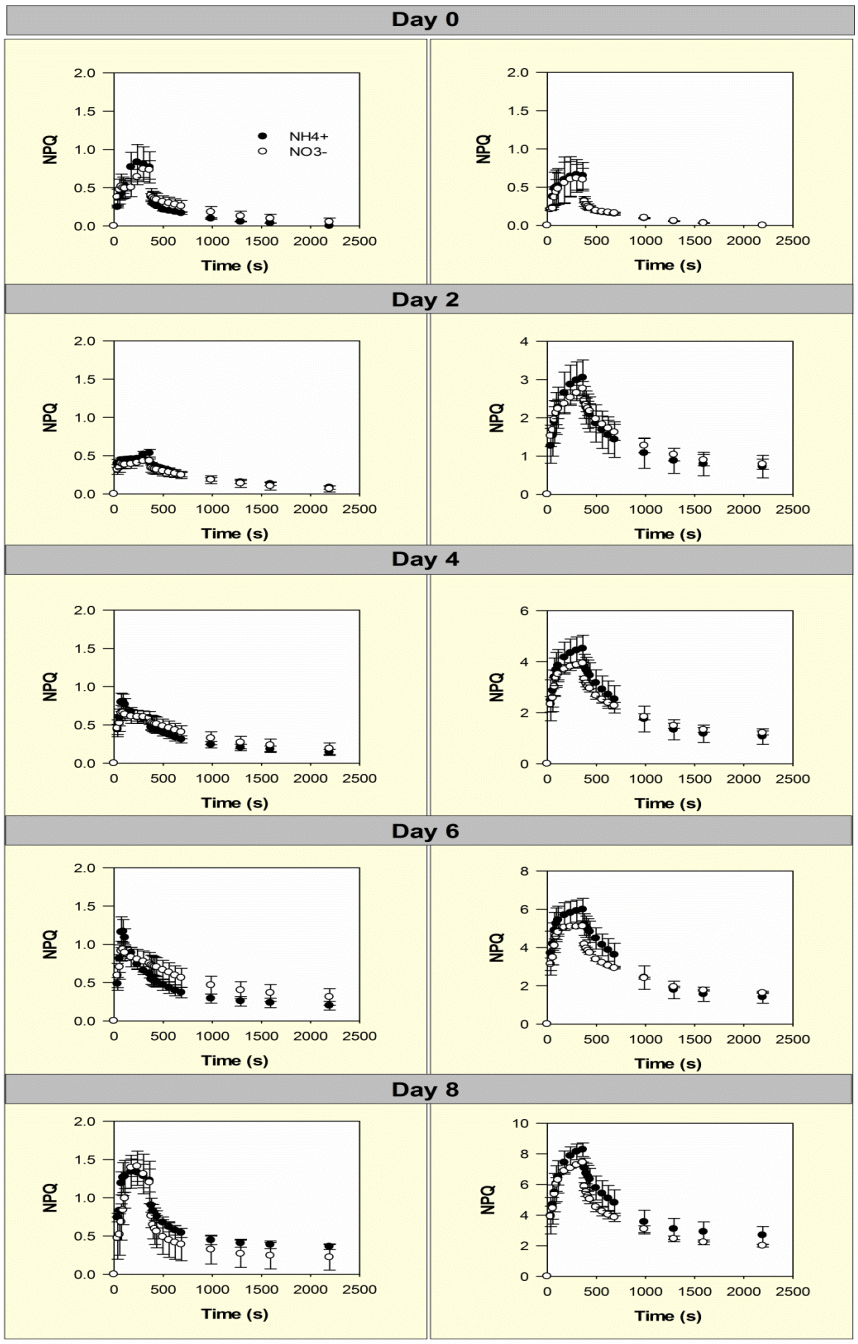


Figure 14. Non-photochemical quenching (NPQ) kinetics measured in rice plants previously acclimated for 0, 2, 4, 6 and 8 days under moderate light conditions – 400 µmol m^{-2} s^{-1} (left panel) or high light - 1700 µmol m^{-2} s^{-1} (right panel) and simultaneously supplied with 10 mM nitrate (open circles) or 10 mM ammonium (closed circles). The actinic light used in the induction phase was similar to the actinic acclimatization light of each plant in the growth chamber (ML and HL). In the graphs, gray squares represent the beginning of the photochemical recovery phase in the dark. Black bars represent ± standard error.