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ELIEZER DE ARAÚJO GUILHERME

NITROGEN USE EFFICIENCY, PHOTOSYNTHESIS AND PHOTORESPIRATION IN COTTON PLANTS EXPOSED TO EXCESS ENERGY AND N-DEPRIVATION

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To the Supreme Creator and Sustainer of the universe. To my dear parents, Eliane and Antonio. To my lovely wife Andrea. To my wise son Isaque. To my brothers Alexandre and Bruno.

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“When on the student's graduation the most of the tasks were done by his master, then the educational system failed.”
(Elaborated by the author)

ABSTRACT

Adverse environmental conditions might cause damage to plants whose severity also depends on the inherent characteristics of the plant. High light and nitrogen oscillation in soil are one of the most important abiotic stresses that plants often have to deal with. Therefore, to understand how plants efficiently manage the nitrogen to minimize losses in photosynthesis and productivity under excess light and N fluctuation is crucial to crop production. First, a research was conducted to test the hypothesis that increased assimilatory nitrate reduction is able to stimulate photorespiration and these two processes together are capable to enhance photosynthesis in presence of high light. Cotton plants in hydroponic medium were supplied with 10 mM NO_3^- (high nitrate supply-HN) or NO_3^- - deprivation (ND) for short-term and subsequently exposed to high light-HL or low light-LL. HL induced photoinhibition in both N-treatments but strongly enhanced nitrate assimilation and photorespiration rates in HN. Despite these constraints caused on PSII integrity, HN-plants displayed higher electron flux through PSII and enhancement in CO_2 assimilation, which was positively related to the photosynthetic nitrogen use efficiency (PNUE). These plants also exhibited increase in nitrate reductase (NR) and glutamine synthetase activities, which were associated with large accumulation of free amino acids and ammonia, in parallel to high NO_3^- reduction rates. The increase in photorespiration was greatly dependent on light intensity and, in a minor extent, on nitrate supply. These results have raised another question: How do HN plants followed by N deprivation efficiently use nitrogen in order to preserve photosynthesis? To solver this issue a second research was conducted on which cotton plants were exposed to three conditions in nutrient solution: (a) previous exposure to high nitrate supply (10 mM) for long-term (8 days); (b) nitrate deprivation (NO_3^- withdrawal) for four days followed by (c) an early N-deficiency during four days. Plants supplied with nitrate excess were able to display increment in shoot nitrogen use efficiency (NUE), whereas PNUE did not change, evidencing that excess N was not able to additionally improve CO_2 assimilation. NR activity was crucial to remobilize stored nitrate through deprivation phase, whereas free amino acids, total proteins, and chlorophylls were essential to N-remobilization. Despite the great decrease in chlorophyll content, PSII and PSI activities were kept stable until the onset of early N-deficiency. In conclusion under high light condition the high nitrate supply stimulates nitrate assimilation, which induces photorespiration and these two processes act synergistically favouring the photosynthetic efficiency, mainly improving PSII efficiency and CO_2 assimilation. On the

other hand, nitrate deprivation plants in non-photoinhibitory conditions present high NUE associated with NR activity and N remobilization to maintain the photosystems integrity even in the initial nitrogen deficiency.

Keywords: *Gossypium hirsutum.* High light. Nitrate assimilation. Nitrogen deficiency. Photoinhibition.

RESUMO

Condições ambientais adversas podem causar danos às plantas cuja gravidade irá depender também das características inerentes à planta. A alta luminosidade e a oscilação do nitrogênio no solo são alguns dos mais importantes estresses para os vegetais. Portanto, entender como esses organismos administram o nitrogênio para minimizar as perdas na fotossíntese e na produtividade em condições de luz excessiva e de flutuação de N é crucial. Primeiramente foi testada a hipótese de que o aumento da assimilação do nitrato é capaz de estimular a fotorrespiração e esses dois processos juntos são capazes de melhorar a fotossíntese sob alta luz. Plantas de algodão foram supridas em hidroponia com 10 mM NO_3^- (alto nitrato-HN) ou privação de NO_3^- (ND) em curto prazo e subsequentemente expostas a alta luz - HL ou baixa luz - LL. A fotoinibição foi induzida por HL em ambos os tratamentos, mas houve intensificação da assimilação de nitrato e da taxa de fotorrespiração em HN. Apesar dos danos no PSII, as plantas HN apresentaram maior fluxo de elétrons e aumento na assimilação de CO_2, o que foi positivamente relacionado com a eficiência de utilização do nitrogênio para a fotossíntese (PNUE). Estas plantas também exibiram aumento nas atividades de redutase de nitrato (RN) e glutamina sintetase, associado ao grande acúmulo de aminoácidos livres e amônia e alta diminuição de NO_3^-. O aumento da fotorrespiração foi bastante dependente da luz e, em menor escala, do nitrato. Esses resultados levantaram outra questão: como as plantas HN seguido de privação de N fazem o uso do nitrogênio tendo em vistas a preservar a fotossíntese? Uma segunda pesquisa foi realizada em que plantas de algodão foram expostas a: (a) exposição prévia a alta oferta de nitrato (10 mM) em longo prazo (8 dias); (b) privação de nitrato (retirada de NO_3^-) por quatro dias, seguida de (c) deficiência precoce de N durante quatro dias. Plantas com excesso de nitrato exibiram incremento na eficiência de uso do N (NUE) da parte aérea, enquanto o PNUE não mudou, evidenciando que o excesso de N não foi capaz de melhorar adicionalmente a assimilação de CO_2. A NR foi fundamental para remobilizar o nitrato armazenado na fase de privação, enquanto aminoácidos livres, proteínas totais e clorofilas foram essenciais para a remobilização de N. Apesar da grande diminuição na clorofila, os PSII e PSI mantiveram-se íntegros mesmo durante a deficiência inicial. Conclui-se que sob condições de excesso de luz, o alto suprimento de nitrato estimula a assimilação de nitrato, que induz a fotorrespiração, e esses dois processos agem sinergicamente, favorecendo a eficiência fotossintética, principalmente melhorando a eficiência do PSII e a assimilação de CO_2. Por outro lado, plantas em privação de nitrato em

condições não fotoinibitórias apresentam alta NUE associada à atividade de NR e remobilização de N para manter a integridade dos fotossistemas mesmo na deficiência inicial de nitrogênio.

Palavras-chave: Alta luz. Assimilação de nitrato. Deficiência de nitrogênio. Fotoinibição. *Gossypium hirsutum*.

ABBREVIATION LIST

A	Net CO_2 assimilation rate
AA	Free amino acids
OEC	Oxygen evolution complex
Chl	Chlorophyll
C_i	Intercellular CO_2 concentration
CEF	Cyclic electrons flow
E	Transpiration
EKII	Minimum saturating irradiance for ETRII
ETC	Electron transport chain
ETRII	Electron transport rate from PSII
$ETR_{max}I$	Maximum electron transport rate from PSI
$ETR_{max}II$	Maximum electron transport rate from PSII
Fm	Maximum fluorescence in the dark
Fo	Minimum fluorescence in the dark
Fv/Fm	Maximum quantum yield of PSII
GOGAT	Glutamate synthase
GS	Glutamine synthetase
HL	High light
HN	High nitrate supplying treatment
LHC	Light-harvesting complex
J_c	Electron flux to Rubisco carboxylation
J_{max}	Maximum electron transport rate for RuBP regeneration
J_o	Electron flux to Rubisco oxygenation
ND	Nitrate deprivation treatment
NPQ	non-photochemical quenching
NR	Nitrate reductase
NUE	N use efficiency
PNUE	Photosynthetic nitrogen use efficiency
PPFD	Photosynthetic photon flux density
QA	Quinone A
QB	Quinone B

Pr	Photorespiratory CO_2 evolution
PSII	Photosystem II
qP	Photochemical quenching coefficient
RbcL	Rubisco large subunit
RD	Dark respiration
REF	Reference light
ROS	Reactive oxygen species
TBARS	Thiobarbituric acid reactive substances
TSS	Total soluble sugars
Vcmax	Maximum Rubisco carboxylation rate
Y(I)	Effective quantum yield of PSI
Y(II)	Effective quantum yield of PSII
Y(NA)	Limitation of acceptor side of PSI
Y(ND)	Limitation of donor side of PSI

CONTENTS

1 INTRODUCTION

As sessile organism, plants need to be very effective in dealing with unfavorable environmental conditions such as drought, low or high temperature, low or high light and low or high nutrients supply. In most cases the abiotic stress causes several negative effects in plants, for instance in metabolism, growth, development and productivity. In generally when plants are subjected to stressful situations might occur excess energy which causes reactive oxygen species (ROS) over production that in turn leads to impairment of photosynthesis called photoinhibition. To avoid these damages, plants normally trigger a series of mechanisms in order to dissipate the excess energy. Some pathways that require high energy might act as photoprotection mechanism once they are strong sinks of electrons and ATP. In this context it has been demonstrated that nitrogen assimilation and photorespiration are important to photosynthesis efficiency under some stress conditions, such as salinity and drought. However, little is known about how these processes might help photosynthesis under high light, a very common condition in tropical regions.

Nitrogen is the mineral nutrient most required by plants because it is part of several molecules important to life, such as amino acids, proteins, DNA and chlorophylls. Almost half of the nitrogen present in leaves is used to compose the photosynthetic machinery. Being 20% only for Rubisco, the main enzyme of carbolaxylation phase. In soils the nitrogen levels might fluctuate a lot, on which a part is absorbed by plants and another part is lost by some process such as denitrification returning to atmosphere or undergoing leaching and erosion polluting the environment. Therefore, the nitrogen cost for crop production is very high and are far beyond monetary costs, reaching environmental and even social spheres. Thus, to understand how plants efficiently manage the available N in order to improve photosynthesis, to grow, develop and produce is a crucial issue for the planet. However, there are still few studies related to this subject taking into account real situations such as high N levels after fertilization followed by N deprivation reaching an initial deficiency in plant.

How does nitrogen use might protect photosynthesis under high light? How does plants efficient use N in a real condition of high N levels after fertilization followed by N deprivation reaching an initial deficiency in plant? These are extremely important matters that have caught the attention of scientists for decades. Although these issues are well studied there are still many questions to be answered. This thesis aims to help answering these

questions adding important information in the assembly of this great puzzle called scientific knowledge.

2 LITERATURE REVIEW

The cotton plant (Gossypium hirsutum L.)

For many centuries cotton has been widely used practically everywhere in the world. Since it has several applications, for instance in clothing manufacture and in the health field the importance of cotton is incontestable (RIELLO, 2013; ALBERTINI, 2017). Besides that, cotton plants are relatively resistant to drought, high light and others stressful conditions which allow its cultivation in several regions with some climatic restriction such as water limitation and high solar irradiance (RIELLO, 2011; LEE and FANG, 2015; WENDEL and GROVER, 2015). Therefore due the several utilization of cotton and the cotton plant potential to produce in limited environments the cotton cultivation has become an important economic activity for the world (ALBERTINI, 2017). Many studies have been made with cotton plants in order to improve its tolerance to some biotic and abiotic stress. In fact many works have been successful and a wide variety of genetic materials already available to the farmers (ABDURAKHMONOV *et al.*, 2016; DU *et al.*, 2018).

The cotton plant has C3 metabolism presenting high photorespiration which might reach more than 50% of photosynthesis mainly in high light and high temperature conditions. Especially in tropical regions, both mitochondrial respiration and photorespiration might be highly stimulated in this plant, which reduces their photosynthetic coefficient (Kp). (MOTA, 1976; HEARN and CONSTABLE, 1984; BELTRÃO *et al.*, 1993; LARCHER, 2000). In this species the impact of light is dependent of several factors such as the stage of development and the presence of environmental stress. For example, in the reproduction phase large amounts of light are required due to the high demand for photoassimilates to both vegetative and reproductive growth (GUINN, 1974; ZHAO and OOSTERHUIS, 1998). However, as in any other plant the excess light might lead to various disorders such as photoinhibition that may reflect on productivity (LI *et al.*, 2009). The nitrogen excess is also a factor that might affect cotton productivity by stimulating the vegetative phase to the detriment of the reproductive phase (ROSOLEM and MIKKELSEN, 1989; BOQUET *et al.*, 1994). Despite

the importance of these subjects little is known about the nitrogen use efficiency (NUE) by cotton plants in order to protect photosynthesis under high light conditions.

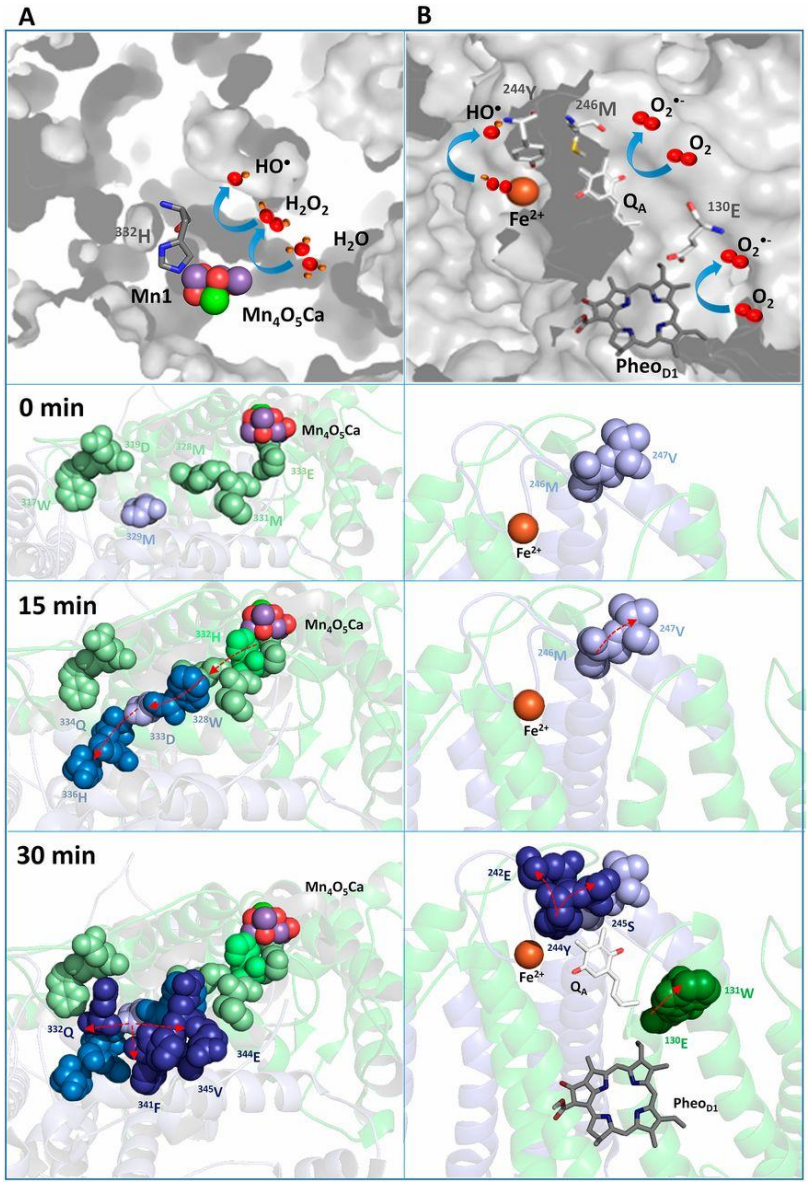
Photoinhibition processes

The photochemical phase of photosynthesis is performed by two very complex structures called photosystem I (PSI) (BUSCH, 2011) and photosystem II (PSII) (KLUGHAMMER and SCHREIBER, 1994; CARDONA, 2012). Both photosystems have light collecting systems called the light-harvesting complex (LHC) (LIU, 2004; ROCHAIX, 2014; CROCE, 2014). The light energy (photons) captured by the antennas is transferred by resonance to the reaction center of photosystem II where the charge separation event occurs. This process allows the breakdown of the water molecule in the oxygen evolution complex (OEC) (BARBER, 2002; UMENA *et al.*, 2011). In sequence, the electrons removed from water are transported by an electron transport chain (ETC) in the thylakoid membrane to reduce NADP+. This transport process generates a proton gradient used for ATP synthesis. Then, these energetic molecules might be used for the carboxylation reactions in the Calvin-Benson cycle (ARNON *et al.*, 1954; HILL and BENDALL, 1960; PAUL and FOYER, 2001). However, in many conditions the rate of formation of ATP and NADPH occurs at a rate higher than their use which may lead to excess energy in chloroplasts (GOH *et al.*, 2012).

It is well known that under some stresses the plants normally undergo photosynthesis impairment where the Calvin-Benson cycle is generally more affected than the photochemical phase in the early stages. This imbalance between the two phases of photosynthesis might produce electrons excess and consequently reactive oxygen species (ROS) (TAKAHASHI and MURATA, 2008). When in excess ROS might affect some proteins of photosynthesis leading to a process called photoinhibition (KALE *et al.*, 2017; JIMBO *et al.*, 2018). Some works have shown that one of the main targets of photoinhibition is PSII, especially in the OEC (TAKAHASHI and BADGER, 2011; GOH, 2012; KALE *et al.*, 2017). Although the PSI can be inhibited in some specific conditions (SONOIKE, 2011; HUANG *et al.*, 2018). By a natural and constant process the PSII is damaged and repaired, and therefore photoinhibition only happens when the rate of damage is greater than the intensity of repair (MURATA and NISHIYAMA, 2018; JIMBO *et al.*, 2018). In an elegant research Kale *et al.* (2017) showed that the photoinhibition process caused by ROS in PSII occurs through a cascade of amino acid oxidation along the D1 and D2 proteins. These data

reinforces the idea that the PSII damage process is also quite complex, requiring still many studies for its full elucidation (Figure 1).

Figure 1 - Proposed schemes for amino acid oxidation in the vicinity of Mn_4O_5Ca cluster and the nonheme iron, Q_A, and $Pheo_{D1}$. (*A*) At the Mn_4O_5Ca cluster, $HO^{\bullet}$ is formed by reduction of H_2O_2 by Mn1. The H_2O_2 is formed by incomplete oxidation of water. Incomplete water oxidation could occur either because of uncontrolled water access to the Mn_4O_5Ca cluster via a water ingress channel, which is modulated by chloride, or defects in a proton exit pathway, which is modulated by chloride. It should be noted that these possibilities, and others, are not mutually exclusive. D1:^{332}H, which is coordinated to Mn1, may be a primary target of this short-lived $HO^{\bullet}$ and was among the first modified amino acid residues modified in the photoinhibitory timecourse. Hydrogen abstraction from D1:^{332}H would lead to the formation of a carbon-centered histidyl radical. In the presence of O_2, the formation of protein peroxyl radicals occurs. These radicals may lead to hydrogen abstraction from adjacent D1 residues (plausibly from ^{328}M, ^{331}M, and ^{333}E). In the early stage of photoinhibition, propagation of radical reactions on the D1 protein could continue from D1:^{328}M to other nearby residues (such as D1:^{319}D or D2:^{329}M). In the later stages of photoinhibition, D2:^{329}M may act as a bridge that facilitates radical propagation in the C terminus of D2 protein. It should be noted that given the half life of $HO^{\bullet}$ ($\sim 1 \times 10^{-9}$ s), it could potentially diffuse and abstract hydrogen directly from other nearby residues. (*B*) At the nonheme iron, $HO^{\bullet}$ is formed by reduction of bound peroxide generated by the reaction of $O_2^{\bullet-}$ with nonheme iron. $O_2^{\bullet-}$ is formed by reduction of O_2 by either $Q_A^{\bullet-}$, $Pheo_{D1}^{\bullet-}$, or both. D2:^{244}Y, coordinated to bicarbonate ligand of the nonheme iron, may be a primary target of short-lived $HO^{\bullet}$. As noted above, the half life of $HO^{\bullet}$ could potentially allow migration and hydrogen abstraction directly from other nearby residues. $O_2^{\bullet-}$ formed by reduction of O_2 may also directly oxidatively modify nearby residues (such as D1:^{130}E or D1:^{131}W). In *A* and *B*, oxidized D1 residues are shown as green spheres and oxidized D2 residues are shown as blue spheres. Darker shades of green and blue represent later oxidation events. Arrows show possible propagation pathways of radical reactions from residues in the proximity of the Mn_4O_5Ca cluster, the nonheme iron, Q_A, and $Pheo_{D1}$ to residues that are more distant from these sites. Note that not all oxidized residues identified in Table S1 are illustrated in this figure.



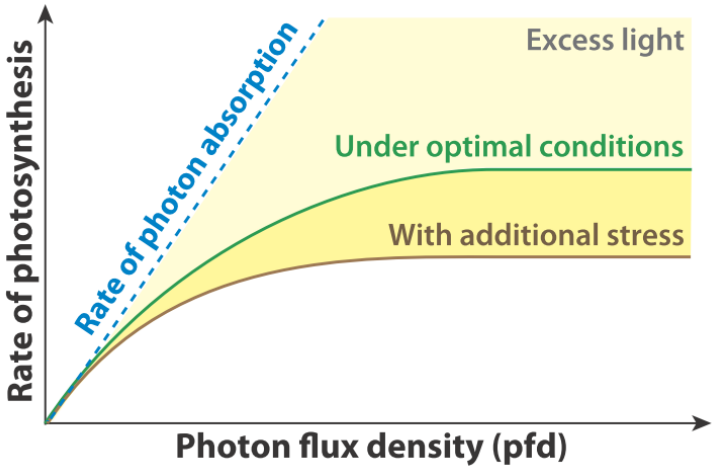
Source: KALE *et al* (2017)

When light becomes a problem

In some circumstances the plant light utilization capacity is less than the amount of incident light leading to a phenomenon called excess light. Therefore, in this concept, excess light is not only associated with the amount of light, but also with the plants ability to utilize the incoming energy in each circumstance (Figure 2) (LI *et al.*, 2009). This problem is extremely increased in tropical regions where there is high solar radiation practically every day. In addition this is the fact that these regions are generally affected by stresses such as drought, salinity, heat and nutrient deficiency that might reduce the energy utilization efficiency. To reduce the light excess formation the plants might trigger some protection

mechanisms such as the movements of leaves and chloroplasts (TAKAHASHI and BADGER, 2011).When these mechanisms are not sufficient, excess energy formation occurs in chloroplast leading to ROS production (KALE *et al.*, 2017; JIMBO *et al.*, 2018). To avoid excess ROS formation plants use several mechanisms to dissipate energy such as water water cycle and cyclic electron flow (FLEXAS and MEDRANO 2002; HORTON and RUBAN, 2005; ASADA, 2006; BAGARD et al., 2008; TAKAHASHI and MURATA, 2008). Even with all the photoprotection mechanisms the photosynthetic apparatus might undergoes photoinhibition under excess light (LI *et al.*, 2009). When this process occurs in a high degree of severity or remains for a long time the productivity might be reduced (TAKAHASHI and BADGER, 2011). Therefore, despite light be essential to plant production it might become a problem in some circumstances.

Figure 2 - Light response curves for photosynthesis compared with the rate of light absorption.



Source: LI *et al* (2009)

Importance of nitrate supply and assimilation and photorespiration for photosynthesis under adverse environmental conditions

Generally the nitrogen absorbed by roots reaches the shoot in nitrate or amino acids forms (LIN *et al.*, 2000). When reaching the mesophyll cells, NO_3^- can be stored in the vacuoles or reduced to nitrite in the cytosol by nitrate reductase enzyme (NR), which reduces NO_3^- to NO_2^- (nitrite) with NADH consumption. Noctor and Foyer (1998) suggest that a part of the NADH used for NR is derived from cytosolic reduction of NAD+ by malate produced in chloroplasts from oxaloacetate reduction using photosynthetic NADPH as an electron

donor. Thus, NR might play an important role as an electron sink under electron excess condition in chloroplasts. After the reduction of nitrate to nitrite, this molecule is rapidly transported to chloroplasts and reduced to NH_4^+ by the nitrite reductase enzyme (NiR), which consumes six electrons by reduced ferredoxin that comes from the photochemical phase of photosynthesis (LEA *et al.*, 1999). The NH_4^+ is then assimilated through its fusion into a glutamate molecule for glutamine formation by glutamine synthetase enzyme (GS) using ATP. Subsequently the glutamine amide group is transferred to a 2-oxoglutarate molecule to produce two glutamate molecules by glutamine: 2-oxoglutarate amino transferase enzyme (GOGAT) using 2 electrons from NADH or reduced ferredoxin. The coordinated action between the GS and GOGAT enzymes was known as the GS / GOGAT cycle. (MIFLIN *et al.*, 1977; LEA *et al.*, 1990).

The importance of nitrate assimilation for photosynthesis improvement has been shown in some studies, mainly under stress conditions such as salinity and high light (ARAGÃO *et al.*, 2012a; KLOTZ *et al.*, 2015; HUANG *et al.*, 2016; BUSCH *et al.*, 2018). However, despite being heavily studied these issues are still poorly understood. One hypothesis is that under electrons excess the nitrate assimilation might act as a strong electrons sink and avoiding excess ROS formation (ARAGÃO *et al.*, 2012a; KLOTZ *et al.*, 2015). When submitted to salinity, *Jatropha curcas* plants showed increase in photoinhibition of PSII, reduction of CO_2 assimilation and lower biomass production when NO_3^- / NH_4^+ ratio in nutritive solution was reduced. In the same work, with NR inhibition and salinity, it was observed higher H_2O_2 content, higher lipid peroxidation and greater electrolyte leakage in leaves when compared to control (ARAGÃO, 2012b). Furthermore, under low N conditions plants tend to increase the activity of some energy dissipating mechanisms, such as the non-photochemical quenching (NPQ) in a probable attempt to alleviate the lack of electron sink generated by N assimilation (EINALI *et al.*, 2013; SAROUSSI *et al.*, 2016). Sun et al (2016) found that high N supply might improve photosynthesis efficiency also under oscillating high light.

Another hypothesis is that high nitrate assimilation improves photorespiration and both processes act together to benefit photosynthesis (FORDE and LEA, 2007; HUANG *et al.*, 2016; BUSCH *et al.*, 2018). Working with tobacco subjected to low and high N supply Huang *et al.* (2016) found that high N supplying is capable to improve CO_2 assimilation (2-fold) and electrons transport rate to PSII (2.5-fold) under high light during light curve performance. These results were strongly related to improvement in photorespiration (2-fold)

and water water cycle (WWC) (2-fold). The authors suggest that under excess light high N supply improves photorespiration and both processes induce WWC and on this way helping to avoid oxidative stress, since this last pathway is also an important electrons sink. However, WWC activity was estimated by mathematical equation which makes the conclusions questionable (MIYAKE and YOKOTA, 2000; MAKINO *et al.*, 2002). Some data show that the regulation processes between nitrate assimilation and photorespiration might be a two-way path. Since under inhibition of photorespiration by high CO_2 occurs decrease in nitrate assimilation (BLOOM, 2015). All these data clearly show a complex and efficient integration between nitrogen assimilation and photorespiration in order to protect photosynthesis under stress condition.

Photorespiration occurs in the presence of O_2, as this molecule might be used by enzyme ribulose-1,5-bisphosphate carboxylase / oxygenasse (Rubisco) as substrate. The photorespiratory process produce one molecule of phosphoglycerate (PGA) and one molecule of 2-phosphoglycolate for each assimilated O_2. PGA follows the normal pathway in Calvin-Benson cycle, while 2-phosphoglycollate follows a series of reactions until partial recovery of glyceraldehyde-3-phosphate (PETERHANSEL, 2011). After Rubisco oxygenation the 2-phosphoglycolate is converted to glycolate releasing Pi (SOMERVILLE and OGREN, 1979). Then glycolate is carried to peroxisomes where it is oxidized to produce glyoxylate and hydrogen peroxide (H_2O_2). Glyoxylate receives an amino group to produce glycine and releasing 2-oxoglutarate. The glutamate used in this reaction normally comes from nitrogen assimilation or reassimilation pathways (IGARASHI *et al.*, 2003; LIEPMAN and OLSEN, 2003; IGARASHI *et al.*, 2006). After, glycine is transported to mitochondria where undergoes several reactions to produce serine with release of one CO_2 and one NH_4^+ for each two glycine consumed (BAUWE and KOLUKISAOGLU, 2003). Then serine is carried to peroxisomes were suffer several reactions until 3-phosphoglycerate regeneration that follow the normal pathway in Calvin-Benson cycle (SOMERVILLE and OGREN, 1980; GIVAN and KLECZKOWSKI, 1992; LIEPMAN and OLSEN, 2001; BOLDT *et al.*, 2005; BARTSCH *et al.*, 2008; FOYER *et al.*, 2009).

Normally the released ammonium in mitochondrial photorespiration phase is assimilated in chloroplast by GS / GOGAT cycle. During high photorespiratory rates the levels of released ammonium is about 10-fold higher than ammonium from nitrate assimilation (NOCTOR and FOYER, 1998; WINGLER *et al.*, 2000). Therefore, despite the assimilation process by GS / GOGAT consumes few electrons per ammonium (only two) the

high rates of photorespiratory ammonium assimilation lead to great energy consumption. In the presence of photorespiration, the energy consumption per CO_2 fixed is about 5.375 mol ATP and 3.5 mol NADPH while in absence of photorespiration these levels are much lower (3 mol ATP and 2 mol NADPH) (WINGLER *et al.*, 2000). As it is clear nitrogen assimilation and photorespiration are metabolically interconnected processes. First the nitrogen that comes from the soil is assimilated to glutamate and in turn is used in photorespiration to produce glycine and 2-oxoglutarate in peroxisome (FORDE and LEA; 2007). Then the released ammonium in mitochondrion is assimilated by GS / GOGAT cycle that might use a portion of 2-oxoglutarate from peroxisome (NOCTOR and FOYER, 1998).

For many decades photorespiration has been studied as a villain of photosynthesis and a great amount of works have been made in order to reduce photorespiration in C3 plants (PETERHANSEL and MAURINO, 2011; BETTI *et al.*, 2016; SCHULER *et al.*, 2016; ORR *et al.*, 2016; LONG *et al.*, 2018; RAE *et al.*, 2017). However, in the past few decades some researchers have discussed about the positive impact of photorespiration in photosynthesis even in C4 plants (WINGLER *et al.*, 2000; TAKAHASHI *et al.*, 2007; ZELITCH *et al.*, 2009; BAUWE *et al.*, 2010; VOSS *et al.*, 2013; SOUTH *et al.*, 2019). A recent research with alternative photorespiratory pathways showed a significant increase in photosynthesis efficiency and on biomass productivity in field (SOUTH *et al.*, 2019). Some researchers have shown that the elimination of toxic intermediates (such as glycolate) promoted by photorespiration is extremely important for CO_2 assimilation efficiency (LU *et al.*, 2014; DELLERO *et al.*, 2016). This process is closely associated with the consumption of glutamate from nitrogen assimilation to produce and export serine and glycine (BUSCH *et al.*, 2018). It has also been shown that photorespiration might stimulates nitrogen assimilation (RACHMILEVITCH *et al.*, 2004; BLOOM *et al.*, 2014; BLOOM, 2015) and thus improving photosynthesis as shown above.

An interesting old work with tobacco plants enriched or reduced in GS2, a key enzyme of photorespiration, shown in this mutant high photorespiration and in parallel high tolerance to high light stress. In fact after 200 min of exposure to high light GS2-enriched plants had a significantly higher electron transport rate and a significantly lower percentage of photoinhibition than the control plants. When these plants were exposed to 24 hours of high light, while the control plants suffered serious visual damage the plants enriched in GS2 remained visually intact (Figure 3) (KOZAKI and TAKEBA, 1996). Another research with Arabidopsis mutants deficient in others key photorespiration enzymes showed important

results not only in photochemistry but also in the Calvin-Benson cycle. These mutants showed a decrease of about 75% and 50% in CO_2 assimilation and in the maximum quantum efficiency of PSII respectively. In another hand, the NPQ levels were much higher in the mutants compared to control, showing the high need for energy dissipation when photorespiration is inhibited (TAKAHASHI *et al.*, 2007).

Figure 3 – Comparison of photooxidation damage in a transgenic tobacco plant leaf enriched for GS2 (right) and a control leaf (left) after exposure do high-intensity light (2,000 µmol m^{-2} s^{-1}) for 24 h.

Source: KOZAKI and TAKEBA (1996)

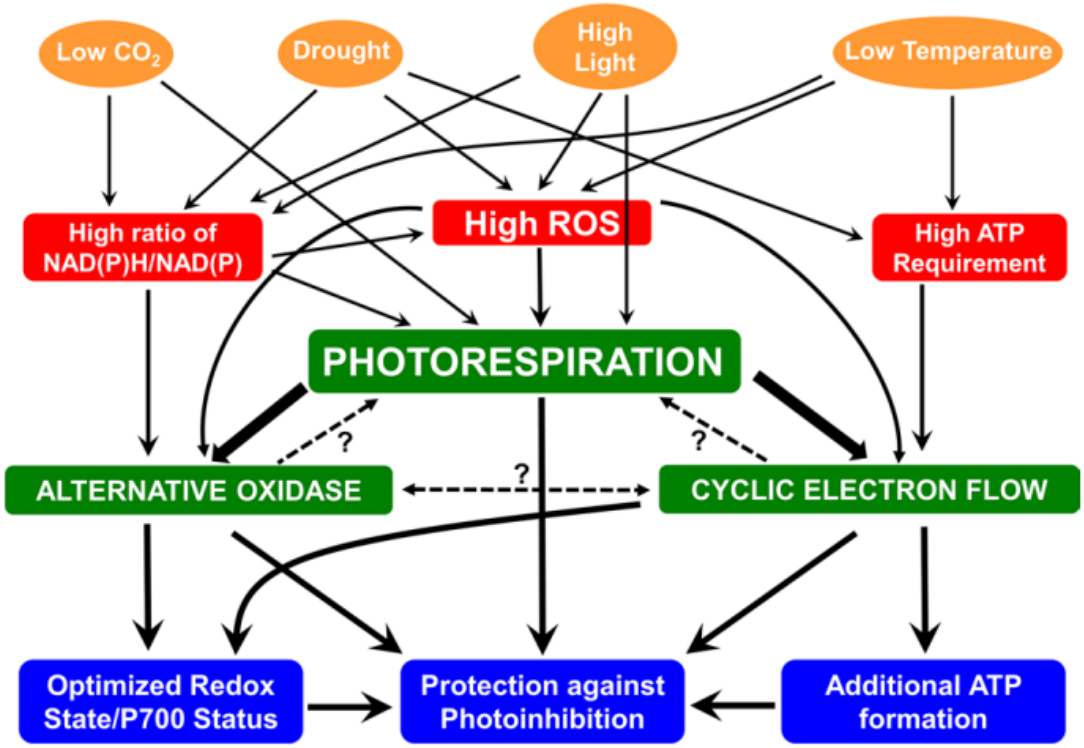
An elegant research showed that 2-phosphoglycolate has a regulatory role in the Calvin-Benson cycle as well as in the allocation of photosynthates between ribulose 1-5-biphosphate regeneration and starch synthesis. The authors suggest that this may represent one of the control loops that sense the ratio of photorespiratory to photosynthetic carbon flux and in turn adjusts stomatal conductance, photosynthetic CO_2 and photorespiratory O_2 fixation, and starch synthesis in response to changes in the environment (FLÜGEL *et al.*, 2017). Another recent study has shown that photorespiration might regulate the electrons transport in PSII in a quite efficient way. In this research it was found that glycolate is a key molecule in this regulation process. When its concentration is increased, glycolate might replace the CO_2 molecule present in the PSII in the region next to non-heme iron. This replacement reduces the efficiency of electrons transfer from Quinone A (QA) to Quinone B (QB) and, consequently, reduces the rate of electron transport in chloroplast ETC which in

turn reduces ROS and ATP excess formation. This regulatory mechanism constitutes an important protection mechanism mainly when both CO_2 assimilation, photorespiration and nitrogen assimilation are not able to consume all input energy (MESSANT *et al.*, 2018).

In addition, many studies have shown that photorespiration might positive regulate some protection mechanisms such as the cyclic electrons flow (CEF) and the alternative oxidase (AOX) activity under abiotic stress (IGAMBERDIEV *et al.*, 2001; BYKOVA *et al.*, 2005; STRODTKÖTTER *et al.*, 2009; YOSHIDA *et al.*, 2011; WATANABE *et al.*, 2016; ZHANG *et al.*, 2017; SUNIL *et al.*, 2019). AOX is an important component of the members of mitochondrial energy dissipation pathway. Despite being a mitochondrial enzyme, AOX has an important role in redox homeostasis in chloroplasts, mainly by modulating NADPH/ATP ratio, ATP/ADP ratio and carbon use efficiency (SAHA *et al.*, 2016; VANLERBERGHE *et al.*, 2016; WANG *et al.*, 2018; SUNIL *et al.*, 2019). Under stressful conditions the photorespiration might regulate AOX activity (IGAMBERDIEV *et al.*, 2001; BYKOVA *et al.*, 2005) or vice versa (STRODTKÖTTER *et al.*, 2009; YOSHIDA *et al.*, 2011; WATANABE *et al.*, 2016; ZHANG *et al.*, 2017). This shows an efficient integration between these two processes aiming regulating the redox state and consequently protecting the cell against excess energy.

Although there is controversy among scientists regarding CEF many researchers suggest that it is an efficient mechanism to protect against excess energy. While the linear electron transport leads to the generation of ATP and NADPH the CEF, mediated by PSI accomplishes only the synthesis of ATP. The equilibrium between ATP/NADPH production and consumption gets disturbed and leads to photoinhibition or photodamage under abiotic stress. The balance might be restored by upregulation of CEF, which dissipates the excess energy in thylakoids via an enhanced energy-dependent non-photochemical quenching (YAMORI and SHIKANAI, 2016; ALRIC and JOHNSON, 2017; SHIKANAI and YAMAMOTO, 2017; MURATA and NISHIYAMA, 2018). Some researchers show a high connectivity between photorespiration and CEF where this first process might efficiently and positive regulate CEF to help avoiding oxidative stress (FOYER *et al.*, 2012; HUANG *et al.*, 2015; TAKAGI *et al.*, 2016; WADA *et al.*, 2018). Therefore, photorespiration is important for photosynthesis protection not only by its activity *per se* but by regulating other important processes related to redox state such as CEF and AOX. In an important review Sunil *et al.* (2018) have made a comprehensive scheme showing how this integration might occur in different circumstances (Figure 4).

Figure 4 - A schematic diagram of the beneficial role of photorespiration and its complementation by CEF and AOX to protect plants against abiotic stress. Any abiotic stress increases the level of ROS. Parallely, due to the disturbance in metabolism, the cellular compartments become over-reduced and thylakoids over-excited. The cells would then need more ATP for metabolism as well as repair processes. Under abiotic stress, the photorespiratory pathway is activated. In turn, photorespiration upregulates CEF and AOX. All three phenomena of photorespiration, CEF and AOX work together to optimize redox state of the cell, protect against photoinhibition and supply ATP for repair mechanisms. While the stimulation of CEF and AOX by photorespiration is well established, it is not clear, if there is a feedback modulation. Similarly, the extent of cross-talk between CEF and AOX is also not completely known. Solid arrows represent information available in the literature. The dotted arrows represent suggestions, which need to be verified experimentally.



Source: SUNIL *et al* (2018)

Although there are many integrative mechanisms already elucidated involving photosynthesis, photorespiration and nitrate assimilation (HEYNEKE and FERNIE, 2018; ROACH and KRIEGER-LISZKAY, 2019) we are still far from solving all the questions. There are still few studies involving improvement of photosynthesis efficiency by nitrate assimilation and photorespiration, mainly in photoinhibition condition promoted by high light. So many questions remain unanswered, especially involving physiological and biochemical mechanisms that regulate nitrate assimilation and photorespiration related to

photosynthesis improvement in a condition of excess energy and high nitrate supply. Therefore, further studies involving Cartesian, physiological and systemic approaches need to be performed in order to increase our understanding in this subject.

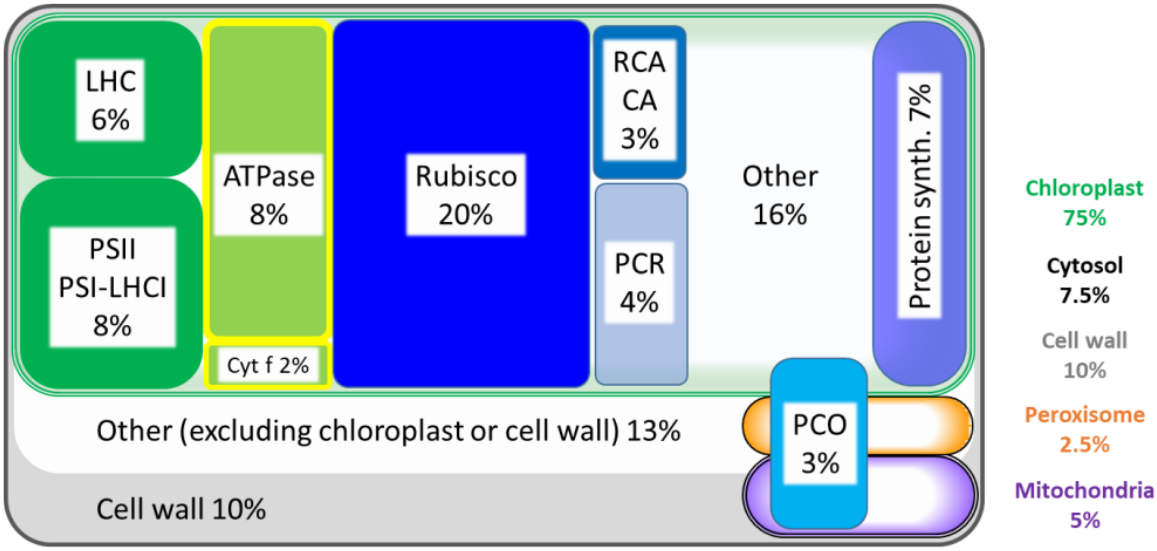
Why to study nitrogen use efficiency?

Nitrogen is the mineral nutrient most required by plants, as it is part of molecules fundamental to life, such as amino acids, proteins, DNA and chlorophyll. Therefore, every year the farmers need to apply great amounts of nitrogen fertilizers that are quite expensive (EVANS and CLARKE, 2018). In aerated soils nitrate is the main form available for plants (CAMPBELL, 1999; TABATA, 2014). However, due its chemical features nitrate might be easily lost from soil. Another important N-source is the ammonium that in general comes from fertilization and organic matter decomposition. This N-form is usually consumed by some soil microorganisms as an electron donor by a process called nitrification, where ammonium is oxidized to nitrate (KIRK and KRONZUCKER, 2005; LI *et al.*, 2008). Therefore, the N-sources in cultivated soils generally might vary over large concentration ranges in a few days (TABATA, 2014). Due this high oscillation in nitrogen concentration on soil, plants need to efficiently manage their N status in order to grow, develop, produce and face adverse conditions such as abiotic stress (XU, 2012). Despite the great importance of this issue little is known about how plants efficiently use available nitrogen starting from a high-supply condition and gradually progressing to a deprivation condition.

According to some studies the nitrogen distribution within the cell in mature C3 leaves is: chloroplast 75%, mitochondria 5%, peroxisomes 2.5%, cytosol 7.5% and cell walls 10%. A great amount of total-N cellular is destined only for Rubisco (about 20%) and approximately 25% for photochemical phase of photosynthesis. That is, at least 45% of the total nitrogen in leaf cells is destined for photosynthesis (Figure 5) (LI *et al.*, 2017; MAKINO and OSMOND, 1991; ONODA *et al.*, 2017; WANG *et al.*, 2015). These data clear show the great importance of nitrogen to photosynthetic machinery. As a consequence, the nitrogen cost of photosynthesis is extremely high (EVANS and CLARKE, 2018). In fact the costs with nitrogen fertilizers to food production are an issue that needs to be carefully considered. Assuming an average grain nitrogen content for wheat, rice and maize of 1.9% (JAKSOMSAK *et al.*, 2017; RAPP *et al.*, 2018; URIBELAAREA *et al.*, 2008), harvest grain

accounts for one quarter of global N fertilizer. These data only correspond to wheat, rice and maize production and does not take into account the losses of nitrogen from leaching, erosion and denitrification. In addition, there are costs associated with environmental damage caused by the fertilizers excess applied in soils. All these costs generate a great demand for improvement in nitrogen use efficiency (EVANS and CLARKE, 2018).

Figure 5 - Leaf nitrogen budget



Source: EVANS and CLARKE (2018)

The scientific problems

Although there are many studies involving the issues raised here the vast majority of them investigated separately nitrate supply, nitrate assimilation and photorespiration. In addition, generally these experiments were done with different plant species and under different environmental conditions. Even more scarce are researches involving all of these processes under photoinhibition promoted by high light. These and other factors make difficult to get reliable conclusions about the interactions between all of these processes. Regarding NUE there are few studies to evaluate in more detail how the N-nutrition dynamics work in distinct physiological phases begging with high nitrate supply, following to progressive N deprivation until reaching an early deficiency phase.

Therefore, some questions raised here need to be solved involving nitrogen use efficiency, photosynthesis and photorespiration in plants exposed to excess energy and N-oscillation. Could nitrate assimilation and photorespiration improve photosynthesis under a

photoinhibitory condition caused by excess light? Other issues are raised here with respect to NUE in different N-status: how does the N-nutrition dynamics works in distinct physiological phases begging with high nitrate supply, following to progressive N deprivation until reaching an early deficiency phase? What are the relationships among NUE especially nitrate reductase activity and photosynthesis in these distinct physiological phases? As we can see here these issues are not simple to solver and we cannot analyse it only using a Cartesian view but also using a systemic view. Since photosynthesis, nitrogen assimilation and photorespiration are closely interconnected.

3 OBJECTIVES

The general objective of this thesis is to evaluate how nitrogen use efficiency, photosynthesis and photorespiration work in a synergistic and integrated way in plants exposed to excess energy and N-oscillation.

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

1. To test the hypothesis that under high light the high nitrate supply stimulates nitrate assimilation which in turn stimulates photorespiration and both processes improve photosynthesis efficiency mainly acting as a sink of electrons excess.

2. To evaluate in a deeper way the NUE dynamics in distinct physiological phases begging with high nitrate supply until reaching an early deficiency phase by using biochemical, physiological and systemic approaches.

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4 INCREASE IN ASSIMILATORY NITRATE REDUCTION AND PHOTORESPIRATION ENHANCES CO_2 ASSIMILATION UNDER HIGH LIGHT-INDUCED PHOTOINHIBITION IN COTTON

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Abstract

The mechanisms by which nitrate assimilation might favour photorespiration and how these pathways could cooperatively improve photosynthesis are still little understood, especially under excess light. Here, we tested the hypothesis that increased assimilatory nitrate reduction is able to stimulate photorespiration and these two processes together are capable to enhance photosynthesis in presence of high light. Cotton plants were supplied with 10 mM NO_3^- (high nitrate supply-HN) or NO_3^--deprivation for short-term and subsequently exposed to high light-HL or low light-LL. HL induced photoinhibition in both N-treatments but strongly enhanced nitrate assimilation and photorespiration rates in HN. Despite these apparent constraints caused by HL on PSII integrity, HN-plants displayed higher electron flux through PSII, in parallel to enhancement in CO_2 assimilation, which was positively related to the photosynthetic nitrogen utilization efficiency. These plants also exhibited increase in nitrate reductase and glutamine synthetase activities, which were associated with large accumulation of free amino acids and ammonia, in parallel to high NO_3^- reduction rates. The increase in photorespiration was greatly dependent on light intensity and, in a minor extent, on nitrate supply. The changes in the alternative electron sink strength towards nitrate assimilation and photorespiration was corroborated by decrease in the acceptor side limitation of PSI. In addition, the correlation study evidenced that CO_2 assimilation, nitrate reductase activity, and photorespiration rates were greatly correlated. Thus, our data clearly show that nitrate assimilatory reduction and photorespiration are mutually up regulated by high light, favoring CO_2 assimilation and photochemical activity under a photoinhibitory condition.

Key words: *Gossypium hirsutum;* nitrate supply; photosynthesis; photochemical activity; photoinhibition.

Introduction

Light utilization, photosynthetic efficiency, photorespiration and nitrate assimilation in higher plants are processes strongly interconnected and subjected to complex regulation. However, the physiological integration among these processes is poorly understood. This network is especially important under specific environmental conditions such as high light, which might differently affect some of these components. Indeed, in this circumstance, excess energy might affect PSII integrity but in opposition, it could favour synergistically nitrate assimilation and photorespiration. The enhancement of these processes, especially under high nitrate supply, can stimulate the sugar consumption and amino acid exportation from chloroplasts (BUSCH *et al.*, 2018). Together, these features can improve CO_2 assimilation in presence of excess energy through reduction of the triose-P limitation (TPU). Moreover, nitrate assimilation and photorespiration might contribute for CO_2 assimilation avoiding energy imbalance in chloroplasts induced by excessive reducing power and ATP accumulation (BUSCH *et al.*, 2018).

In nature, oscillating conditions of light, nitrate supply and photorespiration are common and they represent a potential impact for photosynthetic efficiency. Integrative approaches to understanding these effects are required but unfortunately they are lacking, especially in crop species. A suitable balance between energy input in the photochemical apparatus and consumption by the assimilatory biochemical routes (output) is essential for photosynthetic efficiency (FOYER *et al.*, 2012). Indeed, in the most of environmental circumstances, plant leaves are exposed to light intensities much higher than that necessary for CO_2 reduction in Calvin-Benson cycle and this extra energy can generate reactive oxygen species (ROS) over-accumulation (JIMBO *et al.*, 2018). In turn, excess ROS might affect PSII structure and activity, causing photoinhibition and photosynthesis impairment (KALE *et al.*, 2017), which can trigger generalized adverse effects on plant growth.

Plants must be able to dissipate excess energy in chloroplasts by alternative electron sinks, especially by the water-water cycle, photorespiration and nitrate assimilation to avoid ROS accumulation (BAUWE *et al.*, 2010). Nitrate reduction and ammonia assimilation besides act as alternative sinks for excess photosynthetic electrons, can also stimulate photorespiratory reactions essentially by introducing Glu from GS/GOGAT reactions and stimulating Gly/Ser recycling (BUSCH *et al.*, 2018). Moreover, nitrogen assimilation is a crucial process for the synthesis of all proteins, nucleic acids, chlorophylls

and other important molecules. Commonly, NO_3^- is the predominant N-form available for the most crops and leaf is generally the major site for its assimilatory reduction (CAMPBELL 1999). This pathway integrates cytosol and chloroplasts via activities of nitrate reductase, nitrite reductase and GS/GOGAT cycle.

Nitrate reductase is the limiting enzyme of the assimilatory nitrate reduction pathway and its activity and gene expression are strongly dependent on light and sugar availability (CAMPBELL, 1998; HUARANCCA *et al.*, 2018). Under high CO_2 concentrations the nitrate assimilation is impaired suggesting that photorespiration is important to enhance nitrate reduction but the underlying mechanisms are poorly known (Rachmilevitch *et al.*, 2004; Bloom *et al.*, 2014; Bloom 2015). In turn, ammonia assimilation through the GS/GOGAT cycle also is stimulated by light, which consumes carbon skeletons derived from sugars in the form of 2-oxo-ketoglutarate (TCHERKEZ *et al.*, 2012; KUSANO *et al.*, 2011). During photorespiration, Glu participates of Gly formation consuming glyoxylate by the glutamate/glyoxylate aminotransferase, simultaneously favoring the elimination of this organic acid and Gly recycling (FORDE and LEA 2007). Moreover, these processes might mitigate CO_2 assimilation impairment as has been demonstrated that glycolate and glyoxylate might inhibit the Calvin-Benson cycle reactions (ZELITCH *et al.*, 2009; Lu *et al.*, 2014), whereas Gly and Ser synthesis might contribute to C export from chloroplasts.

Recently, Busch et al (2018), working with mathematical modeling and experimental validation with sunflower plants concluded that high nitrate supplying is capable to improve CO_2 fixation by favoring photorespiration and C export as amino acids, besides to provide beneficial effects for against excess electrons and improving the ATP/NADPH balance. Although the importance of this study, the underlying biochemical mechanisms involved in the improvement of photosynthesis efficiency by nitrate assimilation and photorespiration are lacking, especially in a high light condition, which may induce PSII efficiency decrease. Thus, some questions involving physiological and biochemical mechanisms that regulate nitrate assimilation and photorespiration related to photosynthesis improvement in a condition of excess energy and high nitrate supply remain unsolved.

In this context, aiming to uncover some of these complex relationships, a pertinent question has been raised here. Could nitrate assimilation and photorespiration, synergistically, improve CO_2 assimilation under an adverse condition for PSII integrity caused by high light? Previously, we have demonstrated in *Jatropha curc*as plants exposed to

salt stress that high intensity of nitrate assimilation was capable to mitigate impairments in photochemical activity and CO_2 uptake (ARAGÃO *et al.*, 2012). In this current study we tested the hypothesis that high nitrate supply for cotton plants under excess light is capable to stimulate the assimilatory nitrate reduction and photorespiration pathways. Our data clearly show that nitrate assimilatory reduction and photorespiratory activity are mutually upregulated by high light, favoring CO_2 assimilation in a photoinhibitory condition. An integrated balance among these processes, favoring photosynthetic efficiency, is proposed and discussed.

Material and methods

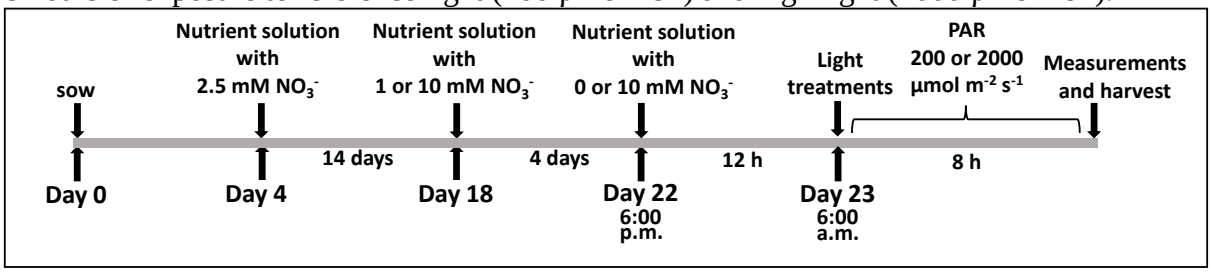
Plant material and growth conditions

Cotton (*Gossypium hirsutum*) seeds from the commercial genotype FM 954GLT, kindly provided by the Bayer Company (Brazil), were employed in the experiments. They were germinated in washed land and seedlings were transplanted to 2.5 L plastic pots filled with Hoagland-Arnon's nutrient solution (HOAGLAND and ARNON 1950), modified for each nitrate concentration. During 14 days, plants were supplied with NO_3^- (2.5 mM) as a sole N-source. After this pre-acclimation period, plants were separated into two groups: 10 mM NO_3^- (high-) and 1 mM NO_3^- (low-supply), which remained during four days under these conditions. These-conditions were performed in order to induce two contrasting nitrate levels but without cause N deficiency in cotton tissues. Plants supplied with 10 mM and 1 mM of NO_3^- received 5.0 mM and 0.5 mM of $Ca(NO_3)_2$, respectively. All the other inorganic nutrients were supplied in equal concentrations in both treatments employing a suitable salt combination. The pH was adjusted to 6.0 every two days and the nutritive solution was completely changed weekly. The plants were grown in a greenhouse under natural conditions (3°44'44.2"S 38°34'29.2"W) as follows: day/night mean temperature of 32°C/25°C, relative humidity average of 65%, maximum photosynthetic photon flux density (PPFD) of 600 µmol m^{-2} s^{-1} at noon and 12 h-photoperiod.

Experiment I: Physiological and biochemical responses to contrasting nitrate levels in presence of low- and high-light

Aiming to evaluate relationships among nitrate assimilation, photosynthesis and photorespiration under high and low light, 22-day-old cotton plants were supplied with two contrasting nitrate levels. Plants previously grown under high nitrate (10 mM NO_3^-) and low nitrate (1 mM) for 4 days were transferred to a controlled growth chamber (28/24 °C day/night temperature and 60% relative humidity) and then nitrate supply was shifted as follow: the 10 mM NO_3^- concentration was kept in the nutrient solution for this plant group (high nitrate supplying treatment - HN), whereas another group was subjected to a short-term nitrate deprivation (ND) by shifting 1 mM NO_3^- concentration to a NO_3^--free solution. These plants remained for 12 h at darkness and afterwards, they were exposed to reference (low) light (200 µmol m^{-2} s^{-1} - REF) or high light (2000 µmol m^{-2} s^{-1} - HL) for 8 hours, performing a total of 20 h in these two contrasting nitrate supplying conditions (Figure 6). Under these conditions the plants experienced a short-term nitrate deprivation but without suffering a N-deficiency. Afterwards, photosynthesis, metabolic nitrate content and nitrate reductase activity measurements were performed in fully expanded leaves. Subsequently, these leaves were immediately frozen with liquid N_2 and kept in -80 ^oC until subsequent biochemical analyses.

Figure 6 – Scheme of experiments in response to contrasting nitrate supply. After four days from sow, plants were grown in greenhouse and exposed to nutrient solution with NO_3^- (2.5 mM) as a sole N-source during 14 days. Afterwards, they were pre-acclimated under two contrasting nitrate supplying: low NO_3^- (1 mM - LN) and high NO_3^- (10 mM - HN) for four days. LN and HN plants were transferred to a growth chamber and subjected to NO_3^- deprivation (ND) or supplied with high nitrate (10 mM NO_3^- -HN), respectively, followed by 8 hours of exposure to reference light (200 µmol^{-2} s^{-1}) and high light (2000 µmol^{-2} s^{-1}).



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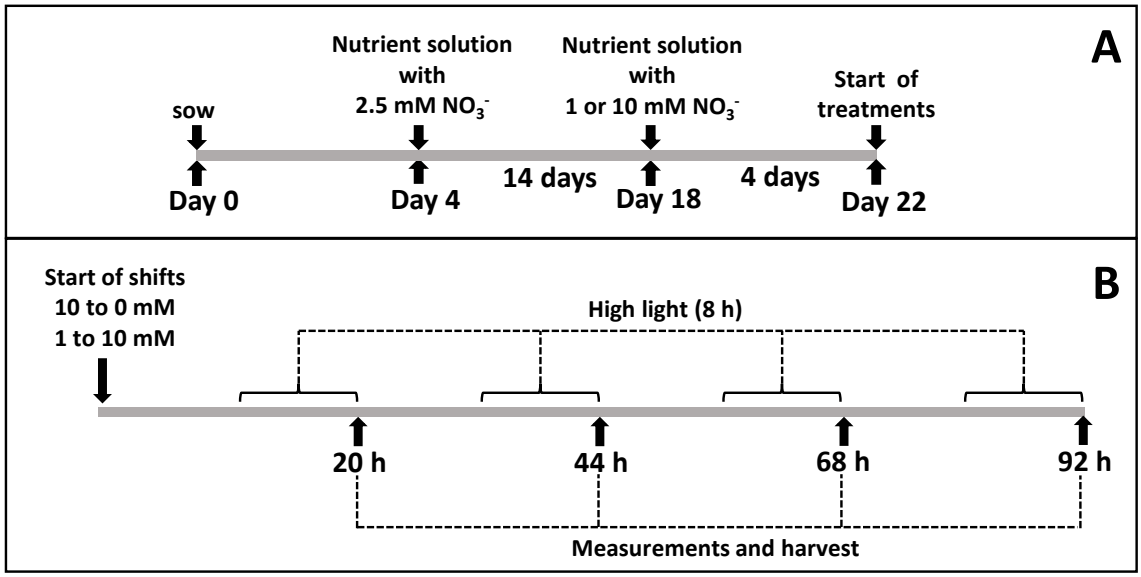
Experiment II - Nitrate assimilation and photosynthetic responses to contrasting NO_3^- supplying during a time-course of 92 hours

In order to elucidate if the effects induced by a transient nitrate deprivation on photosynthesis are mostly dependent on the nitrate assimilation pathway than due to changes in the leaf-N status, 10 mM NO_3^- supplied plants were transferred to a nitrate-free solution for 92h and evaluated in a time-course approach. Conversely, plants previously grown in 1 mM NO_3^- (low nitrate) were transferred to 10 mM NO_3^- solution to evaluate the opposite effect, that is, progressive N accumulation in leaf tissues and its effects on N-nutrition and photosynthesis. In both treatments, the plants were exposed to high light for 8 hours before each harvest. Plants were grown for 18 days in greenhouse conditions, as previously described and, afterwards, transferred to nutrient solution containing 1 mM or 10 mM NO_3^- for 4 days in order to induce contrasting nitrate levels. After, plants were subdivided into two sub-groups: plants grown in 10 mM NO_3^- were shifted to a nitrate-free solution (nitrate withdrawal) and plants that were supplied with 1 mM NO_3^- were cross-shifted to a nutrient solution containing 10 mM NO_3^-. Measurements and leaf harvests were performed after 20, 44, 68 and 92 hours from beginning of the cross-shifting treatments. Before each harvest, plants were exposed to 8 hours of high light (2000 µmol m^{-2} s^{-1}) in a growth chamber as previously described. Photosynthesis, metabolic nitrate content and nitrate reductase activity measurements were performed in fully expanded leaves. After, leaves were harvested, immediately frozen in liquid N_2 and kept in -80 ^oC until further biochemical analyses (Figure 7).

Figure 7 - Scheme of experiments involving shifts in NO_3^- supplying during a time-course. (A) After four days from sow, plants were grown in greenhouse and exposed to nutrient solution with NO_3^- (2.5 mM) as a sole N-source during 14 days. Afterwards, the plants were pre-acclimated under two contrasting nitrate supplying: low NO_3^- (1 mM - LN) and high NO_3^- (10 mM - HN) during four days. (B) LN and HN plants were transferred to a growth chamber and subjected to solutions containing 10 mM NO_3^- and 0 mM NO_3 (nitrate deprivation-ND), respectively, where remained for 20, 44, 68 and 92 hours. Before each harvest, plants were exposed to 8 hours of high light (2000 μmol^{-2} s^{-1}).



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Gas exchange and chlorophyll a fluorescence measurements

In vivo photosynthetic parameters 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 light intensity inside the IRGA chamber during the measurements was 2000 μmol photons m^{-2} s^{-1}. Other environmental conditions were the same for both experiments: 26 °C temperature, 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 up to 10% of the PPFD, in order to maximize stomatal aperture (FLEXAS *et al.*, 2008). A-PPFD curves were performed employing increasing light intensities from 0 to 2000 μmol m^{-2} s^{-1} with five min of exposure per light intensity. Day respiration (R_D) was estimated from A-PPFD curves according to Thornley and Johnson (1990).

A-Ci curves were measured in response to varying intercellular CO_2 partial pressure (Ci) from 0 to 160 Pa according to Sharkey et al (2007). The PPFD employed for A-Ci curves was 1500 μmol m^{-2} s^{-1} (saturating light for photosynthesis of cotton plants). Maximum Rubisco carboxylation rate (V_{cmax}) and maximum electron transport rate for RuBP regeneration (J_{max}) were estimated from these curves, employing the fitting utility version 1.1 provided by Sharkey et al (2007).

The *in vivo* chlorophyll *a* fluorescence parameters were measured using a Dual-PAM 100 fluorometer (Walz, Effeltrich, Germany). Fluorescence parameters were measured using the saturation pulse method (KLUGHAMMER and SCHREIBER 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. PSII and PSI photochemical parameters were measured in response to a rapid light curve from 0 to 2000 μmol m^{-2} s^{-1}, with 1 min of exposure per light intensity. The following parameters were assessed: the maximum quantum yield of PSII [Fv/Fm = (Fm−Fo)/Fm] and effective quantum yield of PSII [Y(II) = (Fm'−Fs)/Fm']. The actual flux of electrons from PSII was calculated according to Miyake et al (2005) using the following equation: ETRII = αII × Y(II) × PPFD, where αII was obtained as αII = 4 × (A + R_D) / [Y(II) × PPFD] from measurements of A and Y(II) in absence of photorespiration (at low oxygen condition).

The PSI photochemical measurements were performed in a DUAL-PAM 100 and the following parameters were measured: limitation on donor side of PSI [Y(ND) = 1 - P700 red], limitation on acceptor side of PSI [Y(NA) = (Pm-Pm')/Pm] as described previously by (Klughammer and Schreiber 1994). In addition, others light curve parameters were estimated according to Platt et al (1980): maximum electron transport rate from PSII (ETR_{max}II) and the minimum saturating irradiance for ETRII (EKII).

The electron flux to Rubisco carboxylation was calculated as J_c=1/3[ETRII + 8(A + R_D)], where R_D is the day respiration parameter calculated from A-PPFD curve. The following photorespiration parameters were calculated: electron flux to Rubisco oxygenation, J_o = 2/3[ETRII - 4(A + R_D)] and the photorespiratory CO_2 evolution, Pr =1/12[ETRII - 4(A + R_D)], according to Valentini et al (1995). The photosynthetic nitrogen utilization efficiency was calculated from total-N content and CO_2 assimilation data and was expressed as μmol CO_2 s^{-1} $mmol^{-1}$ N.

Determination of the NR and GS activities, N-compounds, total chlorophyll and oxidative stress parameters in leaves

Nitrate reductase (NR; EC 1.7.1.1) activity was measured by the *in vivo* method (HAGEMAN and HUCKLESBY, 1971), with minor modifications, as described in details by Silveira *et al.*, (2001a) and the data were expressed as µmol NO_2^- h^{-1} g^{-1} FW. Glutamine Synthetase (GS) activity assays were performed according to described in Lutts et al (1999) and expressed as µmol GGH g^{-1} FW h^{-1}. Metabolic nitrate content was determined by adaptation of the NR activity method already described. The changes included increase of the reaction time for 90 min and absence of nitrate in the reaction medium, as previously described (SILVEIRA *et al.*, 2001b). The contents of nitrate, ammonium and free amino acids were determined in lyophilized leaf tissues. The nitrate concentration was determined by colorimetric salicylic acid method according to Cataldo et al (1975); ammonium content was determined by the phenol-hypochlorite method according to Felker (1977) whereas the content of total free amino acids was measured by ninhydrin method according to Yemm et al (1955). The total-N content was measured according to Baethgen and Alley (1989) and chlorophyll content according to Porra et al (1989). The content of thiobarbituric acid reactive substances (TBARS) was measured by Heath and Packer (1968) method and hydrogen peroxide content was measured using the Amplex®-red kit (Thermo Fisher Scientific®, USA), based on colorimetric measure of resorufin formation in presence of H_2O_2 (ZHOU *et al.*, 1997).

Western blotting determinations for Rubisco large subunit

The soluble protein extract from fully expanded leaves was utilized to perform Rubisco large subunit (RbcL) immunoblotting. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out loading equal amounts of proteins (20 µg) per lane. The denatured proteins were electrophoretically transferred to a nitrocellulose membrane, according to Towbin et al (1979), and polypeptide detection was performed using specific polyclonal antibodies against the RbcL (AS03037; Agrisera, Vännäs, Sweden) according the manufacturer's instructions.

Statistical analysis and experimental design

The first experiment was arranged in a completely randomized design in a 2 x 2 factorial (2 nitrate levels x 2 light regimes), with four replicates. The second experiment was arranged in a completely randomized experiment in a split plot design (2 nitrate levels x 4 harvest times) and three replicates. Data were subjected to analysis of variance by ANOVA and the averages were compared by Tukey test at 5% of probability ($p \leq 0.05$). In order to estimate the relationships between some physiological parameters, selected variables were correlated by the Pearson´s correlation coefficient (r).

Results

Cotton plants supplied with high nitrate followed by exposure to high light suffered photoinhibition indicated by alterations in PSII fluorescence parameters

An experiment was performed to evaluate if nitrate supply could improve photosynthesis and avoid photoinhibitory effects caused by exposure to HL (2000 $\mu mol^{-2}\ s^{-1}$). The results obtained clearly show that maximum quantum yield of PSII (Fv/Fm) of both ND and HN plants, was significantly and similarly decreased (from 0.77 to 0.60) by HL treatment and the photoinhibitory effect reflected in the Fm parameter. These changes were also related to increased Fo values in all plants exposed to HL, as compared to those subjected to reference light. These photochemical changes were strongly related to decrease in total chlorophyll content (Figure 8). Interestingly, the nitrate supply (ND and HN) did not affect the photoinhibitory response caused by HL. In parallel, the TBARS and H_2O_2 contents, two important oxidative stress indicators, were slightly changed by light treatments and they did not evidence signals for negative effects induced by HL and nitrate deprivation (Figure 9).

Figure 8 – Response of (A) maximum quantum yield of PSII, (B) maximal and (C) minimal fluorescence from dark-adapted leaf and (D) chlorophyll content in cotton leaves subjected to NO_3^- deprivation (ND) or supplied with high nitrate (10 mM NO_3^- -HN) followed by 8 hours of exposure to reference light (200 µmol^{-2} s^{-1} -REF) and high light (2000 µmol^{-2} s^{-1} -HL). Data represents averages from four replicates ±SD and different letters represent significant differences among treatments by Tukey's test ($p<0.05$).

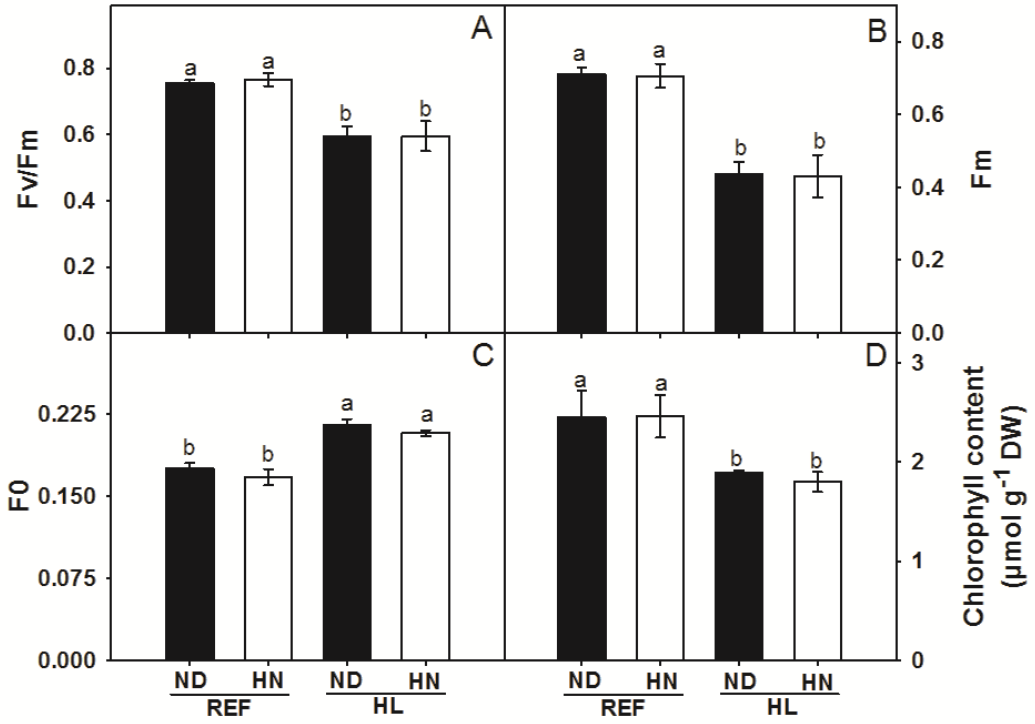

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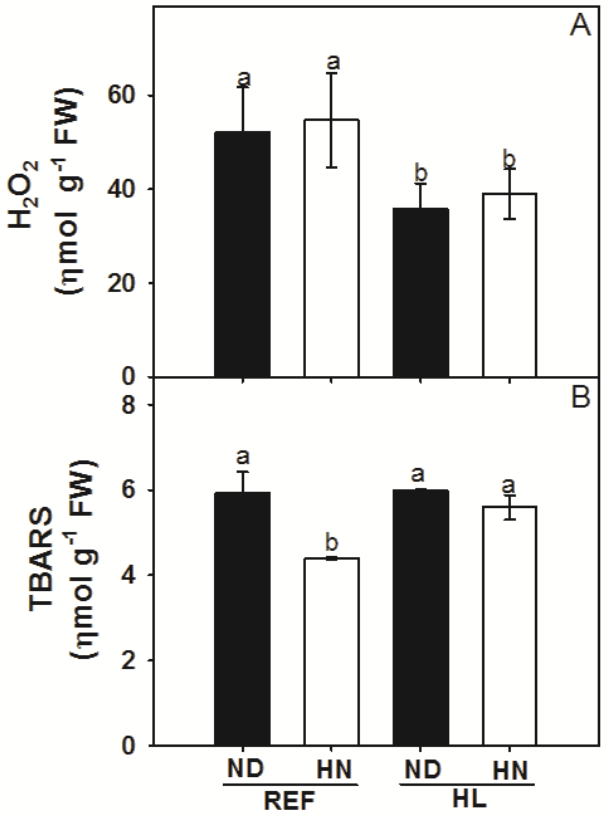
Figure 9 – Contents of (A) hydrogen peroxide – H_2O_2 and (B) thiobarbituric acid reactive substances - TABRS in cotton leaves subjected to NO_3^- deprivation (ND) or supplied with high nitrate (10 mM NO_3^- -HN) followed by 8 hours of exposure to reference light (200 µmol^{-2} s^{-1} -REF) and high light (2000 µmol^{-2} s^{-1} -HL). Data represents averages from four replicates ±SD and different letters represent significant differences among treatments by Tukey's test ($p<0.05$).



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Interestingly, despite the photoinhibitory effects observed at the PSII level, HN supply, in presence of HL, was capable to stimulate the actual quantum yield of PSII. These effects were reflected in increase of ETRII (43%) and in CO_2 assimilation (59%), compared with plants grown in a nitrate-deprived condition (Figure 5 and Table 1). Interestingly, values of PNUE were increased by HL and HN compared to the REF/ND combination, indicating that high nitrate supply *per se* was capable to improve CO_2 assimilation under HL but not in low light (Table 2). The ETRII-stimulation by HN was corroborated by higher values reached by EKII and ETRmaxII, which are photochemical parameters related to light utilization capacity for

photochemical activity (Table 3). In parallel, HN in presence of HL did not affect the donor side limitation but reduced the acceptor side limitation of PSI (Figure 10).

Figure 10 – Changes in (A) electrons transport rate of PSII, (B) limitation of acceptor side of PSI and (C) limitation of donor side of PSI in cotton leaves subjected to NO_3^- deprivation (ND) or high nitrate (10 mM NO_3^- -HN) followed by 8 hours of exposure to high light (2000 μmol^{-2} s^{-1} -HL). Data represent averages from four replicates ± SD and asterisks represent significant differences among treatments by *t*-test ($p<0.05$). Data from ND and HN under REF light were not compared with ND and HN in presence of HL because the Fm (maximal PSII fluorescence) was significantly decreased by effect of HL as showed in Figure 3. In this circumstance, the comparison between the ETRII values is not appropriate.

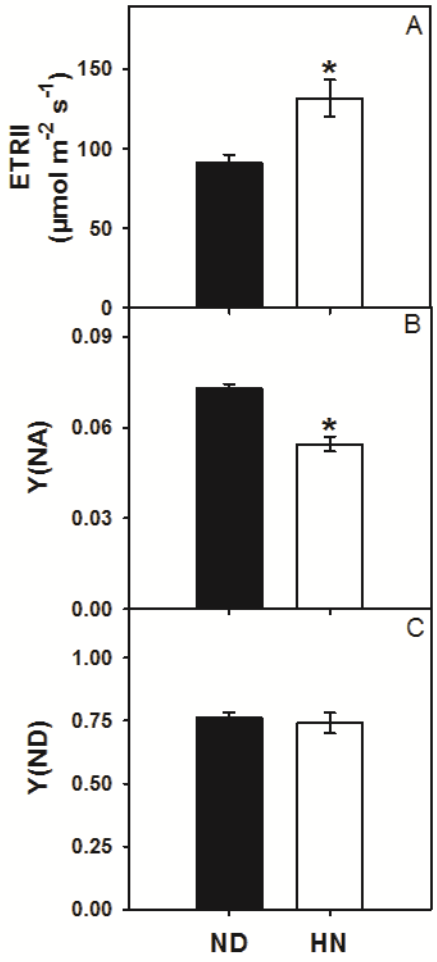

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Table 1 – Changes in photosynthetic, photorespiratory and respiratory parameters in cotton leaves subjected to NO_3^- deprivation (ND) or supplied with high nitrate (10 mM NO_3^- -HN) followed by 8 hours of exposure to reference light (200 µmol^{-2} s^{-1} -REF) and high light (2000 µmol^{-2} s^{-1} -HL). Data represents averages from four replicates ±SD and different letters represent significant differences among treatments by Tukey's test ($p<0.05$).

Variables	REF		HL	
	ND	HN	ND	HN
A (µmol m^{-2} s^{-1})	14.04±1.46b	15.3±1.29b	13.81±1.81b	19.4±2.14a
Vc_{max} (µmol m^{-2} s^{-1})	nd	nd	92±3.0b	129±4.5a
J_{max} (µmol m^{-2} s^{-1})	nd	nd	108±1.8b	144±4.0a
Jc (µmol m^{-2} s^{-1})	42±0.3c	44±1.7c	78±2.5b	119±5.6a
Jo (µmol m^{-2} s^{-1})	9±1.6d	12±1.9c	30±2.8b	50±8a
Jo/Jc ratio	0.14±0.04c	0.26±0.05bc	0.38±0.02b	0.42±0.01a
Pr (µmol m^{-2} s^{-1})	1.14±0.1d	1.44±0.2c	3.73±0.3b	6.26± 0.1a
R_D (µmol m^{-2} s^{-1})	2.0±0.16b	2.5±0.15a	2.4±0.13a	2.7±0.15a

Source: elaborated by the author.

Table 2 – Nitrogen parameters in cotton leaves subjected to NO_3^- deprivation (ND) or supplied with high nitrate (10 mM NO_3^- -HN) followed by 8 hours of exposure to high light (2000 µmol^{-2} s^{-1} -HL). Data represents averages from four replicates ±SD and different letters represent significant differences among treatments by Tukey's test ($p<0.05$).

Variables	REF		HL	
	ND	HN	ND	HN
Total-N content (mmol g^{-1} DW)	2.61±0.20bc	3.48±0.15a	2.22±0.21c	2.88±0.34b
Metabolic nitrate (µmol g^{-1} FW)	0.02±0.005b	2.69±0.25a	0.02±0.003b	2.76±0.13a
Nitrate content (µmol g^{-1} FW)	46.6±8.07c	272.1±28.6a	27.6±1.02c	173.3±7.9b
Ammonium content (µmol g^{-1} DW)	10.5±1.2c	19.9±2.2b	6.28±0.33d	24.03±1.4a
PNUE (µmol CO_2 s^{-1} mmol^{-1} N)	218.2±15.1b	162.2±16.02c	201.3±4.5b	282.4±22.5a

Source: elaborated by the author.

Table 3 – Electron transport rate parameters in cotton leaves subjected to NO_3^- deprivation (ND) or supplied with high nitrate (10 mM NO_3^- -HN) followed by 8 hours of exposure to high light (2000 µmol^{-2} s^{-1} -HL). Data represents averages from four replicates ±SD and different letters represent significant differences among treatments by *t*-test ($p<0.05$).

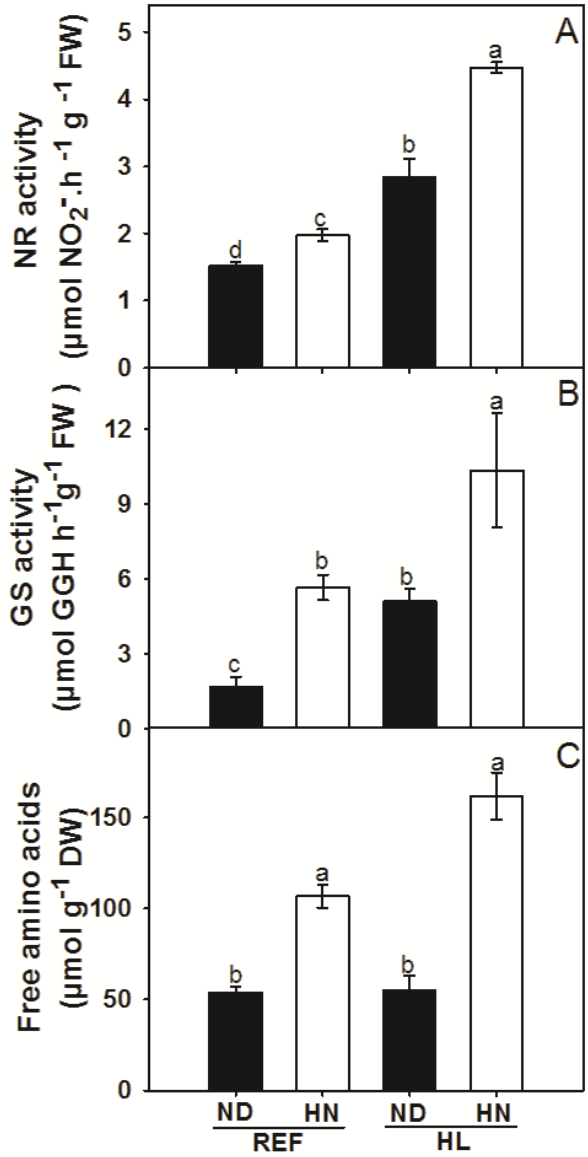
ETRII parameters	ND	HN
ETR_{max}II (µmol m^{-2} s^{-1})	100±2.41b	137±11.4a
EKII (µmol m^{-2} s^{-1})	457.8±26b	713.7±37.7a

Source: elaborated by the author.

High nitrate supply under HL greatly stimulated nitrate assimilation and photorespiration rates contributing for enhancing Rubisco carboxylation

This experiment was performed to verify if HN supply and HL could be capable to stimulate nitrate assimilation and photorespiration rates and, in parallel, stimulating CO_2 fixation. The leaf NR activity was greatly stimulated by HN/HL (increased by 3.0-fold compared to ND/HL), whereas in HN/REF it was slightly increased in comparison to ND/REF treatment. Following a similar trend, GS activity was strongly induced by HN under HL (2.0- fold compared to ND/HL). In parallel, the contents of nitrate, amino acids and ammonium were increased by 6.0-, 3.0- and 4-fold, respectively in that treatment (Figure 11 and Table 2). The leaf metabolic nitrate content, which represents the nitrate flux in leaf cell cytosol, was intensely decreased in ND-plants, reaching values near to zero, as in REF light as well as in HL.

Figure 11 – Performance of (A) nitrate reductase activity, (B) glutamine synthetase activity and content of (C) free amino acids in cotton leaves subjected to NO_3^- deprivation (ND) or supplied with high nitrate (10 mM NO_3^- -HN) followed by 8 hours of exposure to reference light (200 µmol^{-2} s^{-1} -REF) and high light (2000 µmol^{-2} s^{-1} -HL). Data represents averages from four replicates ±SD and different letters represent significant differences among treatments by Tukey's test ($p<0.05$).



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The maximum *in vivo* Rubisco activity (Vcmax) was stimulated in 40% whereas the RuBP regeneration rate (Jmax) increased by 33% in the HN/HL combination compared to HN/REF treatment. In parallel, the electron flux to carboxylation (Jc) increased 52%, whereas the flux to oxygenation improved by 66% and the photorespiration rate (Pr) was enhanced

similarly. Consequently, when plants were exposed to HN/HL the Jo/Jc ratio increased by 61%, which indicates that HN associated with HL was able to induce a greater raise in Rubisco oxygenation compared to carboxylation (Table 1). When this comparison was performed from ND and HN in HL treatment, the increase was only 10%, indicating that the contribution of HL was more important to stimulate photorespiration than high nitrate supply *per se*. This result was corroborated by the increase in the GS activity, a key enzyme of photorespiration, in the HN/HL plants.

Photosynthesis-PPFD curves were fitted to evaluate effects of high nitrate supply in processes related to partitioning of photosynthetic electron flux to different sinks (A, PSII, PSI, Jc and Jo), at distinct light intensities. All photosynthetic parameters were stimulated in HN- compared to ND-plants above 1000 μmol^{-2} s^{-1}, but at 2000 μmol^{-2} s^{-} Jo was greater stimulated as compared to all other parameters (Figure 12 and 13). Indeed, whereas ETRII, Jc and A were increased by approximately 43%, 52% and 59% respectively, Jo increased by 66% at 2000 μmol^{-2} s^{-1} in HN-plants compared to ND-cotton. A Person's correlation was performed to quantify the degree of relationship between some physiological parameters. The obtained data indicated that close and highly significant correlations between P_N x NR (r=0.99**); P_N x Pr (0.99**); ETRII x NR (0.96*); ETRII x Pr (0.97*); Pr x NR (0.99**) have occurred, but it was not significant for GS x NR at $p \leq 0.05$, despite it has displayed a high correlation coefficient (r=0.92) – (Figure 14).

Figure 12 – Response of (A) net CO_2 assimilation and (B) electron transport rate of PSII under different light intensities in cotton leaves subjected to NO_3^- deprivation (ND) or supplied with high nitrate (10 mM NO_3^- -HN) and previously exposed to high light (2000 $\mu mol^{-2}\ s^{-1}$ -HL) for 8 hours.

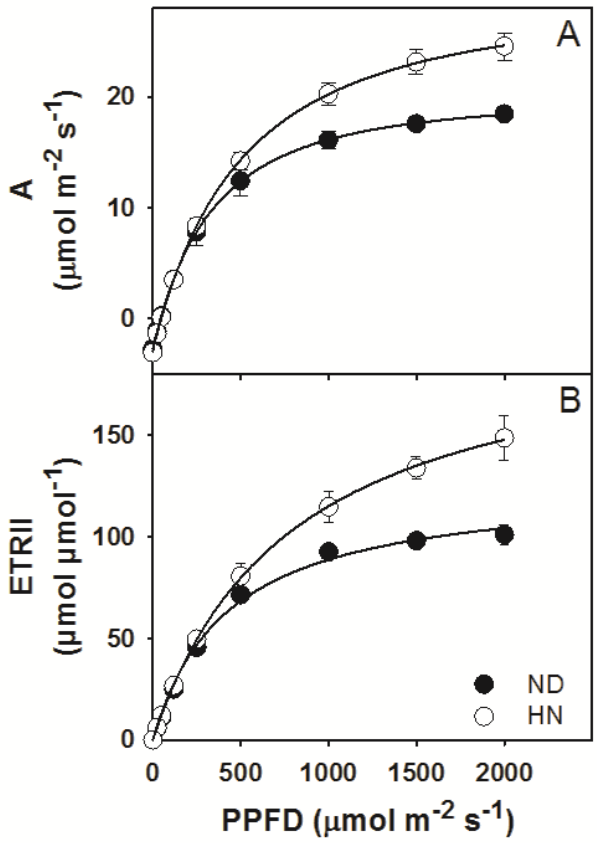

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Figure 13 – Response of (A) electron transport rate to Rubisco carboxylation and (B) electron transport rate to Rubisco oxygenation under different light intensities in cotton leaves subjected to NO_3^- deprivation (ND) or supplied with high nitrate (10 mM NO_3^- -HN) and previously exposed to high light (2000 µmol^{-2} s^{-1} -HL) for 8 hours.

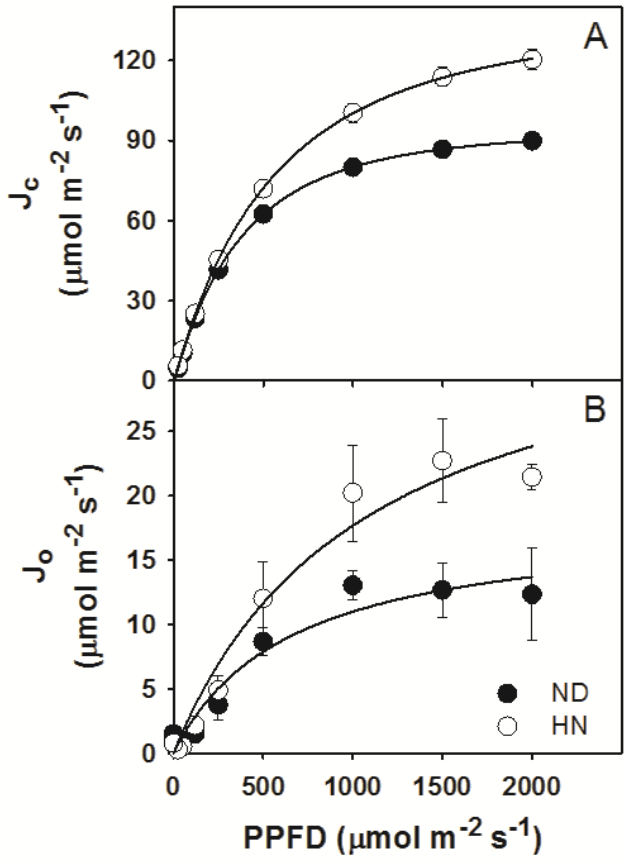

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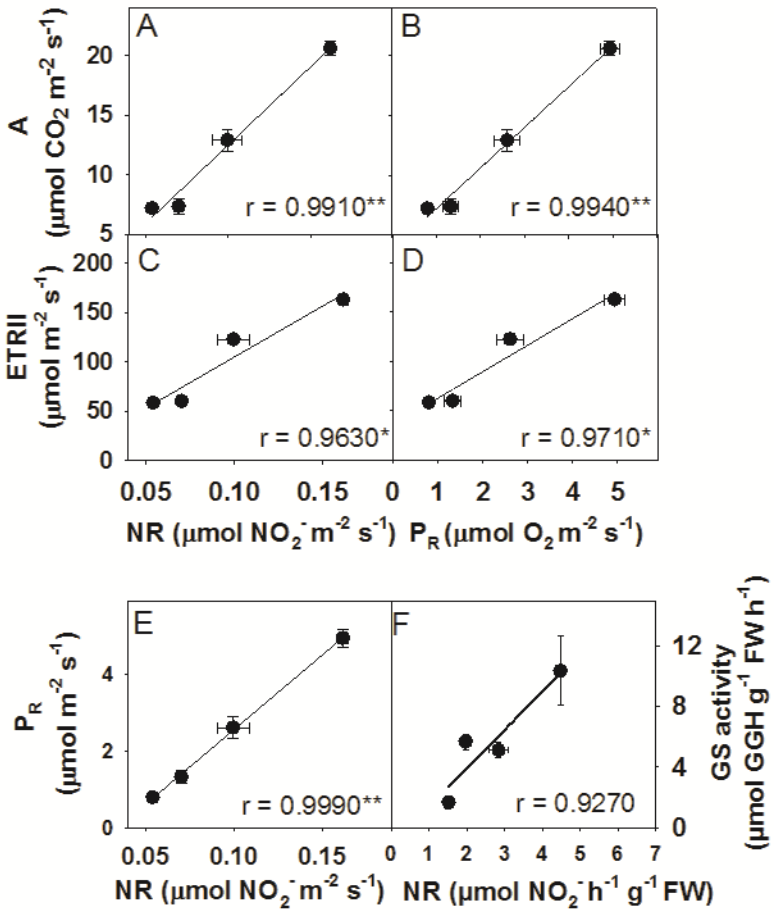
Figure 14 - Correlations between (A) net CO_2 assimilation - A and nitrate reductase activity - NR, (B) A and photorespiration - Pr, (C) Electron transport rate of PSII - ETRII and NR, (D) ETRII and Pr, (E) Pr and NR and (F) GS and NR in cotton leaves subjected to NO_3^- deprivation (ND) or supplied with high nitrate (10 mM NO_3^- -HN) followed by 8 hours of exposure to reference light (200 µmol^{-2} s^{-1} -REF) and high light (2000 µmol^{-2} s^{-1} -HL). ND- and HN-plants were previously supplied with NO_3^- as a sole N-source. The r-value indicate Pearson´s correlation coefficient while ** and * indicate $p \leq 0.01$ and 0.05 respectively.



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Time-course of NO_3^- supply/deprivation revealed that changes in photorespiration and CO_2 assimilation were more dependent on nitrate assimilatory pathway than changes in the leaf N-status

A time-course experiment was performed to elucidate if the effects induced by nitrate supplying on photorespiration and photosynthesis were related to nitrate assimilation

process *per se* or due to changes induced by alterations in the leaf N-status. These shifts allowed reaching a gradual N-starvation and a progressive increase in N-status for 92 h. After 44 hours of shift in nitrate supplying, the values of A, NR activity and chlorophyll contents were similar between the two treatments whereas the total-N was slightly higher in plants supplied with HN (Figure 15). However, the total-N content did not change in deprived plants over the exposure time, whereas plants supplied with HN showed a slight increase after 44 h and this trend between the two treatments remained unchangeable until 68 h. After 92 h, the total-N content dropped significantly in deprived plants (reaching 2.5% in a DW basis), characterizing an N-starvation and light but not an acute N-deficiency (READ *et al.*, 2006). The absence of N-starvation (as indicated by %N) in plants transferred to nitrate-free solution after 68 h was related to slight decrease in A, a prominent reduction in NR activity and to unchangeable values of chlorophyll content. In contrast, the N-starvation that was reached after 92 h was related to significant decreases in A and NR activity whereas the chlorophyll did not change (Figure 15). The minor changes observed in N-nutritional status during the time-course were corroborated by the protein profile revealed by SDS-PAGE, especially in the bands corresponding to Rubisco large and small subunits and by changes in RbcL western blotting bands (Figure 16). These results combined with the visual shoot appearance (Figure 17), indicated that during nitrate withdrawal cotton plants probably experienced a transient nitrate deprivation instead of an acute N-deficiency.

Figure 15 – Changes in (A) net CO_2 assimilation, (B) nitrate reductase activity, (C) total N content and (D) chlorophyll content in cotton leaves response to contrasting nitrate supply. The plants were previously pre-acclimated under two contrasting nitrate supplying: low NO_3^- (1 mM - LN) and high NO_3^- (10 mM - HN) during four days. LN and HN plants were transferred to solutions containing 10 mM NO_3^- and 0 mM NO_3^- (nitrate deprivation-ND), respectively, where remained for 20, 44, 68 and 92 hours. Before each harvest, plants were exposed to 8 hours of high light (2000 µmol^{-2} s^{-1}). Each point represents average from three replicates ± SD and asterisks represent significant differences among treatments by the *t*-test ($p<0.05$).

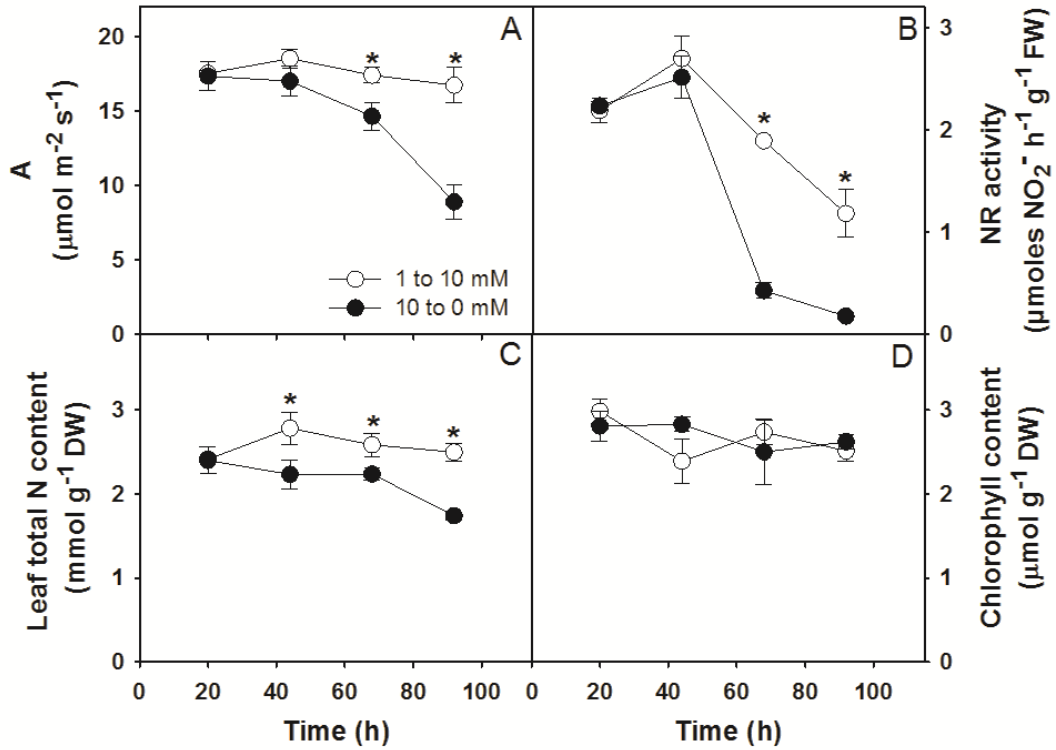

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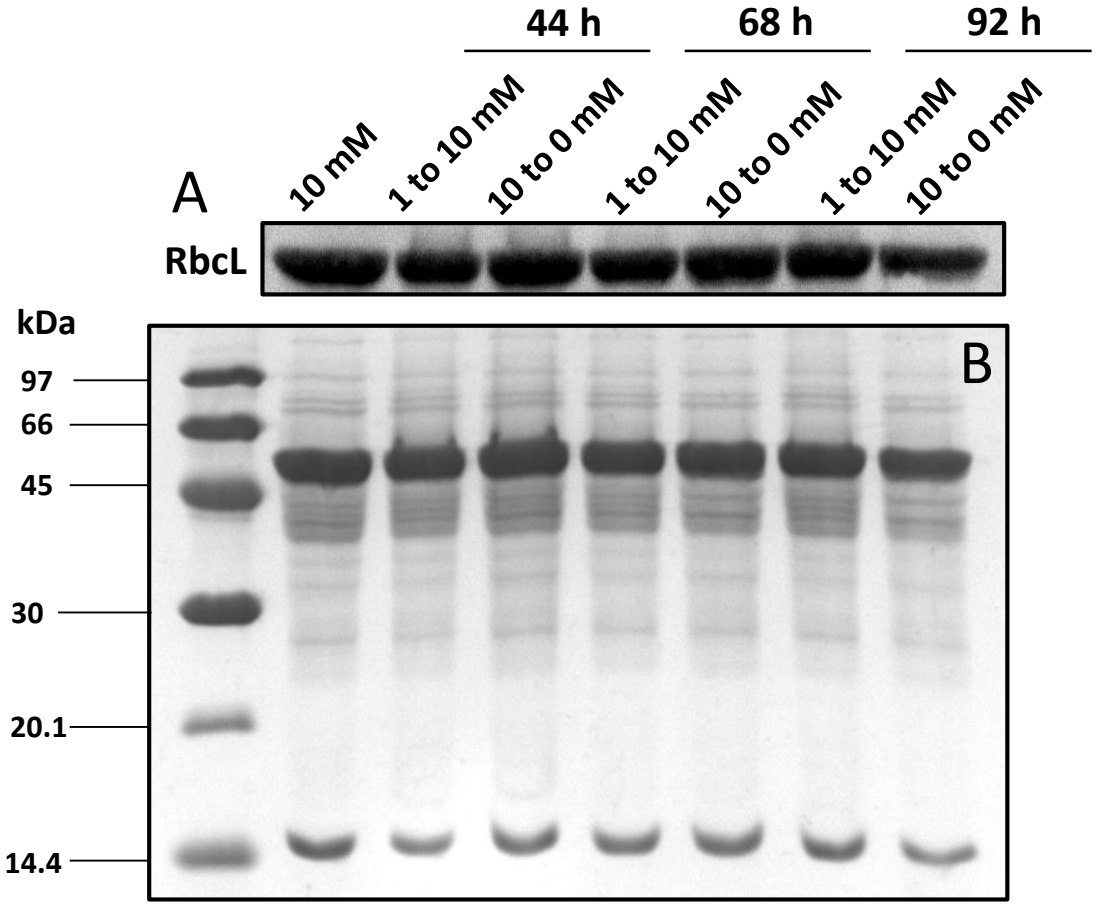
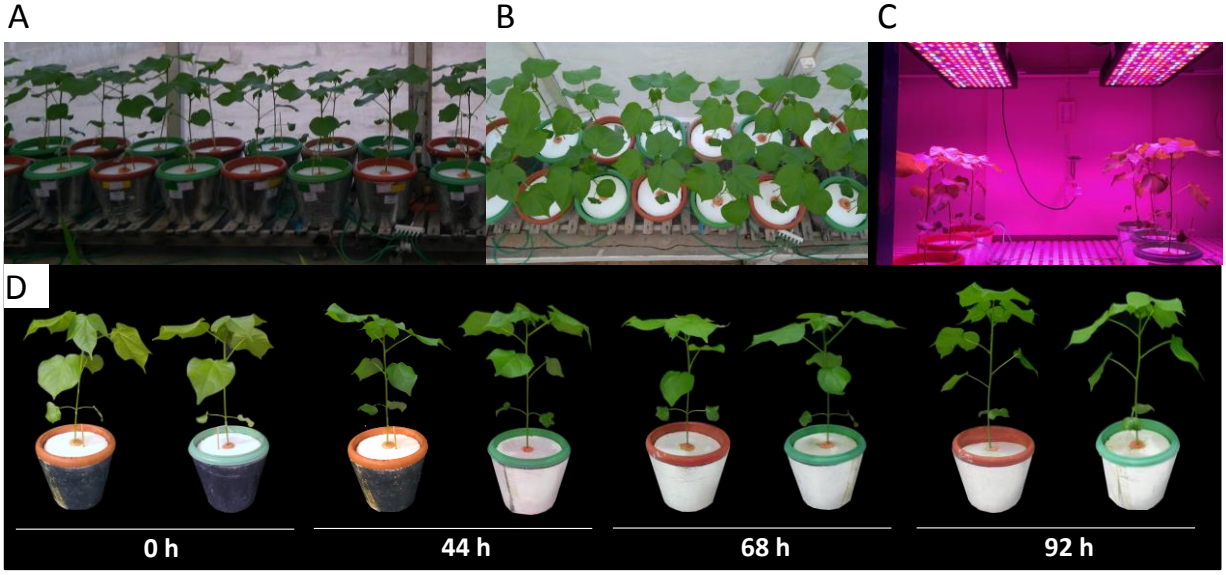
Figure 16 – Changes in the profile of (A) Rubisco large subunit (RbcL) and (B) soluble proteins in cotton leaves exposed to changes in nitrate supply. The plants were previously pre-acclimated under two contrasting nitrate supplying: low NO_3^- (1 mM - LN) and high NO_3^- (10 mM - HN) for four days. LN and HN plants were transferred to solutions containing 10 mM NO_3^- and 0 mM NO_3 (nitrate deprivation-ND), respectively, where remained for 44, 68 and 92 hours. Before each harvest, plants were exposed to 8 hours of high light (2000 µmol^{-2} s^{-1}).



Source: elaborated by the author.

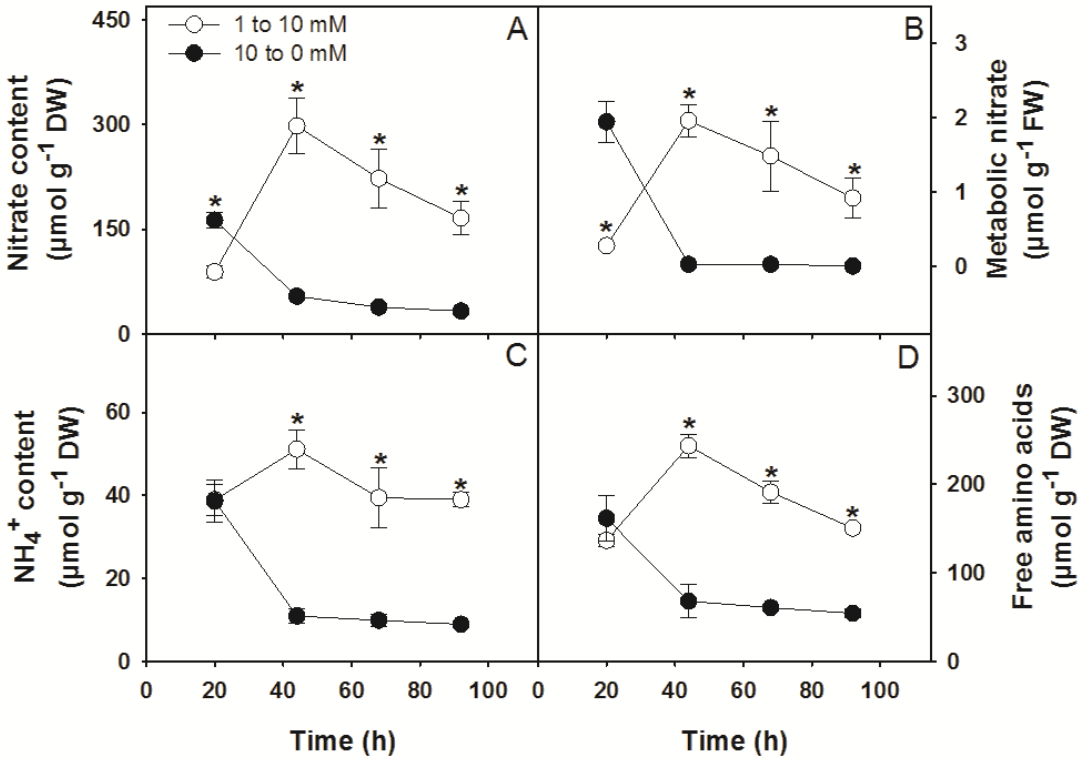
Figure 17 - Shoot morphological characteristics of HN-plants (green pots) and LN-plants (brown pots) under (A) a side view and (B) a top view. (C) High light treatment after shift from 10 to 0 mM NO_3^- (green pots) and from 1 to 10 mM NO_3^- (brown pots). (D) Visual aspects of shoot over the time after the shift of nitrate supply in nutritive solution.



Source: elaborated by the author.

During the time-course the absorbed nitrate was greatly metabolized by NR as revealed by changes in the levels of total nitrate, metabolic nitrate, ammonium and free amino acids (Figure 18). The nitrate content in plants shifted from 10 mM to zero mM in root medium strongly decreased from 170 to 45 µmol g^{-1} DW after 92 h, whereas inversely it increased abruptly from 95 to 280 µmol g^{-1} DW after 44 h in plants transferred to nutrient solution containing 10 mM NO_3^-. Unexpectedly, after this time the nitrate content decreased gradually to 150 µmol g^{-1} DW whereas the metabolic nitrate displayed a similar trend (Figure 18). Overall, the contents of ammonium and amino acids presented similar trend as compared to nitrate in both treatments and these changes occurred in parallel to NR activity.

Figure 18 - Changes in the contents of (A) nitrate, (B) metabolic nitrate, (C) NH_4^+ and (D) free amino acids in cotton leaves in response to nitrate supply. The plants were previously pre-acclimated under two contrasting nitrate supplying: low NO_3^- (1 mM - LN) and high NO_3^- (10 mM - HN) during four days. LN and HN plants were transferred to solutions containing 10 mM NO_3^- and 0 mM NO_3^- (nitrate deprivation-ND), respectively, where remained for 20, 44, 68 and 92 hours. Before each harvest, plants were exposed to 8 hours of high light (2000 μmol^{-2} s^{-1}). Each point represents average from three replicates ±SD and asterisks represent significant differences among treatments by the *t*-test ($p<0.05$).



Source: elaborated by the author.

Discussion

In this study we hypothesized that high rates of nitrate assimilation could stimulate photorespiration and these processes, together, should favor photosynthesis under a photoinhibitory condition induced by high light. We have assumed that these three combined processes should have greatly stimulated the consumption of excessive photosynthetic electrons, ATP and sugars, possibly reducing the inhibitory effects of triose-P limitation on the CO_2 assimilation. This postulation is based on the fact that nitrate reductase, the limiting enzyme of assimilatory nitrate reduction, has its expression and activity positively regulated by light, nitrate flux and sugar availability (CAMPBELL, 1999; HUARANCCA *et al.*, 2018).

Moreover, this process is capable to stimulate photorespiration by introducing Glu, contributing for Gly recycling from glyoxylate (ABADIE *et al.*, 2016). Finally, it is largely known that photorespiration is strongly stimulated by high light.

The mechanisms encompassing the integration and metabolic regulation of photosynthetic CO_2 assimilation, nitrate assimilatory reduction and photorespiration are virtually unknown. Some indirect evidence based on different CO_2 concentration experiments have evidenced that high photorespiration stimulates 2-oxo-ketoglutarate (2-KG) metabolism (TCHERKEZ *et al.*, 2012) and nitrogen assimilation (BLOOM *et al.*, 2014). In this circumstance, Glu is largely utilized for Gly synthesis via glyoxylate transamination (ABADIE *et al.*, 2016), releasing 2-KG in peroxisomes. On the other hand, high nitrate supply might stimulate photorespiratory cycle by supplying Glu and ammonia, inducing glyoxylate and amino acid exportation from chloroplasts. Together, these mechanisms might stimulate CO_2 assimilation by reducing triose-P limitation and excess energy (BUSH *et al.*, 2018) and also might contribute for decreasing the concentration of potential photosynthesis inhibitors, such as glycolate (ZELITCH *et al.*, 2009).

In this current study the most meaningful obtained result is that, indeed, high NO_3^- supply, even in presence of a photoinhibitory irradiance level, is capable to stimulate nitrate assimilation and photorespiration and favoring CO_2 assimilation. Conversely, our data clearly show that potential PSII activity were similarly affected by HL in both N conditions, taken as a basis the observed alterations in the photochemical indicators Fv/Fm, Fm and F0 (YAMANE *et al.*, 1997; FOYER *et al.* 2017), suggesting that transient nitrate deprivation *per se* did not affect the PSII integrity. Moreover, cotton plants exposed to increased N supplement have exhibited some favorable photochemical characteristics, including an increase in PSII efficiency even under a photoinhibitory condition, which reflected in higher rates of electron transport at PSII level and lower limitation in the acceptor side of PSI.

The increase in ETRII rates are probably related to intensification of the strength of alternative sinks for photosynthetic electrons, mainly represented by assimilatory nitrate reduction and photorespiration pathways, as indicated in this study by data of enzymatic and electron flux indicators. This assumption is corroborated in literature by light-curve results, previously noticed for tobacco plants grown in low and high N, followed by exposure to increasing light. The authors have pointed out that plants supplied with +N are able to increase photorespiration and improve carboxylation activity in high light regimes (HUANG *et al.*, 2016). However, a central question has remained open. How increased nitrate supply

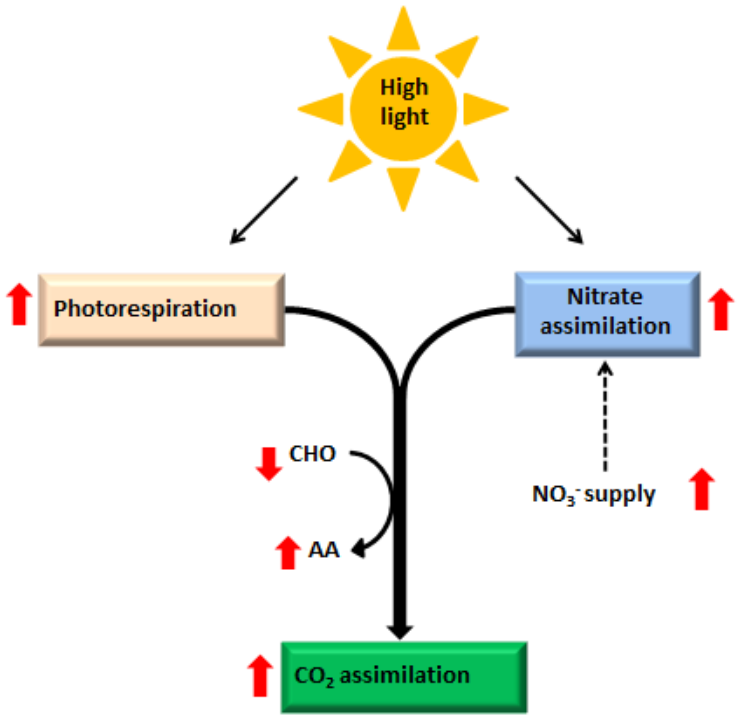
could have generated a simultaneous increase in ETR, photorespiration and CO_2 assimilation in presence of HL? Our data have evidenced that *in vivo* Rubisco carboxylase activity (indicated by Vcmax parameter) is significantly increased by high nitrate supply in presence of HL, suggesting that Calvin-Benson cycle reactions are not impaired by HL under these conditions. Some authors have suggested that these three processes, together, could favour a more adequate energy balance in terms of NADPH and ATP in chloroplasts (BUSH *et al.*, 2018).

A simultaneous increase in electron transport rates for CO_2 fixation, nitrate assimilation, and photorespiration could have contributed for photosynthetic efficiency under HL by different avenues. Initially, this integrated energy consuming mechanism should contribute for an adequate balance in chloroplasts, avoiding accumulation of excess energy and over-accumulation of reactive oxygen species (ROS), protecting the photosynthetic apparatus against photoinhibition and other dangerous effects caused by ROS (FOYER *et al.*, 2017). Indeed, despite HL in this study has induced decreases in Fv/Fm and chlorophyll content, theses responses occurred regardless nitrate supply, and N deprived plants exhibited no signals of ROS accumulation in comparison to N supplied plants (Figure 9). Therefore, the increase in electron sink strength triggered by nitrate assimilation and photorespiration under HL could have favored the PSII activity by a source-sink effect. This assumption is corroborated by observed absence of changes in donor side and decrease of limitations in the acceptor side of PSI.

The biochemical data related to assimilatory nitrate reduction obtained in this study clearly demonstrate two important points for understand the role of this pathway to enhance photorespiration. These results strongly evidence that the effects induced by NO_3^- high supply on photosynthesis and photorespiratory cycle are more dependent on that assimilatory pathway rather than a possible alteration in leaf N-status. This assumption is corroborated by the data obtained from SDS-PAGE and Rubisco western blotting. Despite several reports have previously demonstrated that N deficiency can affect some processes involved with photosynthetic efficiency (BRESTIC *et al.*, 2014; HUANG *et al.*, 2016; MIYAKE *et al.*, 2005), the underlying biochemical mechanisms are scarcely known. Indeed, these authors were not able to elucidate if these observed responses are dependent on *de novo* nitrate assimilation reactions or if they are merely associated to a generalized disturbance in protein metabolism.

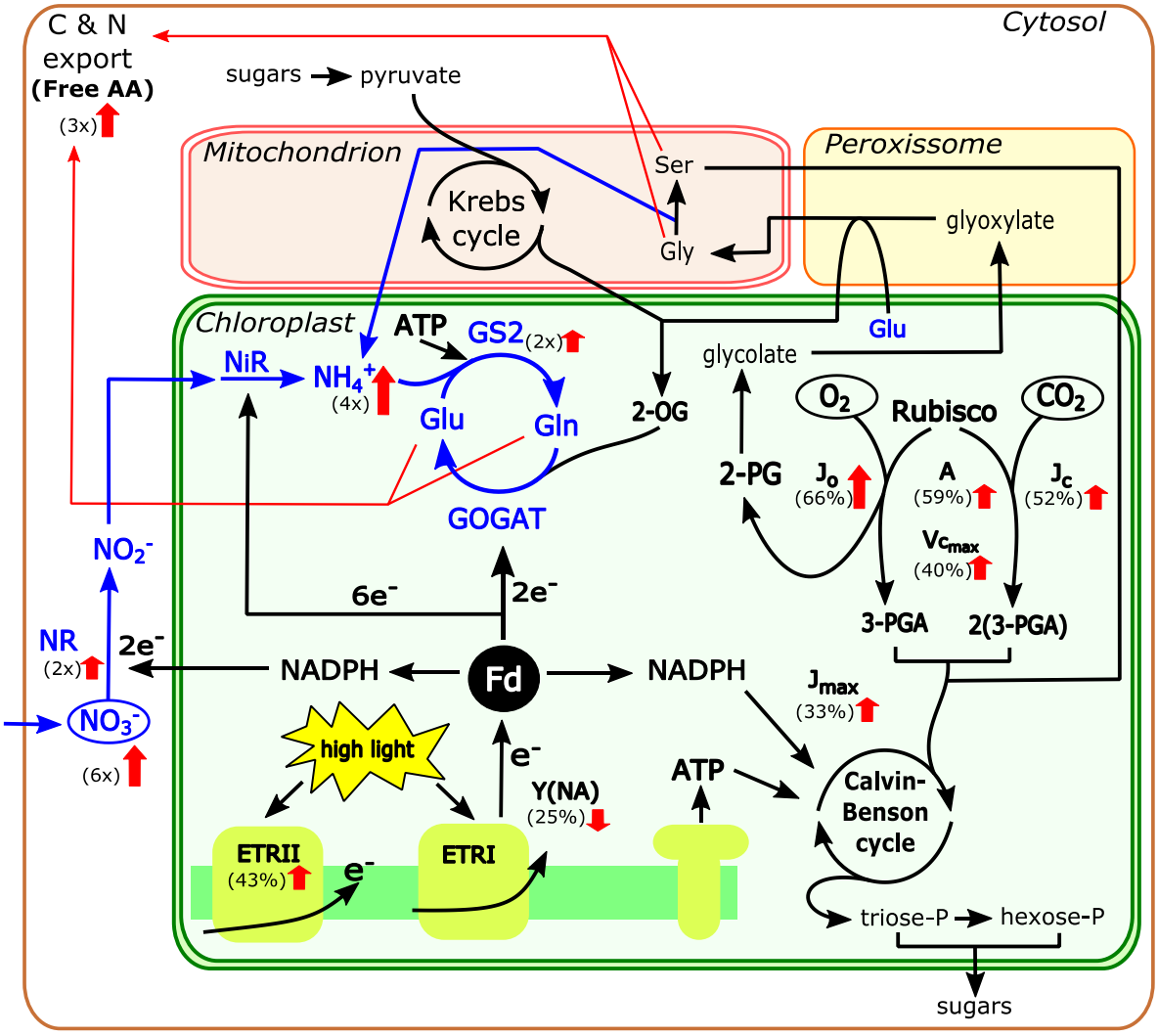
The data obtained in this study from the Pearson´s correlation analysis reinforce that, indeed, integrative events involving high light, photorespiration, nitrate assimilation, which ultimately could have favored photosynthetic efficiency did occur. The Figure 19 schematically summarizes the central idea proposed here. Very high correlation coefficients (above 0.999**) obtained between NR versus A and NR versus Pr, and Pr versus A are noted in this study, evidencing that there is cause-effect relationship among these processes. Indeed, several specific studies have demonstrated that nitrate assimilation is favorable to photosynthesis (BUSCH *et al.*, 2018) and that photorespiration is positively related to nitrate reductase activity (BLOOM *et al.*, 2015). The Figure 20 shows an integrative view involving the main data obtained in this work.

Figure 19 – Simplified scheme showing relationships among high light utilization, photorespiration, nitrate assimilation and CO_2 assimilation. It's proposed that HL stimulates photorespiratory and nitrate assimilation pathways that are mutually and synergistically up regulated, enhancing CO_2 assimilation, probably by sugar consumption and amino acid exportation.



Source: elaborated by the author.

Figure 20 - A proposed scheme to explain the photosynthesis enhancement in response to increased nitrate assimilation and photorespiration in leaves exposed to high light. Nitrate flux and nitrate reductase activity (NR) in cytosol are greatly stimulated by increased nitrate supply in presence of high light. The activities of nitrite reductase (NiR) and GS/GOGAT cycle are stimulated as indicated by increase in the contents of ammonium, amino acids and GS activity, generating enhancement in the consume of electrons and ATP in the photorespiratory cycle. In parallel, high nitrate supply stimulates ETRII and decreases limitation of the acceptor side of PSI - Y(NA), probably due to increase in the electron consumption as NAD(P)H and reduced Fd by nitrate assimilation and photorespiration. These processes induce a great stimulation in the electron transport towards photorespiration (Jo) and carboxylation (Jc), favoring CO_2 uptake (A).



Source: elaborated by the author.

In conclusion, the results obtained in this study point out to an important novelty involving an integrative view of nitrate assimilation, photorespiration and photosynthesis. High nitrate supply is capable to simultaneously stimulate assimilatory nitrate reduction and photorespiration under a photoinhibitory condition induced by high light. In these conditions, these two processes seem to act synergistically, favoring the photosynthetic efficiency via improvement in the quantum yield of PSII and CO_2 assimilation.

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(Submitted manuscript in Plant and Soil)

5 IMPROVING NITROGEN USE EFFICIENCY IN COTTON PLANTS BY NITRATE REDUCTASE ACTIVITY AND PHOTOSYNTHESIS

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Abstract

Understanding mechanisms underlying N use efficiency (NUE) after luxury consumption and nitrate deprivation is crucial for crop productivity. The aim was elucidating the importance of photosynthesis, nitrate assimilation and N-reserve remobilization to NUE in cotton. Plants were exposed to three conditions in nutrient solution: (a) previous exposure to high nitrate supply (10 mM) for long-term (8 days); (b) nitrate deprivation (NO_3^- withdrawal) for eight days followed by (c) an early N-deficiency during four days. Plants supplied with nitrate excess were able to display increment in shoot NUE, whereas photosynthetic N use efficiency did not change, evidencing that excess N was not able to additionally improve CO_2 assimilation. Nitrate reductase (NR) activity was crucial to remobilize stored nitrate through deprivation phase, whereas free amino acids, total proteins, and chlorophylls were essential to N-remobilization. High NUE was important to kept high root growth rates throughout deprivation and early deficiency phases. Despite the great decrease in chlorophyll content, PSII and PSI activities were kept stable until the onset of early N-deficiency. Cotton plants display high NUE under deprivation and early deficiency. These responses are closely associated with high NR turnover and sustaining of photosynthesis, which contribute to N-homeostasis in different nutritional regimes.

Keywords – *Gossypium hirsutum*, nitrate assimilation, NUE, N-nutrition, reserve remobilization.

Introduction

Nitrogen (N) is an essential nutrient required in high amounts and commonly it is limiting to plant growth and productivity worldwide. Nevertheless, crop management frequently employs excessive quantities of nitrogenous fertilizers that are expensive and can cause harmful effects in humans and animals through groundwater contamination with NO_3^- (MASCLAUX-DAUBRESSE *et al.*, 2010; WANG *et al.*, 2018). Thus, an appropriate fertilization for an equilibrated N-nutrition is economic and environmentally indispensable. In aerated soils, nitrate is most important N-source and plants are able to uptake and store great amounts of this anion in its vacuoles without cause toxicity (IMSANDE and TOURAINE 1994; TEGEDER and MASCLAUX-DAUBRESSE 2018). The excess N in plant tissues might be stored in different chemical forms such as NO_3^-, free amino acids, proteins (especially Rubisco) and chlorophylls (KANT, 2018; LI, *et al.*, 2013; WALKER *et al.*, 2018).

N vegetative reserves are remobilized during plant growth and starvation periods and the efficiency that process is crucial to productivity and survival (DIAZ *et al.*, 2008). The N use efficiency (NUE) is commonly defined as the amount of biomass produced per unit of N absorbed or accumulated in determined part of the plant (SEEMANN *et al.*, 1987; TEGEDER and MASCLAUX-DAUBRESSE, 2018). As biomass is closely dependent on photosynthetic capacity and nitrogen strongly affects photosynthesis (MAKINO *et al.*, 1997), NUE also can be expressed as photosynthetic activity per N unity in leaves (PNUE). These parameters are important to establish relationships between C and N metabolism in some physiological circumstances throughout plant development. Indeed, under field conditions, N availability in soils is extremely oscillating and cultivated plants might be subjected to contrasting conditions such as N-luxury nutrition and deficiency (DEVIENNE-BARRET *et al.*, 2000; TORNKVIST *et al.*, 2019).

Although the importance of understanding NUE underlying mechanisms in these two contrasting physiological circumstances this issue has remained scarcely known and some crucial questions were not solved yet. How plants control their N homeostasis during a high nitrate supply and, inversely, under drastic N-deprivation? In an ideal scenario, crops should exhibit high N use efficiency displaying maximum photosynthesis and/or dry matter production with minimum absorbed N (MAKINO *et al.*, 1997; TEGEDER and MASCLAUX-DAUBRESSE, 2018). Or, alternatively, exhibiting high N storage capacity when exposed to NO_3^- high supply associated with efficient mobilization of reserves during starvation (KANT,

2018). For many years this issue has attracted attention of several plant scientists and most common targets have been focused on improving the efficiency of root N uptake, transport, remobilization and control of the synthesis/accumulation of vegetative reserve proteins such as Rubisco in leaves (KIBA and KRAPP, 2016; STASWICK, 1994).

In opposition to excess N, plants face commonly with low nitrogen levels in soils that inexorably will induce low uptake and deprivation, resulting in low growth, commonly associated with reduced photosynthesis (COUSINS and BLOOM, 2003; HUANG *et al.*, 2016; ZHAO *et al.*, 2017). In this N-limiting situation, the plants are able to remobilize parts of stored N, especially for root growth and younger leaves, in detriment of mature tissues (TEGEDER and MASCLAUX-DAUBRESSE, 2018). Paradoxically, despite its importance, physiological mechanisms that occur in each one of these two extreme phases and its reflexes on other processes, such as photosynthesis, are scarcely understood (BUSCH *et al.*, 2018; GUILHERME *et al.*, 2019). Such knowledge might contribute to an efficient N management in order to accomplish higher use efficiency. In parallel, these studies might contribute to development of accurate methods to determine the threshold levels of N-status in plant tissues, in different nutritional circumstances and especially in those related to the improvement of NUE.

In this study, cotton plants were subjected to three contrasting conditions, begging by a previous high nitrate supply, following to progressive N deprivation until reaching an early deficiency phase. The objective was evaluating the N-nutrition dynamics in these distinct physiological phases, highlighting the relationships among storage, remobilization, and NUE especially associated with nitrate reductase activity and photosynthesis. Cotton plants display N homeostasis throughout storage phase and they are able to sustain this condition after the onset of deprivation and early deficiency. The results are discussed in terms of N use efficiency and plant plasticity in distinct nutritional circumstances.

Material and methods

Plant material and growth conditions

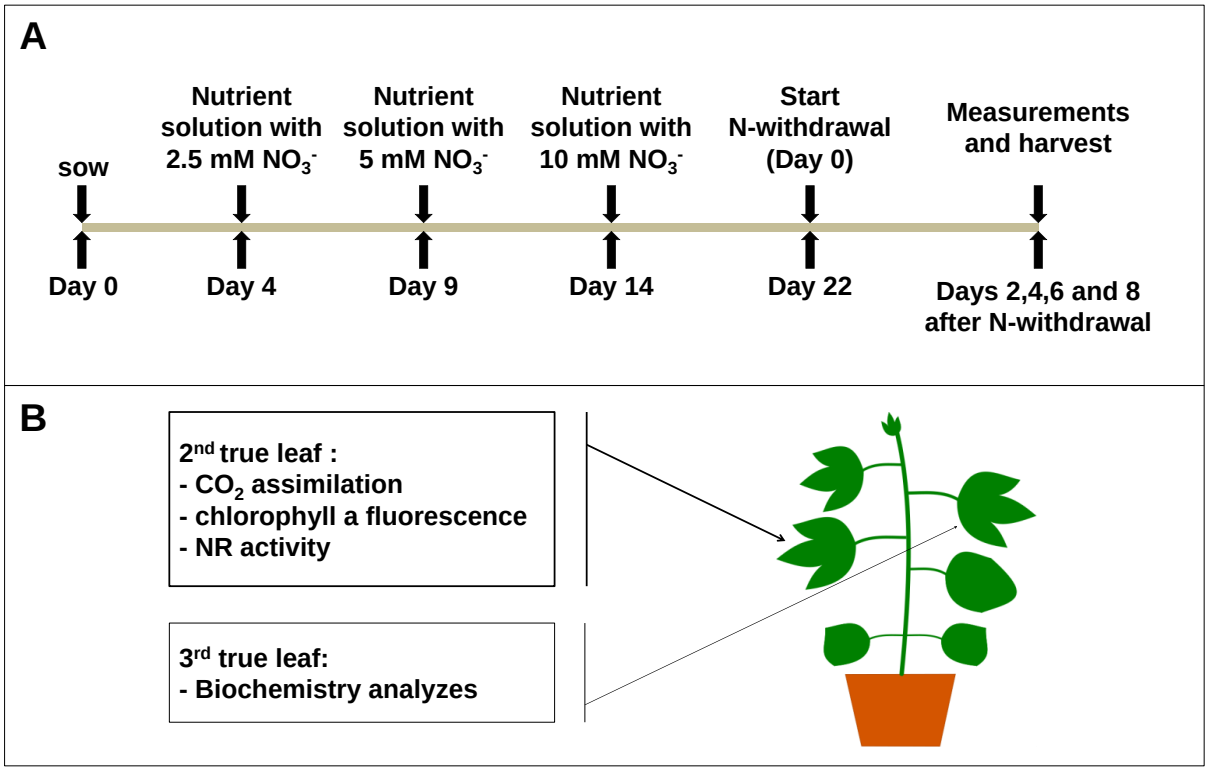
Cotton (*Gossypium hirsutum*) seeds from the commercial genotype FM 954GLT, kindly provided by the Bayer Company (Brazil), were germinated in washed land. Four-days

old seedlings were transplanted to 2.5 L plastic pots filled with ¼ strength Hoagland-Arnon's nutrient solution (Hoagland and Arnon 1950), modified to provide NO_3^- as a sole N-source. The initial NO_3^- concentration was 2.5 mM, which was gradually increased until reach 10 mM, supplied as 5 mM $Ca(NO_3)_2$ 10 days later. The pH was adjusted to 6.0 every two days and the nutritive solution was completely changed weekly. The nutrient solution was maintained under continuous aeration through an air compressor. Plants were grown in a greenhouse under natural conditions (3°44'44.2"S 38°34'29.2"W) as follows: day/night mean temperature of 32°C/25°C, relative humidity average of 60% and maximum photosynthetic photon flux density (PPFD) of 600 µmol m^{-2} s^{-1} at noon and 12 h-photoperiod.

Experiments for evaluate NUE under luxury N consumption, deprivation and early deficiency

After the first phase of nitrate luxury consumption (8 days at 10 mM NO_3^-), 22 days-old plants were exposed to two contrasting conditions. A group was kept under this condition for more 8 days (excess N supplying) and other suffered nitrate deprivation (NO_3^- withdrawal) for 8 consecutive days. In this period, plants initially faced N deprivation during four days as indicated by normal growth rates in both root and shoot. Afterwards, these plants experienced an early N-deficiency as indicated by cessation in shoot growth rates but displaying suitable N-status and absence of any visual symptoms of chlorosis and senescence in old leaves (Figure 21). During the experiments, the concentration of NO_3^- (10 mM) in the nutrient solution of plants supplied with excess nitrate was adjusted every two days. After the time-course throughout 8 days, *in vivo* photosynthesis (photochemical and gas exchange parameters) and NR activity measurements were performed on mature leaf (2^{nd} true leaf). Later, leaf samples (3^{rd} true leaf) were harvested, immediately frozen with liquid N_2 and kept in -80 ^oC until subsequent biochemical analyses (Figure 21).

Figure 21 – Schematic experimental design of cotton plants exposed to nitrate deprivation or supplied with high N (10 mM NO_3^-) for 8 days in a greenhouse. The time-line of the experimental design (A) and indication of leaves used in different measurements (B) are shown.



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Photosynthetic parameters measurements

Net CO_2 assimilation (A), stomatal conductance (g_s), and transpiration rate (E) were measured in fully expanded leaves by using a portable infrared gas analyzer system (IRGA – LI-6400XT, LI-COR, Lincoln, NE, USA). The environmental conditions inside the IRGA's chamber were: 28 °C temperature, air vapour pressure deficit of 1.0±0.2 kPa, air CO_2 partial pressure of 40 Pa and PPFD (photosynthetic photon flux density) of 1,500 µmol photons m^{-2} s^{-1}. The amount of blue light was set up to 10% of the PPFD, in order to maximize stomatal aperture (FLEXAS *et al.*, 2008).

Photochemical parameters were measured using a Dual-PAM 100 (Walz, Germany). For induction/recovery kinetics, 1,000 µmol m^{-2} s^{-1} of PPFD was employed for 5 min (induction), followed by 5 min dark (recovery). The saturation pulse method (KLUGHAMMER and SCHREIBER 2008a) was employed on leaves previously acclimated to dark for 30 min. The intensity and duration of the saturation pulse were 8,000 µmol m^{-2} s^{-1}

and 0.6 s, respectively. The following parameters were assessed: maximum quantum yield of PSII [Fv/Fm = (Fm−Fo)/Fm] and effective quantum yield of PSII [Y(II) = (Fm − Fs)/Fm']. The photochemical quenching coefficient was calculated as qP = (Fm' − Fs)/(Fm' − Fo'). The quinone pool redox state was estimated as 1-qP. In this study, Fm was determined at the onset of light induction kinetics. Fm and Fo are defined as the maximum and minimum fluorescence of dark-adapted leaves, respectively; Fm' and Fs are the maximum and steady state fluorescence in the light-adapted leaves, respectively; Fo' is the minimum fluorescence after the far-red illumination of the previously light-exposed leaves (KLUGHAMMER and SCHREIBER, 2008a). The redox state of the PSI primary donor (P700) was measured and the following parameters were assessed: photochemical quantum yield of PSI [Y(I) = Pm' – P)/Pm], donor side limitation of PSI [Y(ND) = (P – Po)/Pm] and acceptor side of PSI limitation [Y(NA) = (Pm – Pm')/Pm] (KLUGHAMMER and SCHREIBER, 2008b).

Determination of contents of nitrogenous compounds and sugars

Freeze-dried leaf samples were used in all these biochemical analysis. In order to quantify the content of nitrate and free amino acids, samples were incubated with distilled water at 65ºC for 1 h and filtered to obtain the crude extract. Afterwards, nitrate concentration was determined by the colorimetric salicylic acid method, according to Cataldo *et al.* (1975), whereas the content of total free amino acids was measured by the ninhydrin method, according to Yemm *et al.* (1955). The total ammonium content was extracted by using the MCW solution (methanol: chloroform: water 12:5:1, v/v/v) and quantified according to Felker (1977). The total-N content was measured according to Baethgen and Alley (1989) and the total chlorophyll content was determined according to Porra *et al.* (1989). Glucose was quantified in the same extract used for nitrate determination, employing an enzymatic method (Sigma's test kits, Sigma–Aldrich Co., St. Louis, USA) coupled to NADH production, monitored with a spectrophotometer at 340 nm. The content of total soluble sugars and sucrose were quantified according to Dubois *et al.* (1956) and van Handel (1968) respectively, using the same extract of ammonium determination. The starch concentration in the MCW pellet was determined by hydrolyzing with $HClO_4$ (30%, v/v), and the total soluble sugars were measured by the phenol-sulfuric acid method, according to Dubois *et al.* (1956).

Nitrate reductase activity, N efficiency utilization and photosynthetic N use efficiency

The activity of nitrate reductase (NR; EC 1.7.1.1) in leaves was assayed by in vivo method according to Hageman and Hucklesby (1971) as previously reported (GUILHERME *et al.* 2019). Leaf discs were placed in vials containing 5 ml ice-cold incubation medium consisting of 100 mM K-phosphate buffer (pH 7.5) containing 50 mM KNO_3 and 1% (v/v) isopropanol. Leaf discs were infiltrated twice by vacuum, for 2 min each time, at −67 kPa and incubated in the dark for 30 min at 30°C. After incubation, the vials were placed in a boiling water bath for 5 min to stop the enzymatic activity and to extract all nitrite formed. The nitrite released to the medium was determined by colorimetric reaction with 1% (m/v) sulphanilamide with 2.4 N HCl: 0.02% N-naphthyl-ethylenediamine, and readings were taken at 540 nm. Controls were obtained just before the incubation period to discount endogenous nitrite. The nitrogen use efficiency (NUE) and photosynthetic nitrogen use efficiency (PNUE) were assessed as: NUE = shoot dry mass per total-N accumulated (g shoot $mmol^{-1}$ N) and PNUE = A/total-N as $mmol^{-1}$ m^{-2} leaf and was expressed as μmol CO_2 $mmol^{-1}$ N s^{-1}. The NR/NO_3^- ratios were calculated by dividing NR activity per NO_3^- content and it represents an efficiency parameter for nitrate reductase activity.

Experimental design, statistical and correlation network

The experiments were arranged in a completely randomized design in a 2x5 factorial scheme (2 nitrate levels x 5 harvest times) and three replicates, which replicate was represented by a pot containing one plant. The means were compared by *t*-test at 5% probability ($p \leq 0.05$). In order to estimate the relationships between some physiological parameters, selected variables were correlated by Pearson´s coefficient (r). Subsequently, these correlations were fitted in a network arrangement according to edge-weighted spring embedded layout using the Cytoscape software (version 3.7.1), considering an arbitrary threshold of $r < -0.5$ (negative-) or $r > 0.5$ (positive correlation). This layout is based on the "force-directed" paradigm as described in Kamada and Kawai (1989) and this algorithm sets the positions of the nodes in a way that minimizes the sum of forces in the network (connections).

Results

N-deprivation and early deficiency display distinct responses in shoot and root growth, which are related to nitrogen use efficiency

During 8 days, deprived plants displayed two distinct phases in their shoot and root growth. From zero to four days after nitrate deprivation they exhibited a linear dry matter accumulation in both parts (phase I) but during the next 4 days the relative shoot growth rates decreased to near zero (phase II) whereas roots sustained a steady and positive rates similar to plants supplied with high nitrate levels (Figure 22). N-deprived plants also shown higher nitrogen use efficiency from day 2 to 6, but at day 8 it decreased to control levels (Figure 22 C). Therefore, plants N deprived did not exhibit any chlorotic symptom (Figure 24). In fact, these plants fed with N-luxury consumption presented high growth rates in shoot throughout the experimental period. The first growth phase after N deprivation was also characterized by a very large decrease in the contents of all analyzed nitrogen forms in leaves. Indeed, in that phase was noted a pronounced decrease in contents of total-N (3.8 to 1.7 mmol g^{-1} DW), total chlorophylls (5.5- to 2.15 µmol g^{-1} DW) where the accumulated total-N in shoot (mmol N $shoot^{-1}$) remained practically unchanged throughout the experimental period (Figure 23). A similar trend was verified to the contents of nitrate (508- to 30 µmol g^{-1} DW), free amino acids (245- to 70 µmol g^{-1} DW) and ammonium (10- to 4.0 µmol g^{-1} DW) (Figure 25).

Figure 22 - Changes in dry mass of shoot (A), root (B) and (C) NUE in cotton plants exposed to nitrate deprivation or supplied with high nitrate (10 mM NO_3^-) for 8 days in a greenhouse. Plants were previously supplied with 10 mM NO_3^- as a sole N-source for 8 days in order to induce N-luxury consumption. The curves were adjusted by polynomial functions. Data represent the average of three replicates ± SD and asterisks represent significant differences among treatments as indicated by *t*-test ($p < 0.05$).

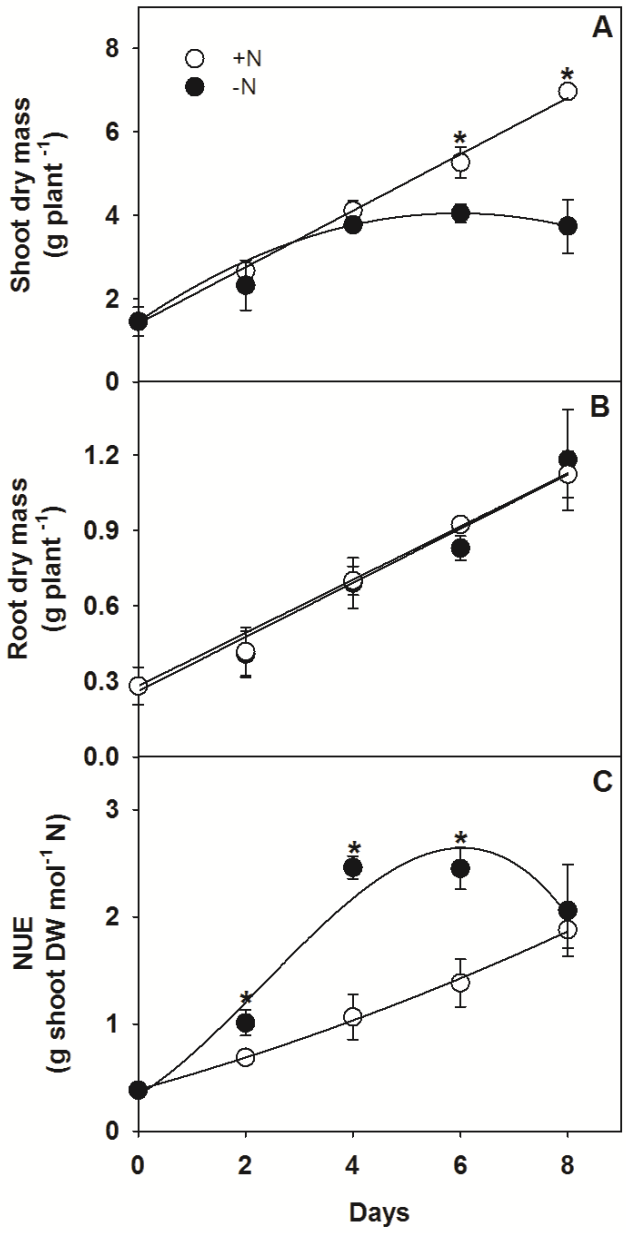

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Figure 23 – Accumulated-N in shoot (A), total-N content in leaves (B) and contents of total chlorophylls (C) in cotton plants exposed to nitrate deprivation or supplied with high nitrate (10 mM NO_3^-) for 8 days in a greenhouse. Plants were previously supplied with 10 mM NO_3^- as a sole N-source for 8 days in order to induce N-luxury consumption. The curves were adjusted by polynomial functions. Data represent the average of three replicates ± SD and asterisks represent significant differences among treatments as indicated by *t*-test ($p < 0.05$).

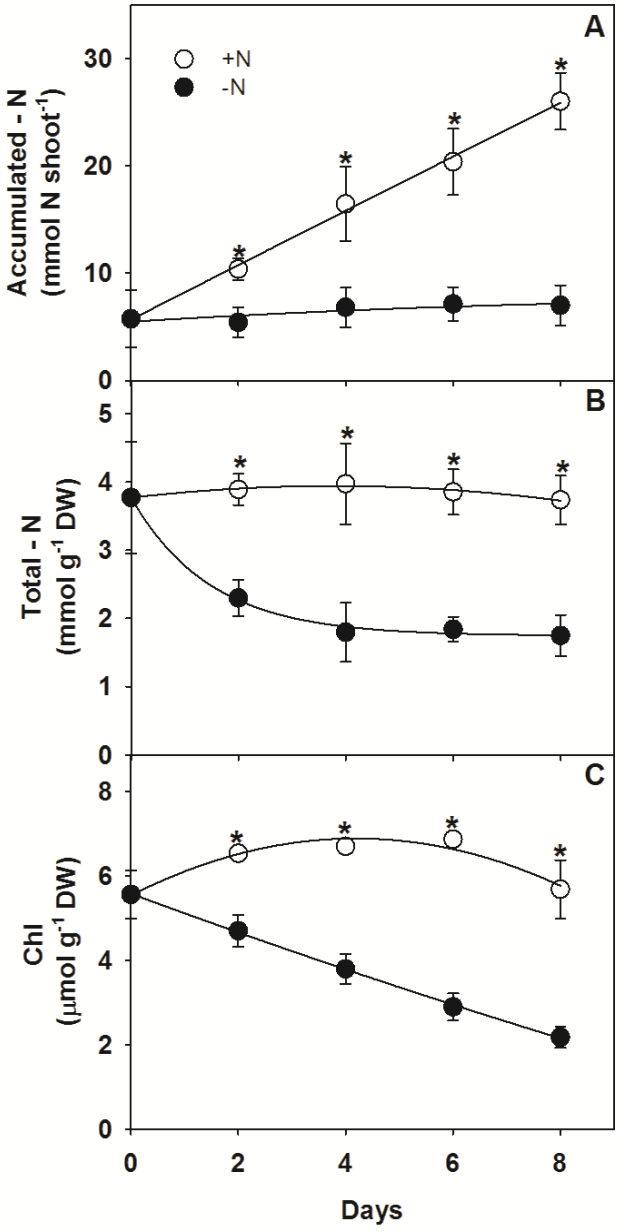

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Figure 24 – Morphological characterization of cotton shoot before nitrate deprivation (zero time, left) and after 8 days of deprivation (-N) or supplying with high nitrate (+N, 10 mM). Deprived plants did not exhibit any visual symptoms of chlorosis and senescence in leaves.

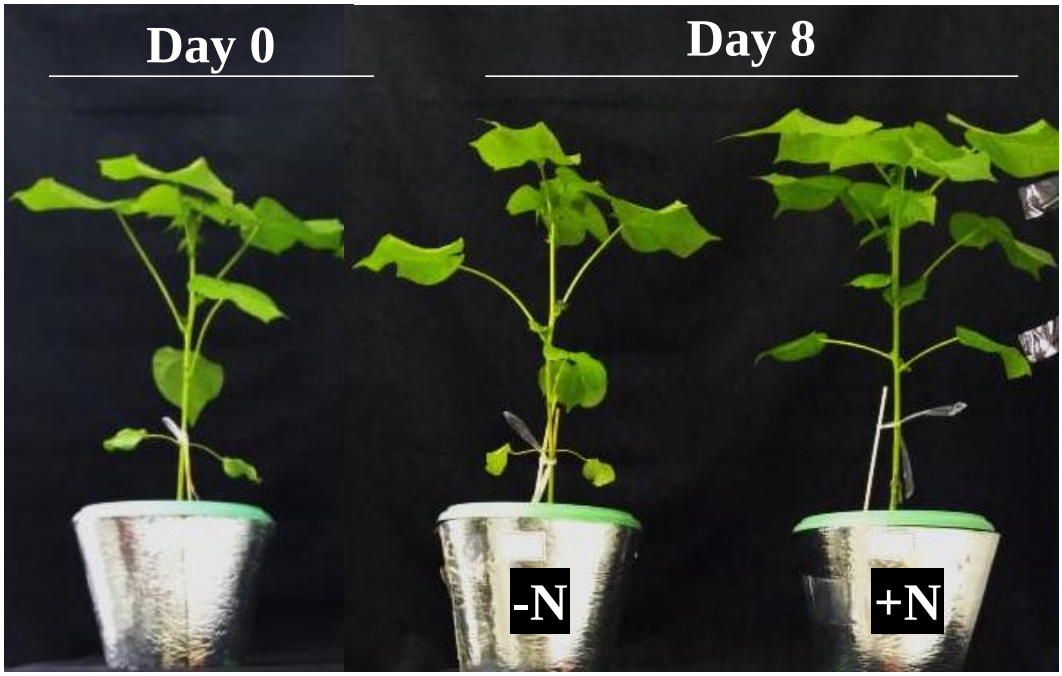

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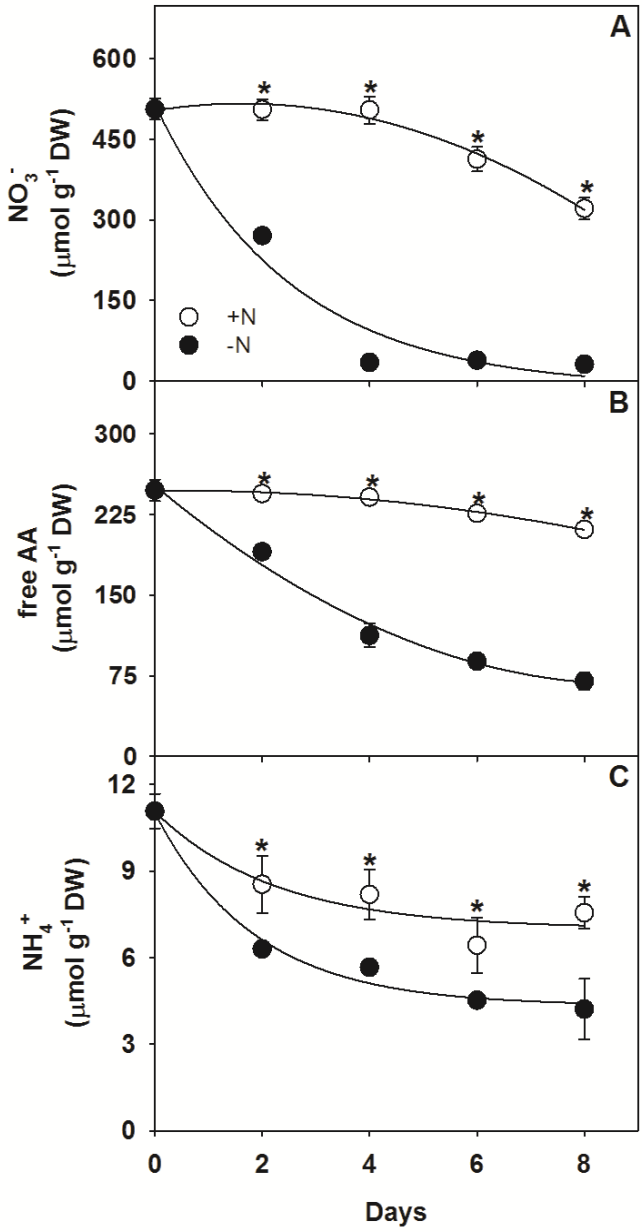
Figure 25 – Contents of nitrate (A), free amino acids (B) and ammonium (C) in leaves of cotton plants exposed to nitrate deprivation or supplied with high nitrate (10 mM NO_3^-) for 8 days in a greenhouse. Plants were previously supplied with 10 mM NO_3^- as a sole N-source for 10 days in order to induce N-luxury consumption. The curves were adjusted by polynomial functions. Data represent the average of three replicates ± SD and asterisks represent significant differences among treatments as indicated by *t*-test ($p < 0.05$).



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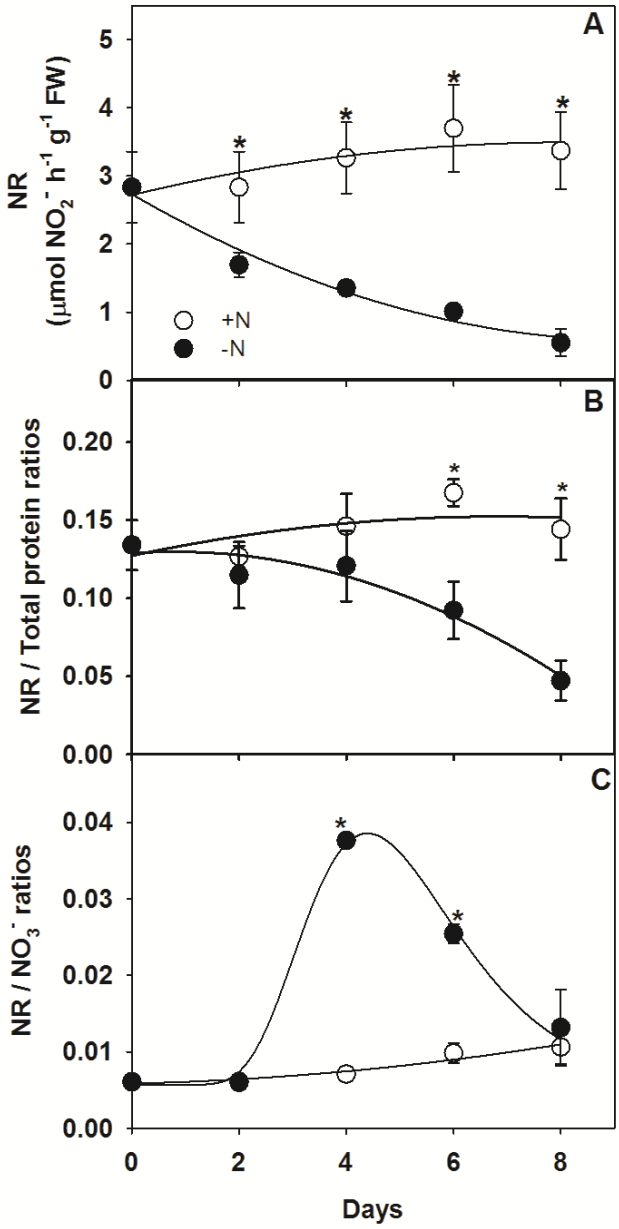
These changes in N fractions were closely correlated with NR activity that decreased 5.6-fold during the entire phase of N starvation (Figure 26). Despite the NR activity has intensely decreased it presented very high efficiency for *in situ* nitrate reduction in leaf tissues since the ratio NR/accumulated-NO_3^- increased intensely from 2 to 4 days of N-

deprivation (by 6.6-fold) and afterwards it decreases similarly to observed for NUE responses (Figure 26). Thus, NR activity was crucial to remobilization of nitrate previously stored in leaves during the luxury consumption phase. As a consequence of this intense N-plasticity and remobilization, these plants were able to display a large increase in NUE in comparison with those supplied with nitrate excess.

Figure 26 - Nitrate reductase activity (A) and nitrate reductase activity/NO_3^- ratios (B) in leaves of cotton plants exposed to nitrate deprivation or supplied with high nitrate (10 mM NO_3^-) for 8 days in a greenhouse. Plants were previously supplied with 10 mM NO_3^- as a sole N-source for 8 days in order to induce N-luxury consumption. The curves were adjusted by polynomial functions. Data represent the average of three replicates ± SD and asterisks represent significant differences among treatments as indicated by *t*-test ($p < 0.05$).



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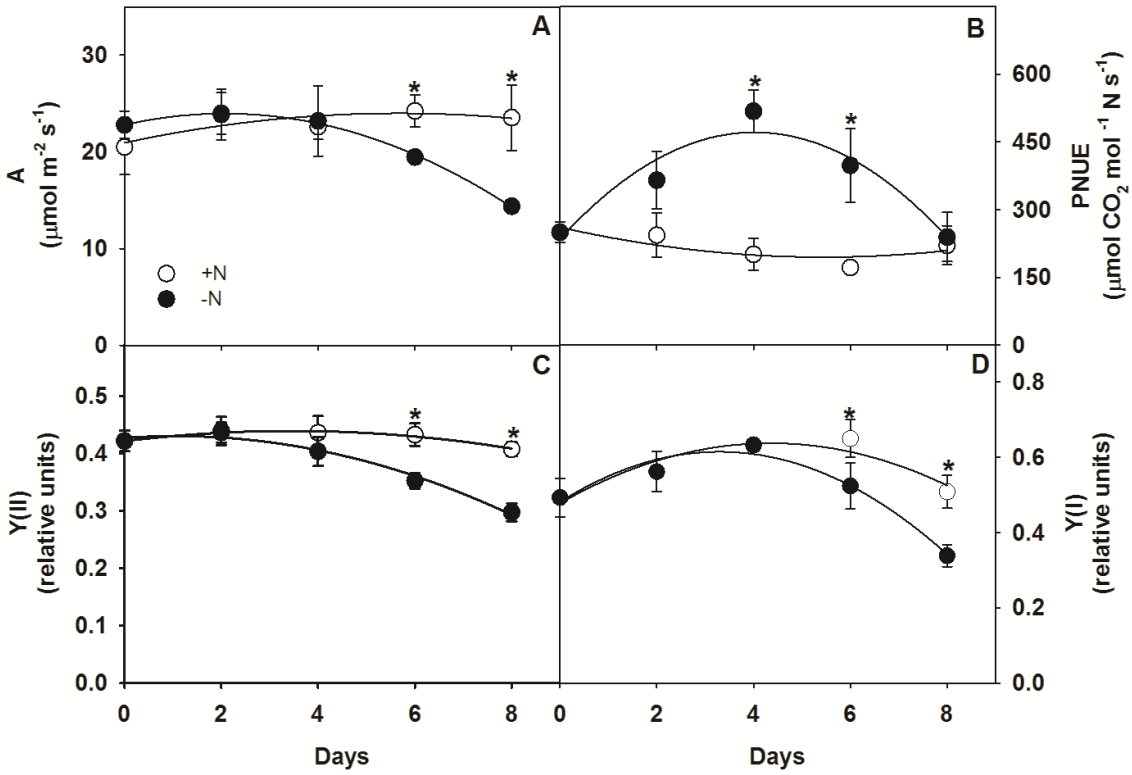
The following phase, from 4 to 8 days of N-deprivation, was characterized by an early N-deficiency period when shoot relative growth was stopped and the contents of all N-forms were kept practically unchanged (Figures 2, 3 and 5). In other words, deprived plants were able to maintain a tight balance between N-availability and growth but preserving the N-total level (represented mainly by total proteins) in leaves. Intriguingly, in contrast to N, during that interval the chlorophyll content significantly decreased despite these plants have not shown any visual signal of chlorosis and senescence in leaves (Figure 24). Indeed, N-deficient early plants visually displayed only a reduction in shoot size as compared to those supplied with high nitrate supplying.

N-deprived plants were able to sustain photosynthesis efficiency until the early deficiency phase

Throughout phase I the N-deprived plants kept its photosynthetic capacity unchangeable as indicated by CO_2 assimilation rates and effective quantum efficiencies of PSII and PSI compared to high nitrate supplied plants (Figure 27). In contrast, the excess N accumulated in leaves of plants supplied with N-luxury consumption was not efficient to induce improvement in photosynthesis, indicating a saturation phase. These results are highlighted by responses displayed by PNUE values (CO_2 assimilation per accumulated N), which were increased in deprived plants but remained practically unchanged in N supplied cotton (Figure 27).

Figure 27 - Net CO_2 assimilation (A), photosynthetic nitrogen use efficiency (B), effective quantum yield of PSII (C) and effective quantum yield of PSI (D) in leaves of cotton plants exposed to nitrate deprivation or supplied with high supply (10 mM NO_3^-) for 8 days in a greenhouse. Plants were previously supplied with 10 mM NO_3^- as a sole N-source for 8 days in order to induce N-luxury consumption. The curves were adjusted by polynomial functions. Data represent the average of three replicates ± SD and asterisks represent significant differences among treatments as indicated by *t*-test ($p < 0.05$).



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The other gas exchange parameters related to photosynthesis, stomatal conductance and transpiration rate did not change by the effect of nitrate supplying over the experimental period (Figure 28). After plants have reached early N-deficiency on the 4th day, they performed distinct responses after that time, which CO_2 assimilation and photochemical activity were significantly decreased in parallel to PNUE (Figure 27). The fluorescence indicators frequently employed to detect photoinhibition and damages on PSII (maximum PSII quantum yield – Fv/Fm, maximum PSII fluorescence – Fm and initial fluorescence – F0) indicated that only F0 slightly decreased at day 8 indicating that PSII integrity was preserved during N-early deficiency (Figure 29).

Figure 28 – Stomatal conductance (A) and transpiration (B) in leaves of cotton plants exposed to nitrate deprivation or supplied with high supply (10 mM NO_3^-) for 8 days in a greenhouse. Plants were previously supplied with 10 mM NO_3^- as a sole N-source for 8 days in order to induce N-luxury consumption. The curves were adjusted by polynomial functions. Data represent the average of three replicates ± SD and asterisks represent significant differences among treatments as indicated by *t*-test ($p < 0.05$).

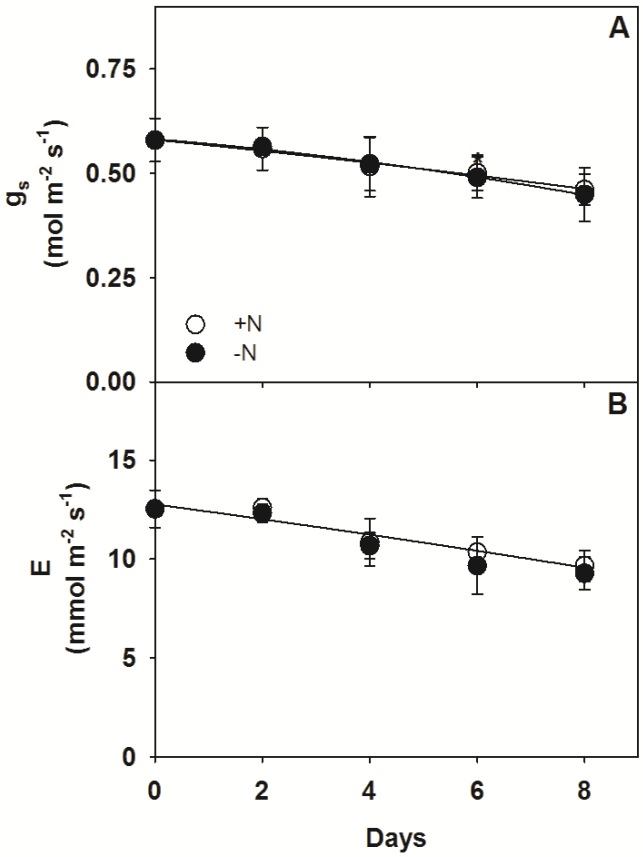

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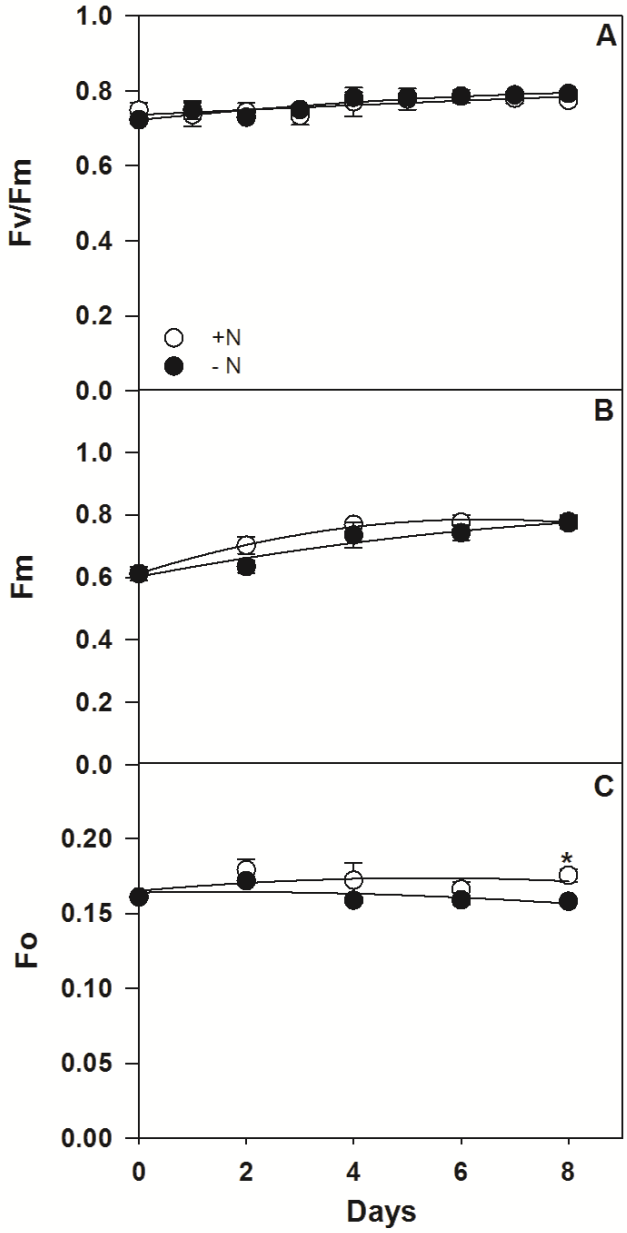
Figure 29 – Changes in maximum quantum yield of PSII (A), maximum (B) and minimum fluorescence (C) in dark-adapted leaves of cotton plants exposed to nitrate deprivation or supplied with high supply (10 mM NO_3^-) for 8 days in a greenhouse. Plants were previously supplied with 10 mM NO_3^- as a sole N-source for 8 days in order to induce N-luxury consumption. The curves were adjusted by polynomial functions. Data represent the average of three replicates ± SD and asterisks represent significant differences among treatments as indicated by *t*-test ($p < 0.05$).



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In order to understand how N-early deficiency could have affected photochemical activity of PSII and PSI, a kinetic study was performed. In comparison to day zero, after 8 days deprived plants presented a fast delay in actual PSII quantum yield but they were able to

instantaneously recovery it after dark exposition (Figure 30). A similar response was observed for PSI kinetics including a significant increase in its donor side limitation [Y(ND)] whereas the acceptor side restriction [Y(NA)] was not affected by N-early deficiency (Figure 31). In parallel, the reduced state of quinones, represented by 1-qP values, was increased in these plants, corroborating the previous results observed in Y(ND) (Figure 31).

Figure 30 - Photochemical kinetics of effective quantum yield of PSII (A, B) and PSI (C, D), measured in cotton leaves before exposure to nitrate deprivation (day zero) and after 8 days of supplying with high nitrogen (10 mM NO_3^-) or N-deprivation. Plants were previously supplied with 10 mM NO_3^- as a sole N-source for 8 days. The actinic light employed for induction kinetics was 1,000 µmol m^{-2} s^{-1}. Photochemical parameters were noted in response to time (20 min), with 10 min of light induction (0–10 min) and 10 min of dark relaxation (10–20 min). Data represent the average from three replicates ± SD.

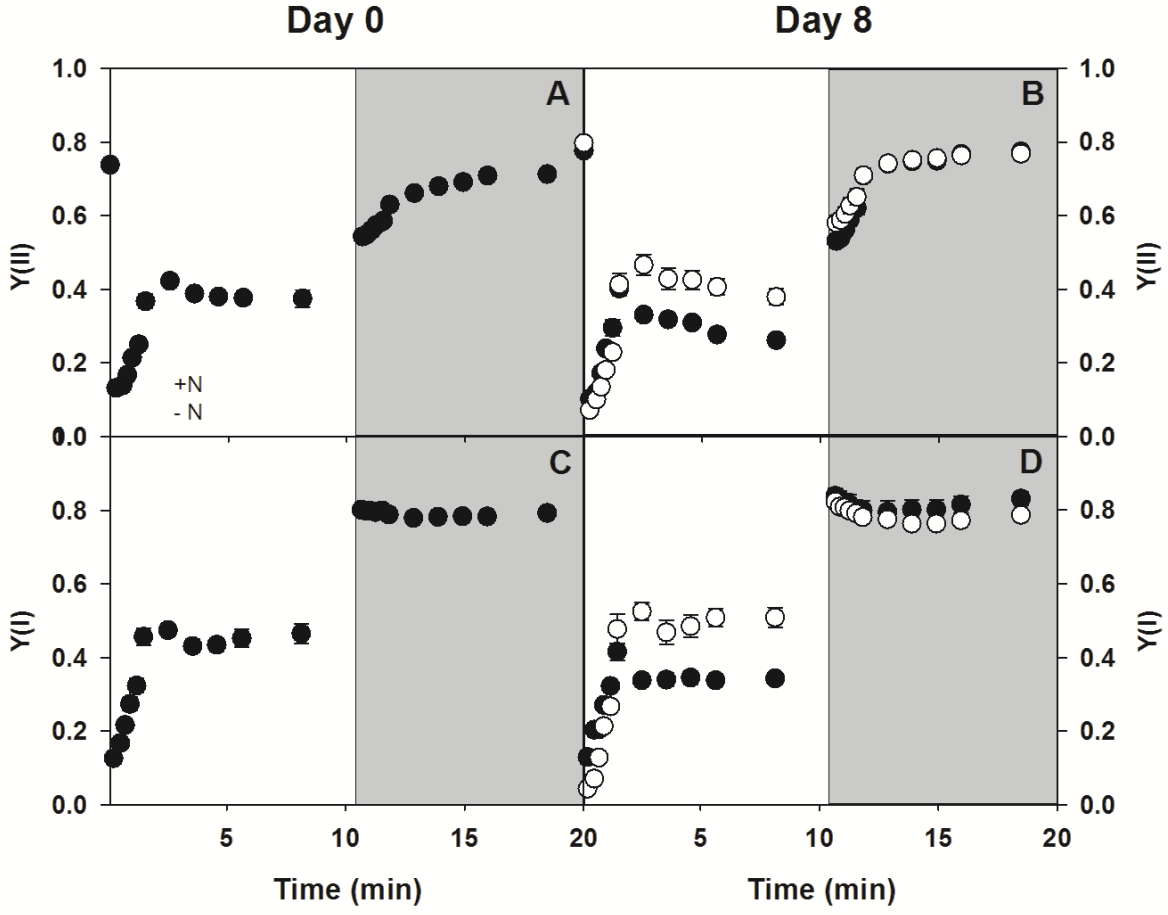

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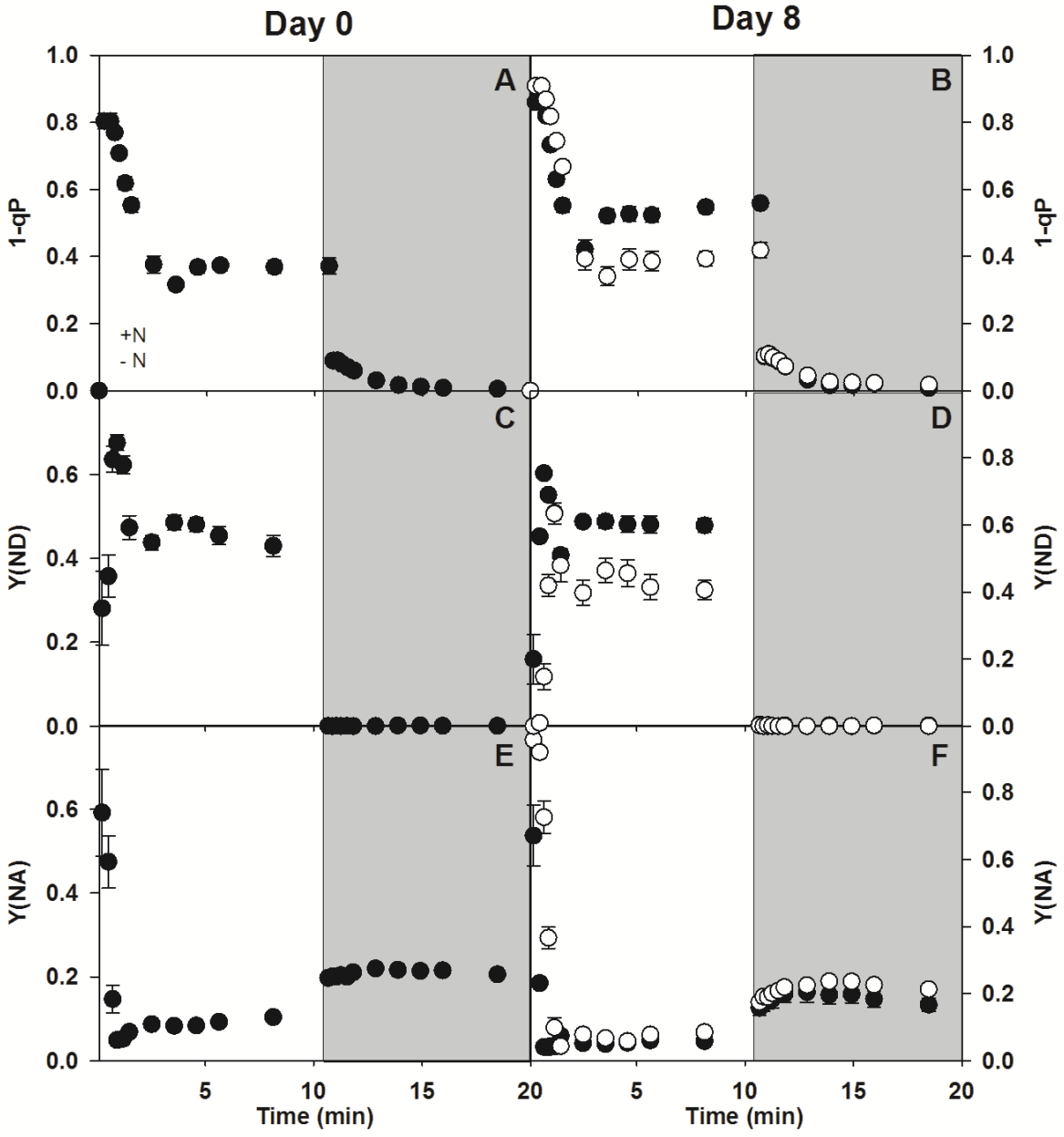
Figure 31 - Photochemical kinetics of quinone pool redox state (A, B), PSI donor side limitation (C, D) and PSI acceptor side limitation (E, F) measured in cotton leaves before exposure to nitrate deprivation (day zero) and after 8 days of supplying with high nitrogen (10 mM NO_3^-) or N-deprivation. Plants were previously supplied with 10 mM NO_3^- as a sole N-source for 8 days. The actinic light employed for induction kinetics was 1,000 µmol m^{-2} s^{-1}. Photochemical parameters were noted in response to time (20 min), with 10 min of light induction (0–10 min) and 10 min of dark relaxation (10–20 min). Data represent the average from three replicates ± SD.



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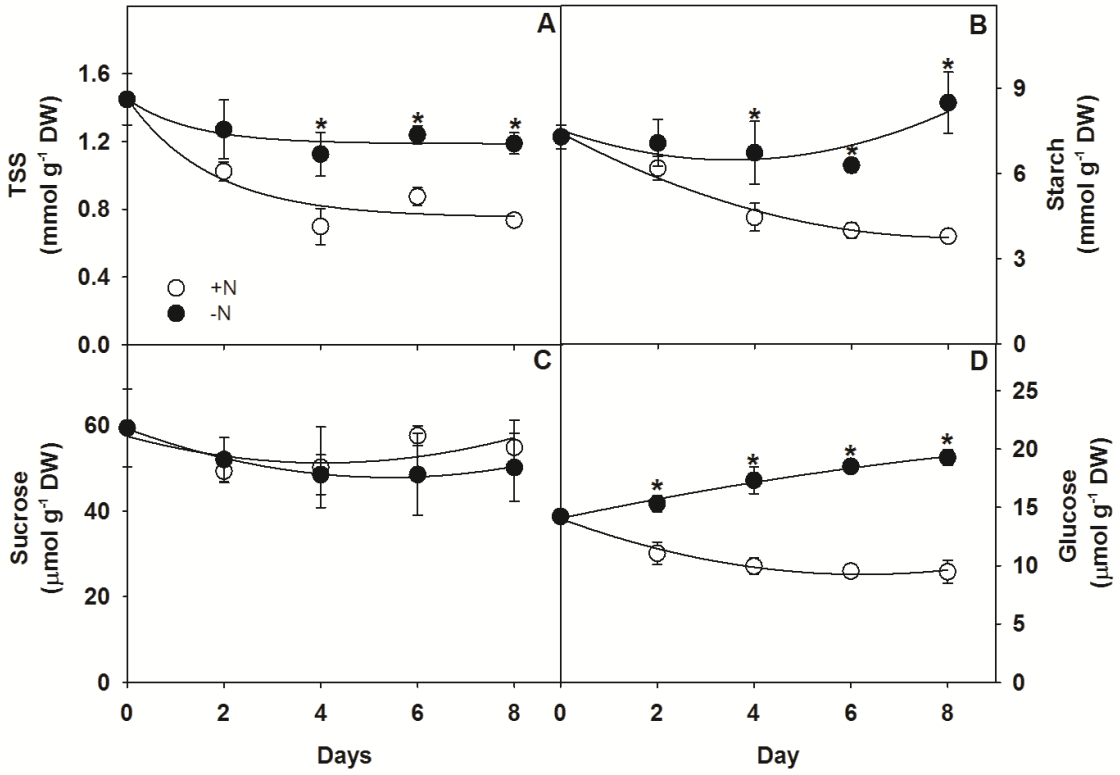
Decrease in leaf N availability induced increases in non-structural carbohydrates but not affected sucrose contents

N-deprivation and early deficiency phases induced similar effects on the levels of non-structural carbohydrates: total soluble sugars (TSS), starch, sucrose and glucose. Except for sucrose, all these C forms were accumulated in leaves whereas plants supplied with high nitrate level presented a trend to decrease starch, TSS and glucose contents throughout time (Figure 32). However, it is possible to evidencing a clear tendency of increase in glucose and starch contents at end of phase II (early N-deficiency), whereas sucrose and TSS were kept unchangeable.

Figure 32 - Total soluble sugars (A), starch (B), sucrose (C) and glucose (D) in leaves of cotton plants exposed to nitrate deprivation or supplied with high supply (10 mM NO_3^-) for 8 days in a greenhouse. Plants were previously supplied with 10 mM NO_3^- as a sole N-source for 8 days in order to induce N-luxury consumption. The curves were adjusted by polynomial functions. Data represent the average of three replicates ± SD and asterisks represent significant differences among treatments as indicated by *t*-test ($p < 0.05$).



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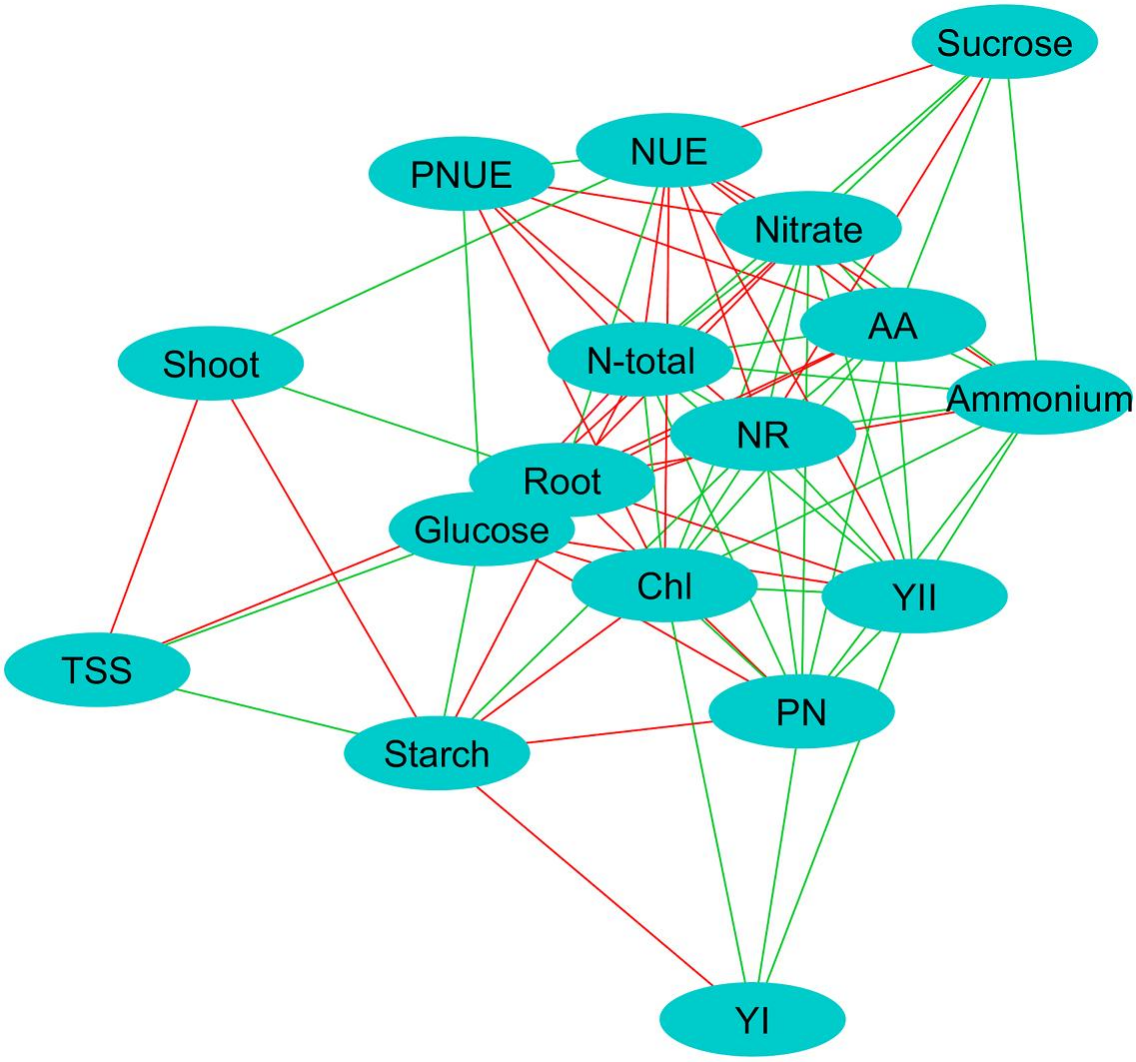
Correlation network analysis of physiological parameters suggested that NR activity and total-N displayed a central role in N-deprivation responses

In order to highlight and integrate the most important parameters obtained in this study, a network was arbitrarily assembled based on Person's coefficients at r >0.5 for positive correlations and r <-0.5 for negative ones (Figure 33). The network analysis revealed that the parameters NR, N-total and chlorophyll displayed a central position (hubs), whereas YI, TSS, shoot growth and sucrose occupied a more peripheral position in the network (Figure 33). These results corroborate that NR displayed a central role in cotton responses to N-deprivation and the results highlight the possible importance of chlorophyll as an N storage molecule.

Figure 33 – Correlation network of physiological parameters measured in cotton plants exposed to nitrate deprivation or supplied with high nitrate (10 mM NO_3^-) during 8 days in a greenhouse. Red lines represent negative correlation with r < - 0.5 and green lines represent correlation with r > 0.5 according to Pearson´s correlation coefficient (r).



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Discussion

In this study were demonstrated new insights into nitrogen use efficiency (NUE) related to photosynthesis and N metabolism in an early phase of an acute N-deficiency in cotton plants previously grown in a luxury N-condition. This issue is important for plant nutrition, physiology and agronomical management of nitrogenous fertilizers since the metabolic mechanisms underlying acclimation to low nitrate availability are scarcely known

(GUILHERME *et al.*, 2019; HIKOSAKA *et al.*, 1994; JIN *et al.*, 2015; SUN *et al.*, 2016; VICENTE *et al.*, 2017). Previously, cotton plants were able to accumulate very large amounts of nitrogenous reserves in leaves, especially in form of proteins (total-N), NO_3^-, free amino acids (AA) and chlorophylls, as indicated by the high contents reached after eight days of continuous supply with high nitrate. Afterwards, following nitrate deprivation, cotton leaves are able to display intense N-remobilization as shown by accentuated decreases in contents of all N-forms, which contributed to support high growth rates of root and shoot, even in the absence of *de novo* N input from root medium.

It is meaningful to highlight the role displayed by NR activity to mobilize great amount of stored nitrate in leaves. Despite that enzymatic activity has progressively decreased during N deprivation, probably as a consequence of NO_3^- flux absence from roots once this is a crucial signal involved in the up-regulation of NR expression, synthesis, and activity (SHANER and BOYER, 1976). In opposition, NO_3^- deficiency in the cytosol is widely known as able to trigger specific proteases lending to NR degradation and consequently decreasing its activity (REED *et al.*, 1980; TORNKVIST *et al.*, 2019). Our data clearly shows that, even in these adverse conditions, NR was able to reduce large amount of NO_3^- as revealed by a prominent increase in the NR/accumulated-NO_3^- ratios. This response highlights the importance and efficiency of that enzyme to remobilize stored nitrate in deprivation conditions, highly contributing to the observed increases in NUE.

Due to its importance for nitrate remobilization and related processes, NR jointly with N content, have occupied a central place (main hubs) in the correlation network assembled from the obtained data in this study (Figure 33). However, it is also important to remark that the prominence of NR was important during all N-deprivation phase, including when the concentrations of this nutrient reached very low levels in leaf tissues. In parallel, the remobilization of N-forms stored in other important biomolecules in that phase, such as proteins, AA and chlorophylls are also important to NUE enhancement (LI *et al.*, 2013; WALKER *et al.*, 2018). The low levels of free ammonia and AA accumulated in leaves during early deficiency phase reveal this high efficiency of remobilization. Indeed, a low accumulation of these compounds is a strong indicator of efficient utilization for protein *de novo* synthesis (PONTE *et al.*, 2014; TORNKVIST *et al.*, 2018), which might contribute to sustaining growth and other physiological processes. Inversely, accumulation of free ammonium and amino acids might indicate disturbances in N metabolism as commonly reported during chlorosis and senescence (DA ROCHA *et al.*, 2012; SILVEIRA *et al.*, 2001).

Another remarkable insight revealed in this study is that even after reaching a N-early deficiency phase, as attested by the threshold level reached by total-N (2.5%) in leaves (READ *et al.*, 2006), cotton plants were able to maintain an N-homeostasis enough to sustain root growth. In parallel, these plants also were able to avoid generalized disturbances in leaves, such as chlorosis and senescence, which are common symptoms of acute nitrogen deficiency in plants (MEI and THIMANN, 1984). Indeed, N is a crucial nutrient for synthesis of proteins and other important molecules including nucleic acids, chlorophylls, and phytohormones. Consequently, nitrate assimilation is also an essential pathway to photosynthesis and, therefore, plant growth (BUSCH *et al.*, 2018; GUILHERME *et al.*, 2019; HUANG *et al.*, 2016; MIYAKE *et al.*, 2005).

In this study chlorophylls play an interesting role, acting initially as a molecule capable to storage high N amount during the luxury consumption phase and subsequently remobilizing it after nitrate deprivation and early deficiency. This supposition is corroborated by the distinct pattern exhibited by chlorophyll decrease during N-deprivation, as compared to photosynthetic and N-metabolism parameters studied. Indeed, despite the content of these molecules have been intense and linearly decreased throughout of these phases, the parameters associated with chlorophyll *a* fluorescence strongly suggest that these pigments were kept in suitable levels during early deficiency (up to 6 days). In fact, this storage role attributed to chlorophylls has already been reported previously for other plant species, but not in the specific physiological circumstances as the reported here (KANT, 2018; LI *et al.*, 2013; WALKER *et al.*, 2018).

After the 6^{th} day of N deprivation, CO_2 assimilation and photochemical activities of PSII and PSI have declined in parallel to an increase in the reduced quinone pool and restriction in the donor side of PSI. This phase coincides with existence of very low NO_3^- levels in leaves and reduced NR activity, probably associated with low amino acids supplying for protein synthesis and low photosynthetic electron consumption by these anabolic pathways (NOCTOR and FOYER, 1998). This circumstance should inevitably advance to long-lived reduced state of quinones pool, generating excess energy in PSII and, consequently, photoinhibition (FOYER *et al.*, 2017). However, until the 8^{th} day from deprivation, the PSII and PSI integrity were maintained, as was shown by Fv/Fm and the instantaneous recover of PSII and PSI activity in dark after illumination period (Figures 9 and 10).

The levels of non-structural carbohydrates starch, total soluble sugars and glucose were accumulated throughout phases of nitrate deprivation and N-early deficiency and similar results have been reported (JIN *et al.*, 2015). In contrast, the sucrose contents in leaves remained similar to those found in plants supplied with excess N, suggesting that sucrose could have been transported to maintain root growth and N-remobilization energetic costs during nitrate deprivation, when photosynthesis remained unchangeable. After reach the early deficiency phase photosynthesis decreased and this response could explain a non-accumulation of sucrose.

In this study, the obtained data reveal that physiological mechanisms underlying N-deficiency are far more complexes than those frequently reported in literature. Indeed, evidences attained here corroborates the idea that plants are able to storage and redistribute efficiently excess N subsequent to a luxury consumption, as commonly might occur after N fertilization in field conditions (KANT, 2018). In this context, nitrate assimilation pathway should display a central role, as it is strongly connected to crucial processes such as photosynthesis and chlorophyll synthesis, ultimately maximizing nitrogen use efficiency (TEGEDER and MASCLAUX-DAUBRESSE, 2018). This investigation highlights also that all physiological responses reported by nitrate deprived-plants evidence that metabolical changes towards acute N-deficiency are, in general, similar to those presented when plants facing other abiotic stress stimulus.

Initially, plants are able to trigger plasticity to optimize acclimation responses to an N-deprivation condition, involving high N remobilization and increased NUE, maintaining its homeostasis and sustaining an adequate growth, at least during a limited time of deficit exposure, in this specific case by approximately 6 days. Afterwards, plants reallocate resources in order to preserve only its essential processes to survive and mitigate nutrient deficiency, for instance maintaining root growth in detriment of shoot (morpho-physiological acclimation). These processes are widely known as dependent on hormonal and genetic control (SUN *et al.*, 2018) and the ability to maintain this homeostasis depends on genetic and environmental factors, including phenotypic plasticity (GRATANI, 2014).

The aggravation of N-deficiency induces several metabolical disturbances since intense and compromising chlorophyll degradation (chlorosis) in parallel to leaf senescence (MEI and THIMANN, 1984). These processes are strongly regulated by cell metabolism and its interaction with the environment, involving several cellular signals, transduction networks and the expression of several genes (HÖRTENSTEINER and KRÄUTLER, 2011; WALKER

et al., 2018). Perhaps, philosophically or anthropomorphically, could be assumed here that, as plants are organisms highly adaptable to diverse environmental pressures, a late survival mechanism to cope with acute N-deficiency could be resumed in the following events: remobilization of luxury storages followed by strategic use of resources to guarantee survival.

Thus, we performed here a descriptive-comprehensive study involving classic physiological mechanisms underlying luxury consumption, nitrate deprivation, followed by an early N-deficiency, employing young cotton plants as a model. Such finds are important to understand the entire process involved in N deficiency, which represents important problem in an agronomical context (CARPENTER *et al.*, 2014; HSIEH *et al.*, 2018). As a future perspective, these finds could contribute to understand metabolic disorders related to N nutrition and some underlying mechanisms related to nitrogen use efficiency. The utilization of other current approaches such as molecular biology, mutant plants and a set of omics tools are crucial to improve the knowledge on this important issue.

Conclusion

In conclusion, this study provides new insights into dynamics of photosynthesis and nitrate assimilation throughout early phases of nitrate deprivation and onset of N-deficiency after luxury consumption in cotton plants. When nitrate well supplied plants undergoes N deprivation they maintain shoot growth and photosynthetic activity until the beginning of N deficiency when shoot growth is paralyzed and the photosynthetic activity tend to progressively decrease. However, even under early N deficiency, plants maintain the photosystems integrity and the root growth. All these processes aiming to face N deprivation are highly related to high NUE associated with large remobilization of stored N and high NR activity.

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6 FINAL CONSIDERATIONS

This thesis studied important issues related to integration between nitrogen assimilation, photorespiration and photosynthesis under excess energy stress and the NUE along distinct N-nutritional phases. One of the key issues addressed here was: how does high nitrate supply improve photosynthesis even in a photoinhibition condition? How do plants manage N during changes in the N-nutritional phases in order to preserve their photosynthetic activity as well as other processes? The main highlights of this work (including the results in APPENDIX A) were: a) High nitrate assimilation rate is more important to improve photosynthesis under high light then the N-status; b) Under high light exposure for long term high nitrate supply (HN) is not enough to avoid photoinhibition of PSII. However, HN plants show greater electron transport and CO_2 assimilation than plants under N-deprivation; c) In this condition the photosynthesis improvement promoted by HN was highly related to increase in nitrate assimilation and photorespiration; d) Under excess light high during short term in a non photoinhibition condition nitrate assimilation is related to improvement in ETRII and CO_2 assimilation while photorespiration is related only to ETRII improvement (Figure 36 and 37) and e) When plants start to suffer N-deprivation and deficiency they show high NUE related mainly to N remobilization and NR activity. In these conditions the plants maintain the photosystems integrity and root growth.

In conclusion, under excess light the high nitrate assimilation rates have impact on photosynthesis protection more than the N status. High nitrate supply stimulates nitrate assimilation that induces photorespiration and this two last processes act synergistically, favoring the photosynthetic efficiency mainly by improving PSII efficiency and CO_2 assimilation under excess energy. However, in a non-photoinhibitory condition the photorespiration responds by improving only the PSII activity under high light. On the other hand, when nitrate well supplied plants face N deprivation in a non-photoinhibitory condition they maintain photosynthetic activity and shoot growth until the beginning of N deficiency, when shoot growth is paralyzed and photosynthesis tend to decrease. However, even under early N deficiency, plants maintain the photosystems integrity and root growth. All these processes aiming to face N deprivation are highly related to high NUE associated with large remobilization of stored N and high NR activity.

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APPENDIX A - OTHER RESULTS OBTAINED DURING DOCTORAL COURSE

Table 4 – Physiological and N-nutritional characterization in 22-day-old cotton plants grown in presence of 1 mM NO_3^- and 10 mM NO_3^- and exposed to high light (2,000 µmol^{-2} s^{-1}) for 8 hours. Data represent averages obtained from three replicates ± SD and different letters represent significant differences among treatments by *t*-test ($p<0.05$).

Variables	1 mM	10 mM
A (µmol m^{-2} s^{-1})	17.5±0.2A	17.4±1.0A
NR activity (µmol NO_2^- h^{-1} g^{-1} FW)	2.33±0.79A	2.29±0.68A
Metabolic nitrate (µmol g^{-1} FW)	0.28±0.04B	1.95±0.3A
Nitrate content (µmol g^{-1} DW)	88.6±9.3B	162.8±11.6A
N-α amino soluble (µmol g^{-1} DW)	136.4±6.9A	161.4±26.0A
Total N content (mmol g^{-1} DW)	2.41±0.031A	2.40±0.15A
Ammonium content (µmol g^{-1} DW)	38.9±3.8A	38.7±5A
Shoot dry mass (g)	1.32±0.15A	1.45±0.35A
Root dry mass (g)	0.24±0.03A	0.28±0.07A
Total dry mass (g)	1.56±0.15A	1.73±0.38A

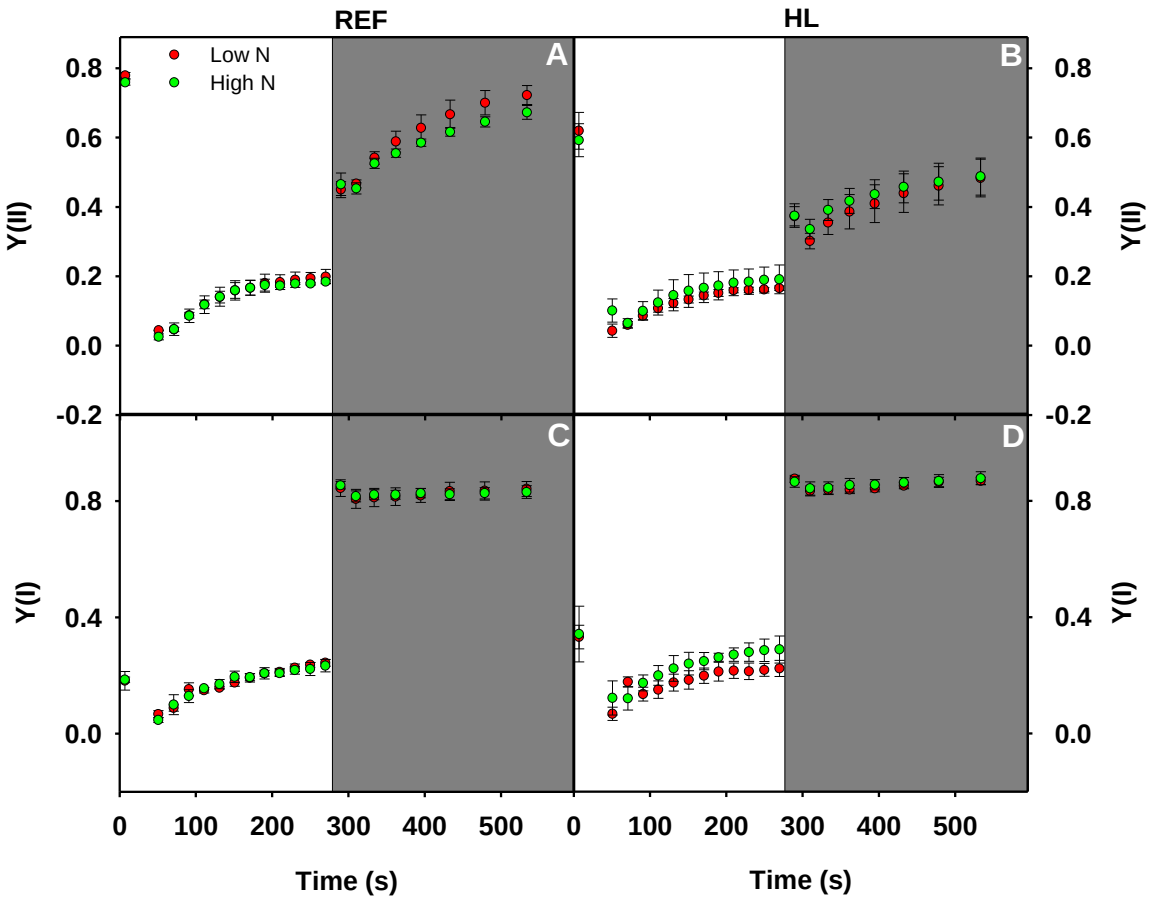
Source: elaborated by the author.

Table 5 - Physiological and metabolic parameters in cotton leaves subjected to NO_3^- deprivation (ND) or supplied with high nitrate (10 mM NO_3^- -HN) followed by 8 hours of exposure to reference light (200 µmol^{-2} s^{-1} -REF) and high light (2000 µmol^{-2} s^{-1} -HL). Data represents averages from four replicates ±SD and different letters represent significant differences among treatments by Tukey's test ($p<0.05$).

Variables	REF		HL	
	ND	HN	ND	HN
Stomatal Limitation (%)	14.26±2.06ab	18.69±2.97a	10.65±0.64b	5.94±0.53c
Metabolic Limitation (%)	18.25±1.75a	–	15.51±3.09a	–
gs (mol m^{-2} s^{-1})	0.28±0.031a	0.14±0.008a	0.26±0.006a	0.238±0.02a
E (mmol m^{-2} s^{-1})	4.87±0.83b	4.00±0.37b	5.42±0.53a	5.10±0.59a
Ci (ppm)	266.8±32.6a	218.6±29.4a	294.6±31.6a	246.7±10.8a
Total soluble sugars (µmol g^{-1} DW)	617.8±42.5b	431.4±18.6c	756.28±23.9a	611.9±35.4b
Glucose (µmol g^{-1} FW)	15.6±0.9c	8.7±0.2d	28.9±2.3a	23.1±1.3b
Fructose (µmol g^{-1} FW)	9.9±0.13b	8.4±1.0b	11.5±0.5a	11.6±0.4a
Sucrose (µmol g^{-1} FW)	9.8±1.3	11.1±2.2	19.6±1.5	23.5±0.61
NPQ	0.17±0.06b	0.09±0.02b	0.8±0.11a	0.79±0.04a
1 - qP	0.10±0.02	0.08±0.01	0.71±0.03	0.66±0.05

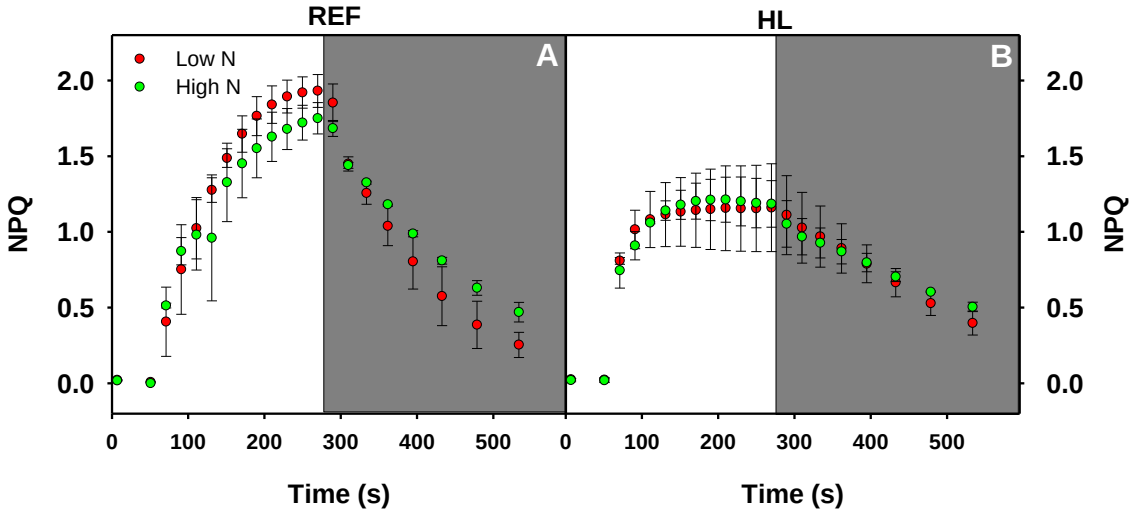
Source: elaborated by the author.

Figure 34 - Induction and relaxation kinetics of effective quantum yield of PSII (A and B) and effective quantum yield of PSI (C and D) in cotton leaves subjected to NO_3^- deprivation (ND) or supplied with high nitrate (10 mM NO_3^- -HN) followed by 8 hours of exposure to reference light (200 µmol^{-2} s^{-1} -REF) and high light (2000 µmol^{-2} s^{-1} -HL). The white side represents the induction period with light (1500 µmol^{-2} s^{-1}) and the gray side represents the relaxation period (dark).



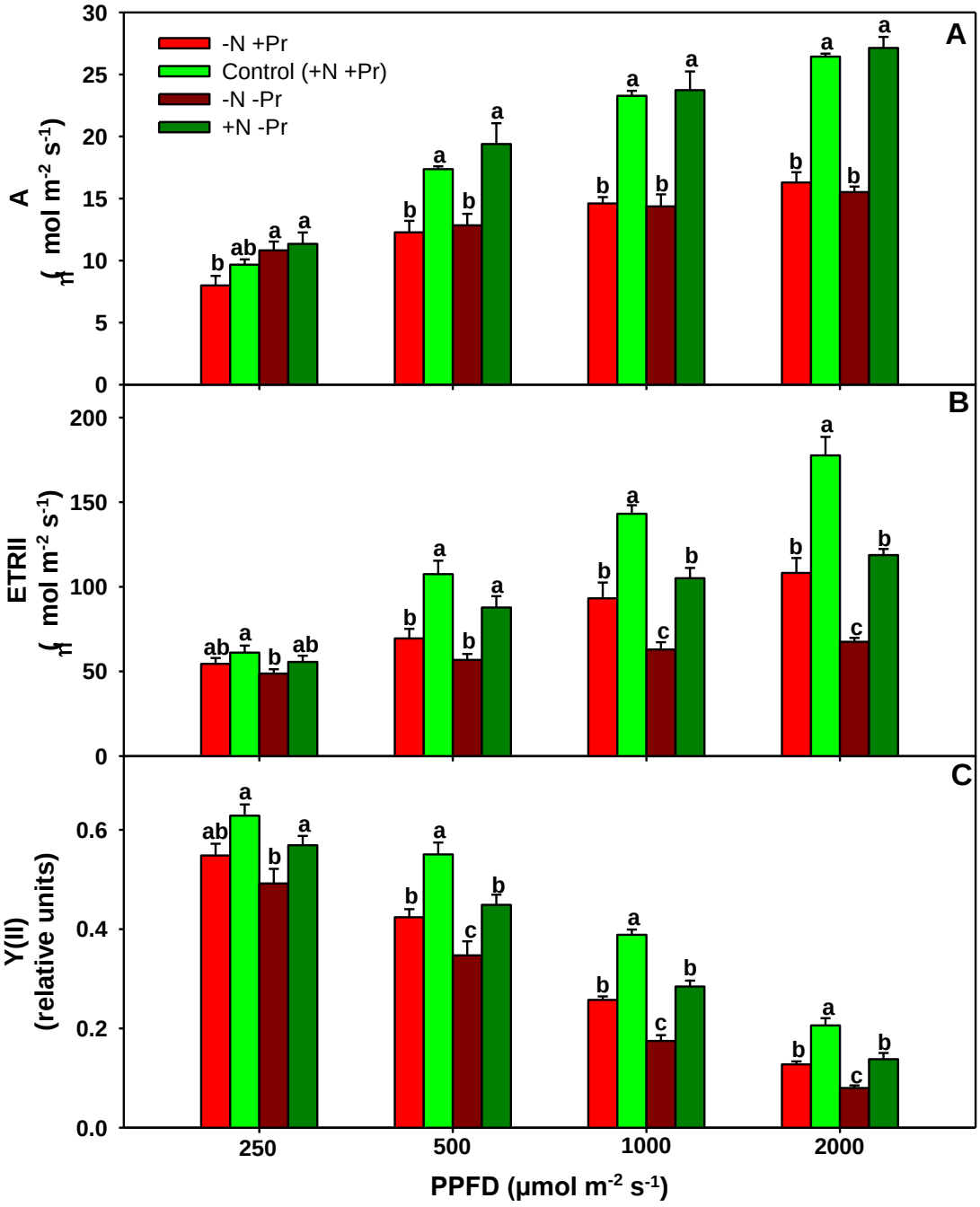
Source: elaborated by the author.

Figure 35 - Induction and relaxation kinetics of non-photochemical quenching (NPQ) in cotton leaves subjected to NO_3^- deprivation (ND) or supplied with high nitrate (10 mM NO_3^- - HN) followed by 8 hours of exposure to reference light (200 µmol^{-2} s^{-1} -REF) (A) and high light (2000 µmol^{-2} s^{-1} -HL) (B). The white side represents the induction period with light (1500 µmol^{-2} s^{-1}) and the gray side represents the relaxation period (dark).



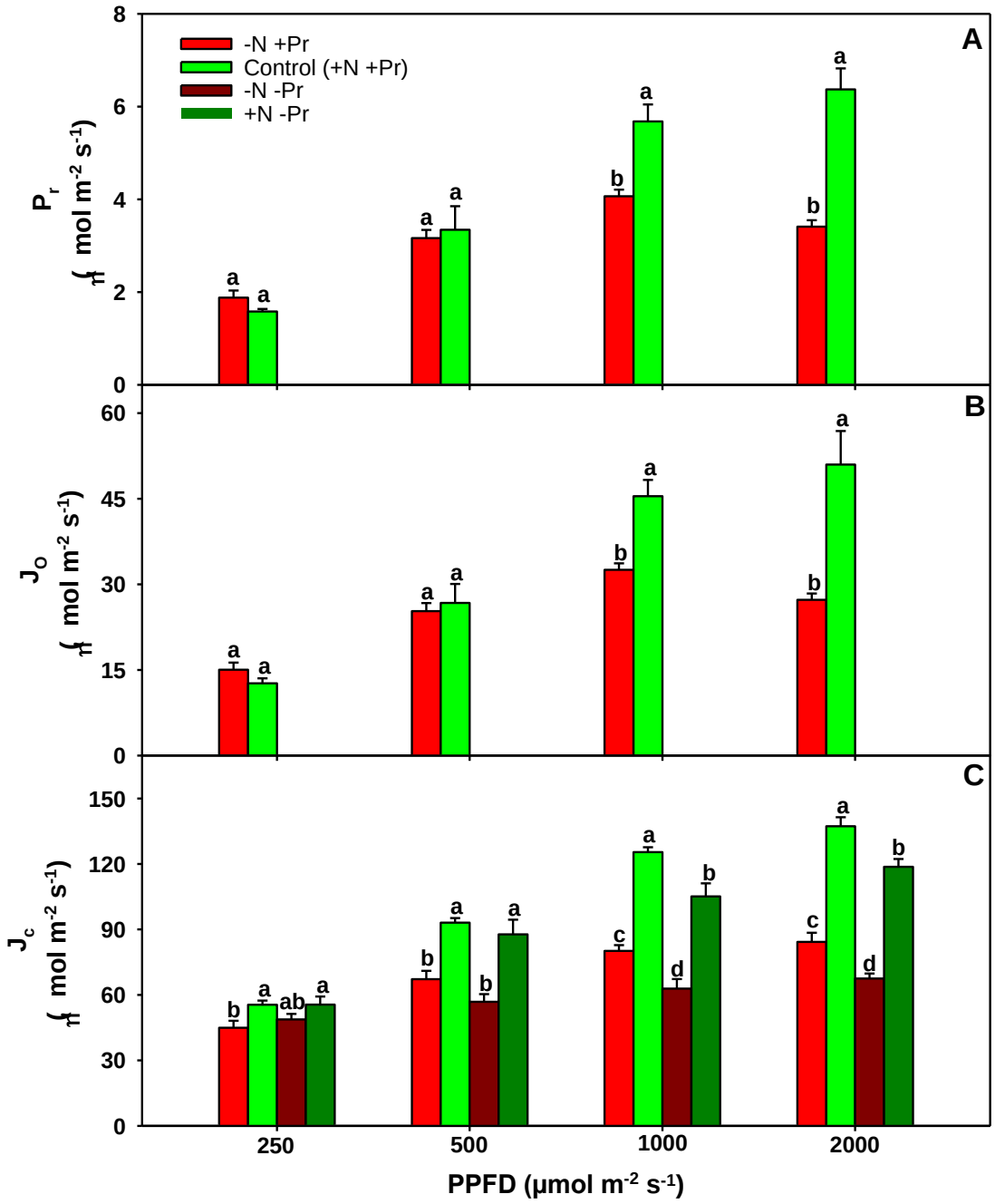
Source: elaborated by the author.

Figure 36 - Performance of CO_2 assimilation (A), electrons transporte rate of PSII (B) and quantum yield of PSII (C) under different light intensities in cotton leaves subjected to NO_3^- deprivation (-N) or high nitrate (10 mM NO_3^- - +N) and ambient O_2 to stimulate photorespiration (+Pr) or low O_2 to inhibit photorespiration (-Pr). The treatments with light intensity and O_2 concentrations were done in the IRGA chamber. For the treatment -Pr was used application of N_2. Data represents averages from three replicates ±SD and different letters represent significant differences among treatments by Tukey's test ($p<0.05$).



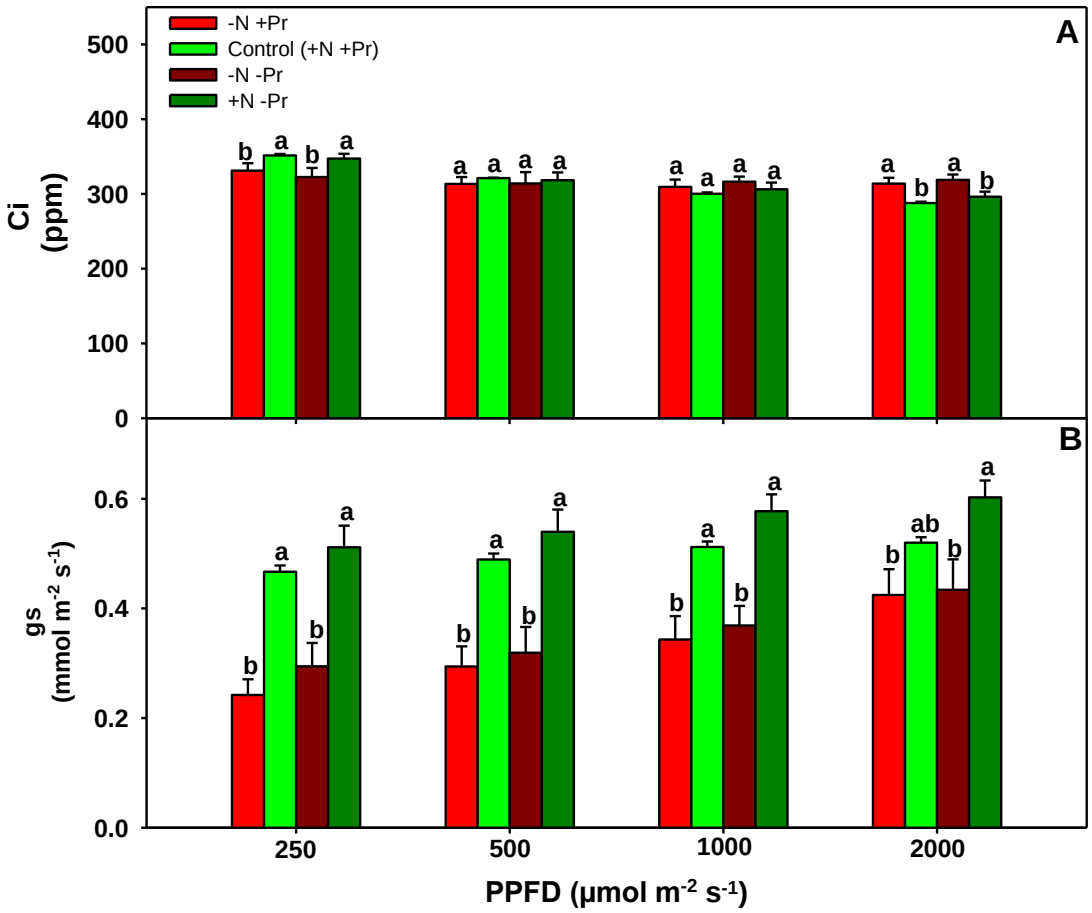
Source: elaborated by the author.

Figure 37 - Performance of photorespiration rate (A), electrons transport rate to Rubisco oxygenation (B) and electrons transport rate to Rubisco carboxylation (C) under different light intensities in cotton leaves subjected to NO_3^- deprivation (-N) or high nitrate (10 mM NO_3^- - +N) and ambient O_2 to stimulate photorespiration (+Pr) or low O_2 to inhibit photorespiration (-Pr). The treatments with light intensity and O_2 concentrations were done in the IRGA chamber. For the treatment -Pr was used application of N_2. Data represents averages from three replicates ±SD and different letters represent significant differences among treatments by Tukey's test ($p<0.05$).



Source: elaborated by the author.

Figure 38 – Changes in leaf internal CO_2 (A) and stomatal conductance (B) under different light intensities in cotton leaves subjected to NO_3^- deprivation (-N) or high nitrate (10 mM NO_3^- - +N) and ambient O_2 to stimulate photorespiration (+Pr) or low O_2 to inhibit photorespiration (-Pr). The treatments with light intensity and O_2 concentrations were done in the IRGA chamber. For the treatment -Pr was used application of N_2. Data represents averages from three replicates ±SD and different letters represent significant differences among treatments by Tukey's test ($p<0.05$).



Source: elaborated by the author.

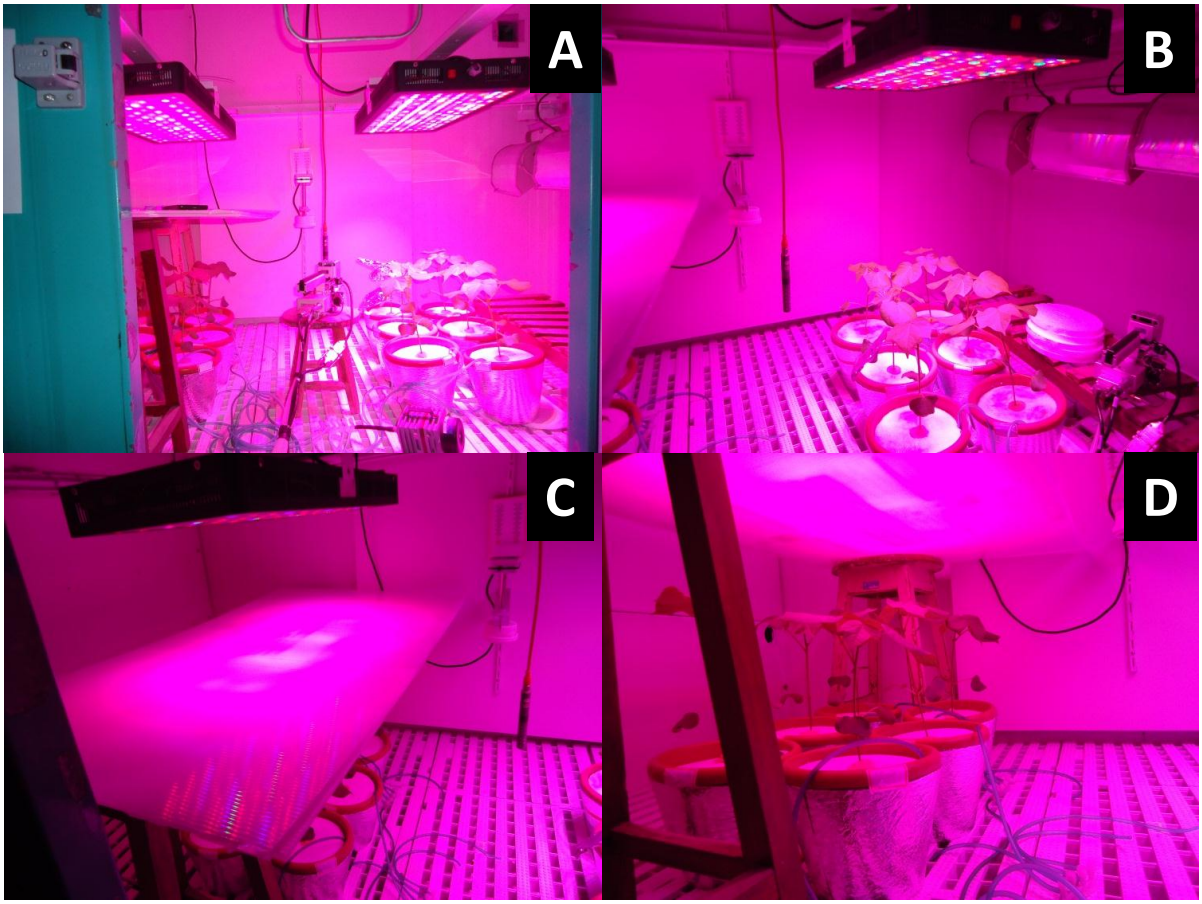
Table 6 - Original data of average and standard deviation (SD) of CO_2 assimilation (A), electrons transporte rate of PSII (ETRII), stomatal conductance (gs), leaf internal CO_2 (Ci), quantum yield of PSII (Y(II)), photorespiration rate (Pr), electrons transport rate to Rubisco carboxylation (Jc) and electrons transport rate to Rubisco oxygenation (Jo) under different light intensities in cotton leaves subjected to NO_3^- deprivation (-N) or high nitrate (10 mM NO_3^- - +N) and ambient O_2 to stimulate photorespiration (+Pr) or low O_2 to inhibit photorespiration (-Pr). The treatments with light intensity and O_2 concentrations were done in the IRGA chamber. For the treatment -Pr was used application of N_2. Data represents averages from three replicates.

Treat	PAR	A	SD	ETRII	SD	gs	SD	Ci	SD	Y(II)	SD	Pr	SD	Jc	SD	Jo	SD
-N +Pr	250	8.0	0.78	54.5	3.43	0.2	0.03	331.2	9.93	0.5	0.02	1.9	0.15	45.0	3.19	15.0	1.24
	500	12.3	0.92	69.5	5.71	0.3	0.04	313.4	9.22	0.4	0.02	3.2	0.18	67.2	3.84	25.3	1.41
	1000	14.6	0.51	93.2	9.38	0.3	0.04	309.4	9.86	0.3	0.01	4.1	0.14	80.2	2.62	32.5	1.13
	2000	16.3	0.83	108.2	8.82	0.4	0.05	313.7	7.83	0.1	0.01	3.4	0.14	84.3	4.20	27.3	1.10
Control +N +Pr	250	9.7	0.43	61.1	4.12	0.5	0.01	351.6	1.91	0.6	0.02	1.6	0.06	55.5	1.93	12.6	0.88
	500	17.4	0.24	107.5	7.90	0.5	0.01	321.3	0.45	0.6	0.02	3.3	0.51	93.1	2.11	26.7	3.33
	1000	23.3	0.41	143.2	4.95	0.5	0.01	300.1	1.91	0.4	0.01	5.7	0.36	125.5	2.21	45.4	2.85
	2000	26.4	0.25	177.6	10.96	0.5	0.01	287.8	1.92	0.2	0.01	6.4	0.45	137.3	4.10	51.0	5.89
-N -Pr	250	10.8	0.72	48.8	2.56	0.3	0.04	322.5	12.23	0.5	0.03	0.0	0.00	48.8	2.56	0.0	0.00
	500	12.8	0.93	56.8	3.51	0.3	0.05	314.1	15.03	0.3	0.03	0.0	0.00	56.8	3.51	0.0	0.00
	1000	14.4	0.98	62.9	4.36	0.4	0.04	316.3	6.93	0.2	0.01	0.0	0.00	62.9	4.36	0.0	0.00
	2000	15.5	0.45	67.5	2.30	0.4	0.06	318.8	7.13	0.1	0.01	0.0	0.00	67.5	2.30	0.0	0.00
+N -Pr	250	11.3	0.92	55.6	3.68	0.5	0.04	347.3	6.46	0.6	0.02	0.0	0.00	55.6	3.68	0.0	0.00
	500	19.4	1.68	87.7	6.75	0.5	0.04	318.5	10.23	0.4	0.02	0.0	0.00	87.7	6.75	0.0	0.00
	1000	23.7	1.50	105.1	6.02	0.6	0.03	306.3	9.02	0.3	0.01	0.0	0.00	105.1	6.02	0.0	0.00
	2000	27.1	0.89	118.7	3.57	0.6	0.03	296.3	6.60	0.1	0.01	0.0	0.00	118.7	3.57	0.0	0.00

Source: elaborated by the author.

APPENDIX B - ORIGINAL PHOTOS OF EXPERIMENTS

Figure 39 - Photos showing how the low (A - left) and high light (A - right and B) treatments were conducted inside the growth chamber. To achieve low light a semi-transparent polycarbonate plate was used above the plants to filter the light (C and D).

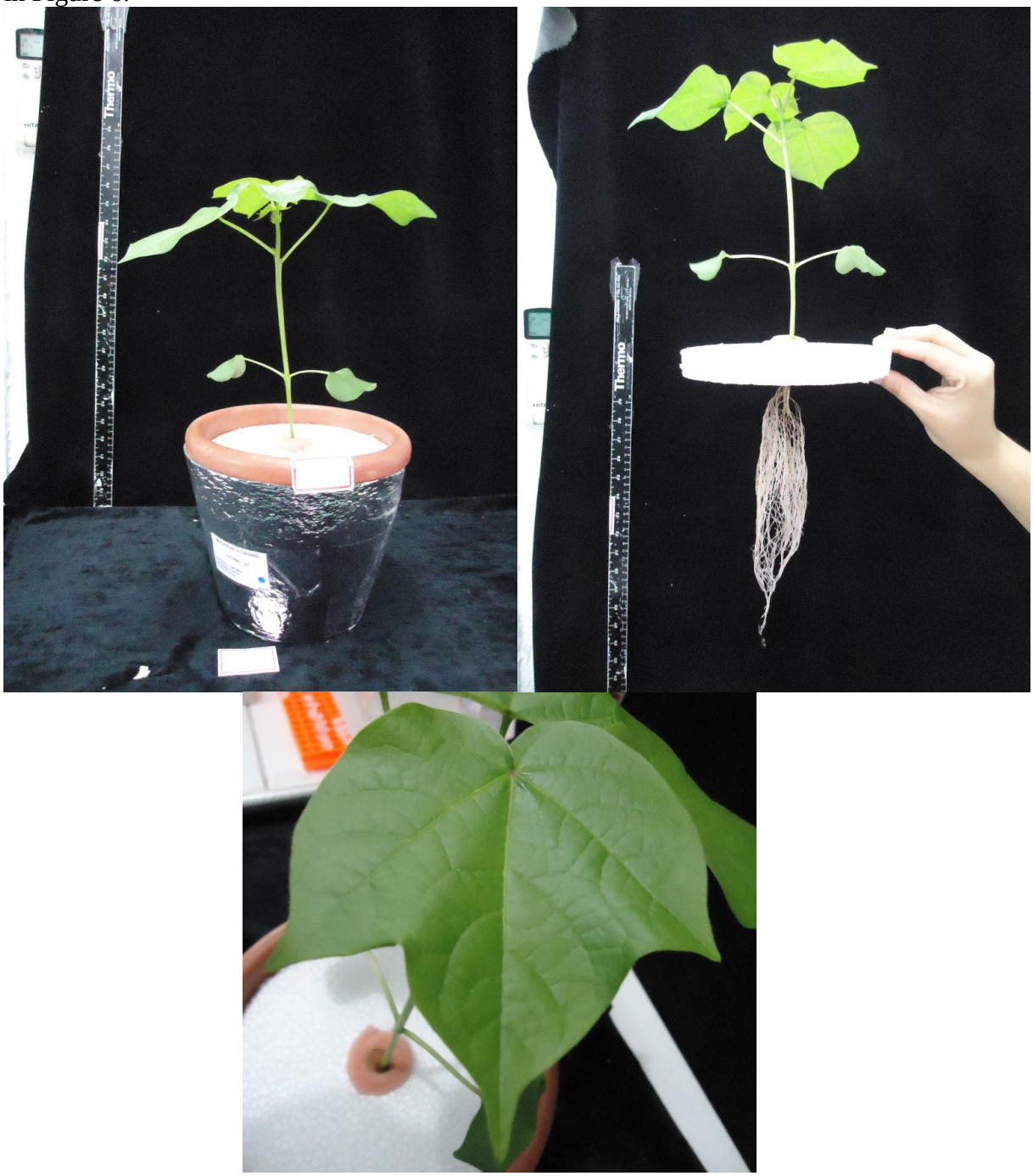


Source: elaborated by the author.

Figure 40 - Cotton plants subjected to NO_3^- deprivation (ND) according to the treatments described in Figure 6.

Source: elaborated by the author.

Figure 41 - Cotton plants subjected to high nitrate (HN) according to the treatments described in Figure 6.

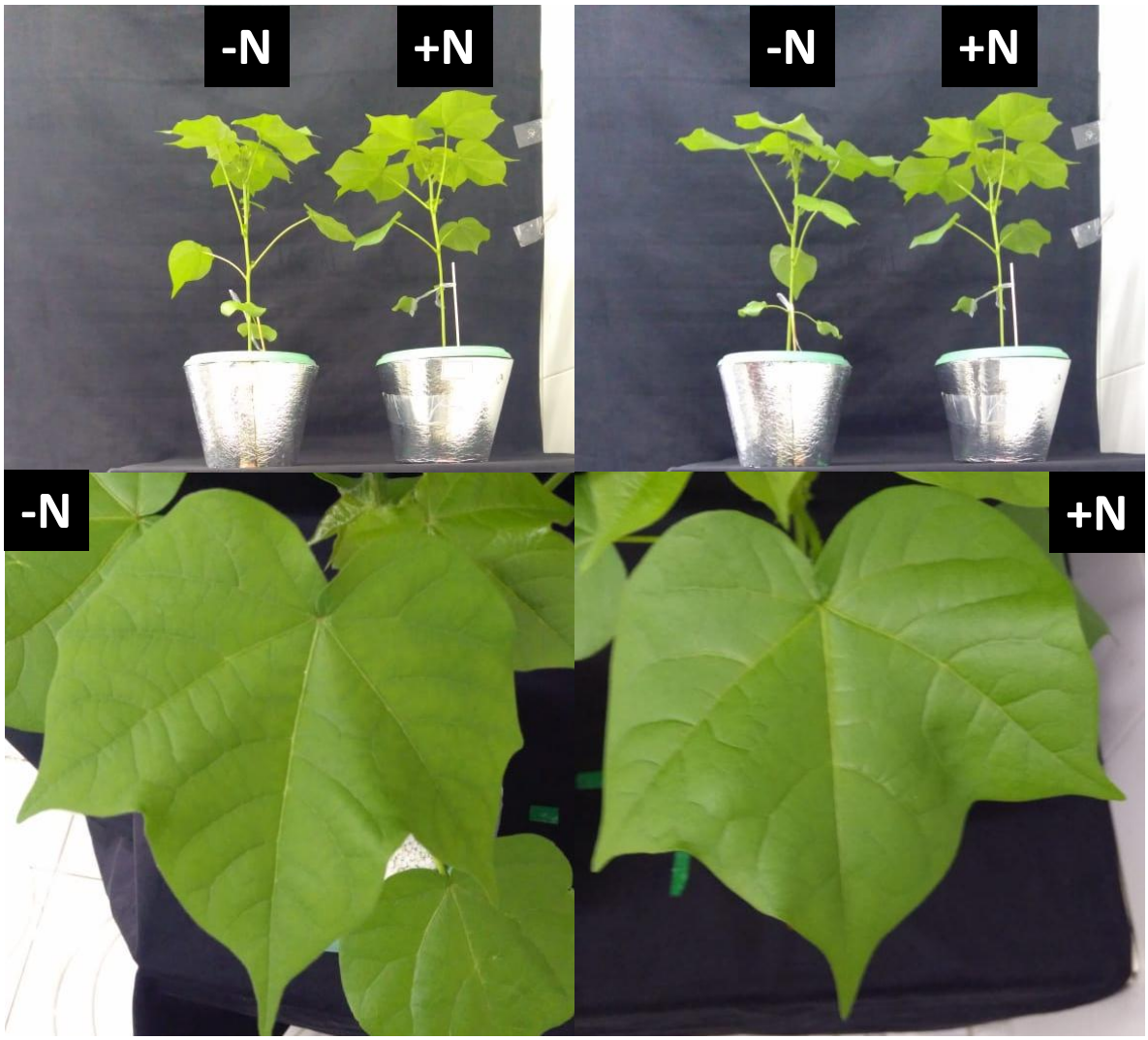


Source: elaborated by the author.

Figure 42 - Cotton plants subjected to to NO_3^- deprivation (ND) or high nitrate (HN) according to the treatments described in Figure 6.

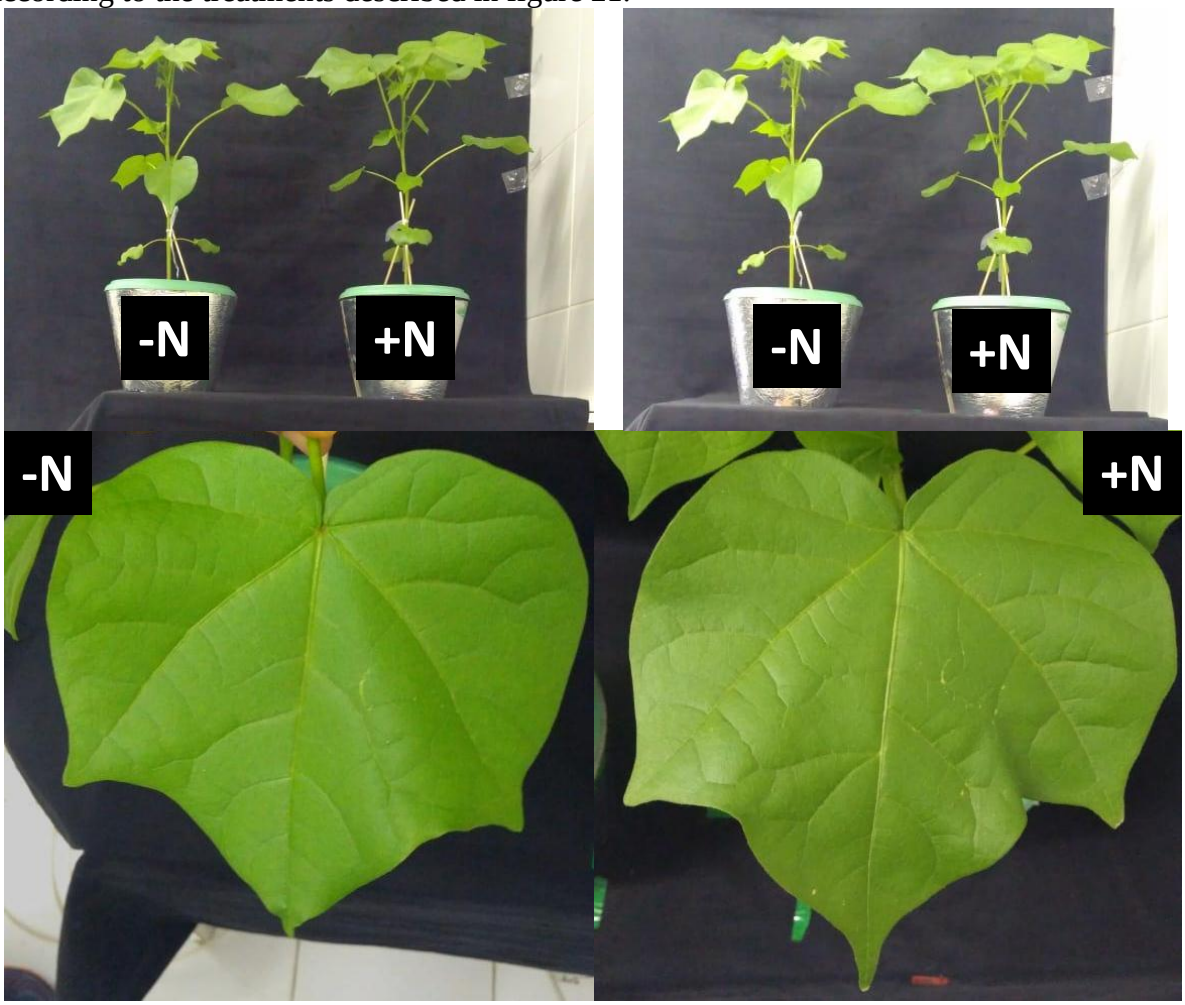
Source: elaborated by the author.

Figure 43 – Cotton plants after 4 days of nitrate deprivation (-N) and high nitrate (+N) according to the treatments described in figure 21.



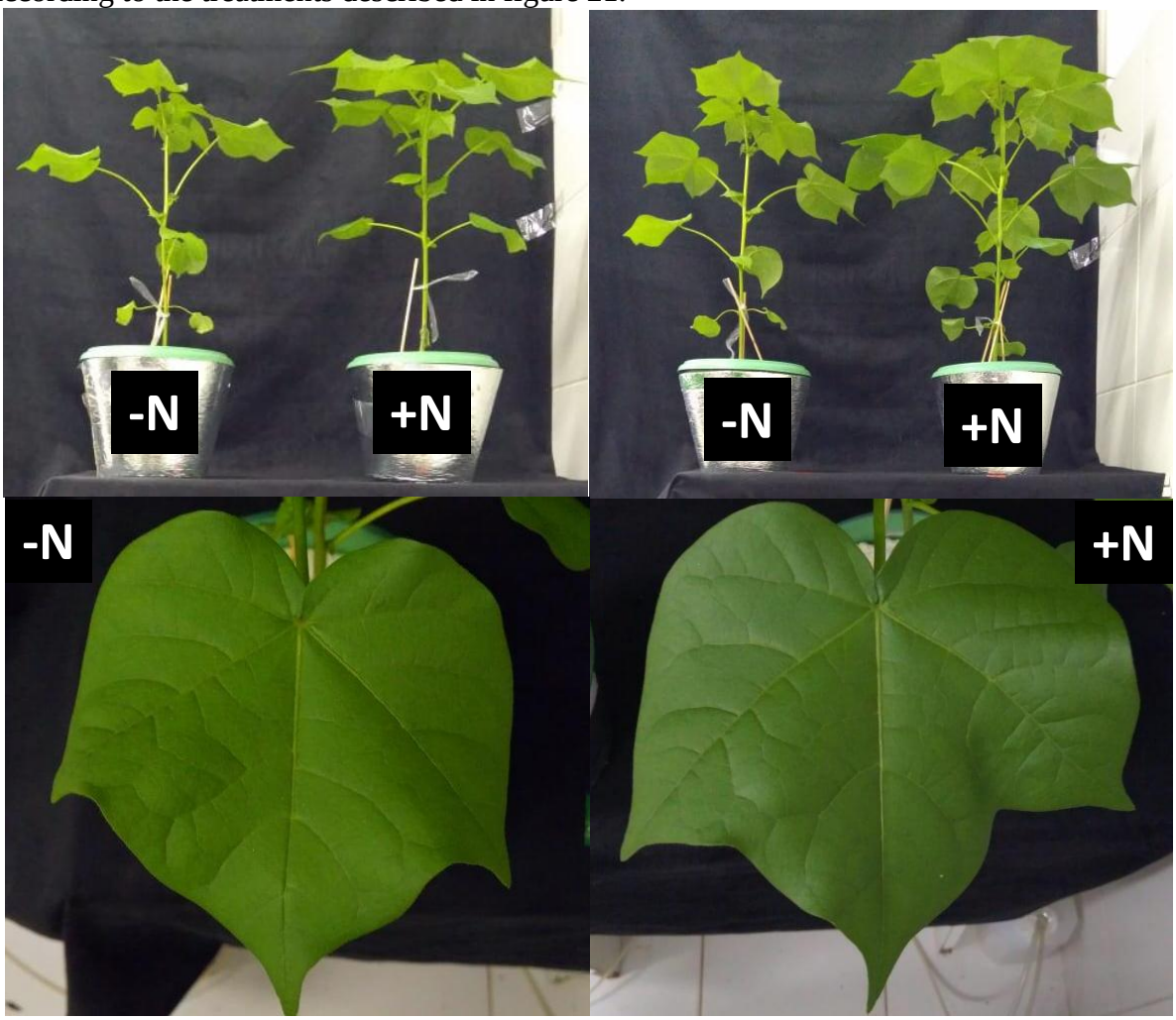
Source: elaborated by the author.

Figure 44 – Cotton plants after 6 days of nitrate deprivation (-N) and high nitrate (+N) according to the treatments described in figure 21.



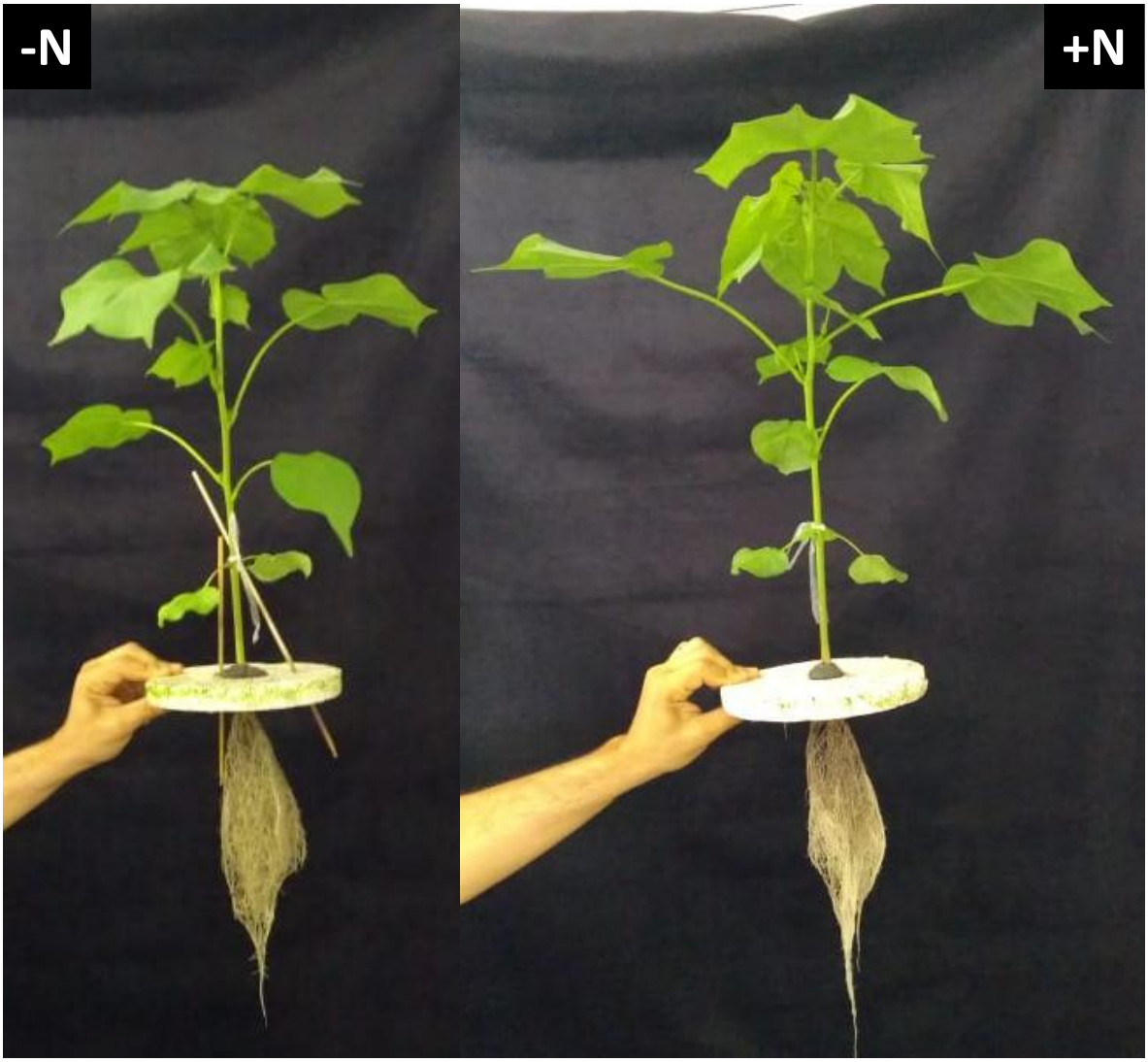
Source: elaborated by the author.

Figure 45 – Cotton plants after 8 days of nitrate deprivation (-N) and high nitrate (+N) according to the treatments described in figure 21.



Source: elaborated by the author.

Figure 46 – Whole cotton plants after 8 days of nitrate deprivation (-N) and high nitrate (+N) according to the treatments described in figure 21.



Source: elaborated by the author.

APPENDIX C – ARTICLES PUBLISHED IN COLLABORATION

SANTOS, A. D. A., SILVEIRA, J. A. G. D., GUILHERME, E. A., BONIFACIO, A., RODRIGUES, A. C., and FIGUEIREDO, M. D. V. B. (2018). Changes induced by co-inoculation in nitrogen–carbon metabolism in cowpea under salinity stress. **Brazilian journal of microbiology**, *49*(4), 685-694.