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NITROGEN SAVING ON TYPE II CELL WALL HAS CONTRIBUTED TO THE ECOLOGICAL SUCCESS OF C_4 GRASSES THROUGHOUT EVOLUTION

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (Área de Concentração – Biologia Celular e Molecular) da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Doutor em Ciências Biológicas.

Orientador: Prof. Dr. Wanderley Dantas dos Santos

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Tese apresentada como requisito parcial para obtenção do grau de Doutor em Ciências Biológicas, do Programa de Pós-Graduação em Ciências Biológicas, da Universidade Estadual de Maringá, sob a apreciação da seguinte banca examinadora:

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BIOGRAFIA

Gabriela Ellen Barreto Bossoni nasceu em Maringá/PR em 02/10/1991. Possui graduação em Ciências Biológicas, com habilitação em Licenciatura pela Faculdade Ingá – Uningá (2012). Concluiu o Mestrado no Programa de Pós-graduação em Ciências Biológicas (Biologia Celular e Molecular) pela Universidade Estadual de Maringá (2015) e iniciou o curso de Doutorado pelo mesmo programa em março de 2015, desenvolvendo o projeto "Nitrogen saving on type II cell wall have contributed to the ecological success of C_4 grasses throughout evolution", no laboratório de Bioquímica Vegetal – BIOPLAN, aonde foi orientada pelo professor Wanderley Dantas dos Santos. Em 2018, desenvolveu estágio de doutorado sanduíche (12 meses) na University of Toronto com o professor Rowan Sage, onde selecionou doze espécies de gramíneas e eudicotiledoneas com metabolismo fotossintético C_3 e C_4 e as cultivou em diferentes concentrações de N. A pós-graduanda tem experiência na área de Bioquímica e Fisiologia Vegetal, atuando principalmente nos temas relacionados à Arquitetura da Parede Celular Primária, Fotossíntese, e Metabolismo do N em plantas.

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“God never takes away something from your life without replacing it with something better”

Billy Graham

APRESENTAÇÃO

Esta tese é composta de três artigos científicos. Inicia com uma revisão "Advances to Improve Nitrogen Use Efficiency Help to Reduce the Intense Use of Nitrogen Fertilizers" que discorre os principais aspectos que contribuem para definir a eficiência no uso do nitrogênio (NUE) e os diversos métodos utilizados para sua mensuração. O segundo é um artigo de pesquisa intitulado "Nitrogen saving on the type II cell wall has contributed to the ecological success of C_4 grasses throughout evolution" que traz resultados inéditos mostrando a influência da parede celular primária tipo II na NUE de gramíneas com metabolismo fotossintético C_4. Por último, um artigo de opinião "Ferulic acid exaptation and N economy in grass cell wall" que revela uma participação da parede celular tipo II das gramíneas C_4 na adaptação e sobrevivência destas durante seu processo evolucionário.

Em consonância com as regras do Programa de Pós-Graduação em Ciências Biológicas, os artigos serão submetidos às seguintes revistas:

Barreto, G. E.; Stata, M.; de Oliveira, M. A. S.; Marchiosi, R.; Ferrarese-Filho, O.; dos Santos, W. D. Advances to improve nitrogen use efficiency help to reduce the intense use of nitrogen fertilizers. Plant Physiology (IF 5.949).

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Barreto, G. E.; Khoshravesh, R.; Ferrarese-Filho, O.; dos Santos, W. D. Ferulic acid exaptation and N economy in grass cell wall. Plant Cell and Environment (IF 6.173).

GENERAL ABSTRACT

INTRODUCTION – Grasses are the most widespread family of plants in agriculture and natural ecosystems. They are used in building, forage, food and bioenergy industry. Within grasses, 45% of species fix the CO_2 using the C_4 photosynthesis, that concentrates CO_2 around the Rubisco reducing photorespiration. Because of their high photosynthetic efficiency, C_4 plants demand less amount of Rubisco in the leaf, economizing N allocated to photosynthesis, and improving their photosynthetic nitrogen use efficiency (PNUE). However, C_4 grasses can present greater PNUE (0.42 µmol mmol s^{-1}) than C_4 eudicotyledons (0.28 µmol mmol s^{-1}), when cultivated in the same N condition. This indicates that grasses must present at least one additional feature in comparison with eudicotyledons that also contributes to their higher PNUE. Non-commelinid monocots and all eudicotyledons contain a primary type I cell wall, while grasses and a few related orders (grouped as commelinids), possess type II cell wall. These two types of cell walls present two main differences in their architectures and composition. First, grasses present glucuronoarabinoxylan (GAX) branched with ferulic acid (FA) as the main hemicellulose (FA-GAX, 20-30% dry weight). Second, grasses have lower content of structural proteins (1% dry weight). Besides ester-linking to GAX, FA can also perform ether-links to lignin, structural proteins and other FA-GAX. Like this, FA works as a molecular 'glue' that cross-links the cell wall polymers, performing roles in the control of the cell wall integrity, cessation of cell growth and protection against pathogen attack. These functions are alike the functions performed by a class of cell wall proteins known as extensins, which in type I cell wall are also in charge of the cell wall integrity, cessation of cell growth, and protection against pathogen attack. However, as FA-GAX does not contain N in their chemical structure, a transference of function from structural proteins to FA-GAX could have contributed to reduce the demand for N in commelinids, which, in turn may have helped grasses to obtain their outstanding success in ecology as well as the unique value they has had to agriculture throughout the development of civilization.

AIMS – To investigate the hypothesis that the N saving in type II cell wall can influence the NUE in C_3 and C_4 plants. For this, the work was carried out in two steps. First, grasses and eudicotyledons were grown in different N concentration to evaluate photosynthesis rate, total N and PNUE. In the second step, the same species were used to determine structural components as N and ferulic acid esterified in their cell wall.

METHODS – Three species of C_3 grasses (*Triticum aestivum* L., *Phalaris*, and *Dicanthelium oligosanthes*), three C_3 eudicotyledons (*Flaveria pringlei*, *Abelmoschus*

esculentus L., and *Atriplex lentiformis*), three C_4 grasses (*Saccharum officinarum* L., *Zea mays*, and *Setaria viridis*), and three C_4 eudicotyledons (*Blepharis ciliares*, *Amaranthus edulis* and *Gomphrena globosa*) were cultivated in glasshouse (University of Toronto), and watered three times a week with Johnson-Hoagland´s solution containing different N concentration (classified as deficit, low, medium and normal N conditions). After thirty days, the young fully expanded leaf was used to measure the photosynthetic rate with a LiCOR 6400 infrared gas analyzer (Lincoln, NE, USA). The leaf chamber conditions were: light intensity 1200 μmol m^{-2} s^{-1} PAR to C_3 plants and 2000 μmol m^{-2} s^{-1} PAR to C_4 plants, humidity 60%, leaf temperature 25ºC to C_3 plants and 30ºC to C_4 plants, flow 300 μmol s^{-1} and CO_2 concentration 1000 ppm. Leaf discs of 1-7 cm diameter were punched out from each species; they were air-dried at 60ºC for 48 h, and ground in a mill. The total N concentration was quantified using an elemental combustion system (ECS 4010). PNUE was calculated using photosynthetic rate per unit leaf area divided by leaf N content per unit area. To structural proteins assays, leaf dried power was washed extensively in methanol/chloroform/water (12/5/3, v/v/v), and citrate buffer at pH 6.8 containing 1% (v/v) sodium dodecyl sulphate (SDS). Then, the pellet was air-dried at 60ºC for 24 h and the dry mass of pellet was assumed to represent the leaf structural biomass. Structural biomass was used to determine the structural N content. To quantify ferulic acid ester-linked in the cell wall, the biomass was homogenized with methanol (50%, v/v) and incubated at 80°C for 90 min. The pellet was dried at 60°C for 24 h. The dry cell wall was re-suspended in 0.5 M NaOH and incubated at 96°C for 2 h. The supernatant was acidified to pH 2.0 with 6 M HCl, centrifuged at 2.180*g*, 4ºC for 15 min and then extracted twice with anhydrous ethyl ether. The ethyl ether extracts were combined and dried at 40°C. The samples were re-suspended in methanol/acetic acid 4% (30/70, v/v) and analyzed with a Shimadzu® Liquid Chromatograph (HPLC). The unpaired two-side *t*-test was applied to evaluate the differences between parameters and values $p \leq 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION – Comparing plants with the C_3 photosynthetic metabolism, the assays revealed that **1)** C_3 grasses and C_3 eudicotyledons tested presented similar amounts of N (total) in their leaves. In general, these results reflected in similar PNUE values to C_3 grasses and C_3 eudicotyledons. **2)** In all N conditions C_3 grasses and C_3 eudicotyledons also did not present differences in the amount of structural N. **3)** Although in N deficit, FA ester-linked to cell wall was 446% higher in C_3 grasses than in C_3 eudicotyledons (reflecting a role of FA in response to N deficit), this higher content of FA into the type II cell wall of C_3 grasses did not contribute with the N economy.

In turn, comparing plants with C_4 photosynthetic metabolism, the tests indicated that **4)** C_4 grasses presented less N (total) content in their leaves than C_4 eudicotyledons. **5)** in N deficit, the lower total N contributed to a the higher PNUE (up 81%) in C_4 grasses when compared with C_4 eudicotyledons. **6)** In addition, in deficit C_4 grasses presented up to 58% less structural N than C_4 eudicotyledons. **7)** In deficit, C_4 grasses showed up to 177% higher amounts of FA ester-linked into their cell wall than C_4 eudicotyledons. **8)** Although the N structural content contributes with a rather small percentage of the total N in the leaves (1 to 10% into structural proteins), this percentage are spread for the whole plant, Rubisco is limited to leaves and green parts of the plant. The reduction in structural N content observed in C_4 grasses can respond for about 20% of the total N savings in these plants when compared with C_4 eudicotyledons, when both groups were grown in deficit of N.

The structural proteins are the major nitrogenous compounds in the plant cell wall. When C is not the main limiting factor to growth, as occurs with C_4 plants, N becomes the main limiting nutrient. Our data suggests that during evolution of C_4 grasses, N limitation in soil imposed a selection pressure to transfer the function performed by structural proteins to FA, reducing demand for N and contributing to a higher NUE. This was possible due the distinct type II cell wall architecture present in C_4 grasses, but not in C_4 eudicotyledons. Our data also suggest that, in turn, although presenting a type II cell wall able to save N, C_3 grass lineages were not selected to save N, since throughout C_3 evolution, carbon continued to be the main limiting factor to growth.

CONCLUSIONS – The reduced content of structural proteins whose function was partially replaced by FA in type II cell wall, contributes to decrease the N allocated into cell wall in C_4 but not in C_3 grasses. Therefore, together with the sensible reduction in Rubisco content (the main N sink in plant leaf) allowed by the limited Rubisco oxygenase activity in C_4 plants, the cell wall architecture of C_4 grasses provides an additional contribution to the higher NUE presented by C_4 grasses.

Short title: Reducing high chemical fertilization using nitrogen use efficiency

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Advances to Improve Nitrogen Use Efficiency Help to Reduce the Intense Use of Nitrogen Fertilizers

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One-sentence summary

Enhancing nitrogen use efficiency emerges as an intelligent alternative for improving crops yield, while reducing costs with fertilizers and environmental unbalance.

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FOOTNOTES

Author contributions

G.E.B, M.T and M.A.S.O wrote the manuscript; R.M and O.F.F completed and reviewed parts of the writing. W.D.S conceived, supervised and reviewed the manuscript agreeing to serve as the author responsible for contact and ensures communication. All the authors read and approved the final manuscript.

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ABSTRACT

Nitrogen is one of most important macronutrients responsible for healthy growth and development in plants. It is incorporated in different molecules in plant cells, such as in proteins, DNA, hormones and natural compounds. The global use of nitrogen fertilizers is associated with better yields of crops. However, large scale nitrogen fertilizer use also intensifies environmental damages, threatens human health, and increases energy inputs and overall costs of production. Alternative approaches include development of efficient biofertilizers and improving nitrogen use efficiency (NUE) in crop species. It is estimated that a 1% increase in plant NUE can save $1.1 billion in fertilizers annually. As a result, researchers have been endeavoring to find ways to improve crop NUE. Moderate success has been obtained by breeding but understanding in detail how different plants cope with low levels of N remains challenging: uptake, assimilation, allocation and mobilization of N varies among different crops and even among distinct cultivars of the same crop. Here we review the basic knowledge related with N metabolism and the recent advances that can aid improvements of crop NUE.

Key words: Ecosystem Conservation, Nitrogen Allocation, Nitrogen Uptake, NUE, Nitrogen Mobilization.

INTRODUCTION

Although nitrogen (N) is abundant in biosphere, comprising about 78% of the Earth's atmosphere, thanks to the high stability of the triple bound between the two N atoms, gaseous nitrogen (N_2) is unavailable to most living beings. Only a restrict number of bacteria can reduce N_2 to ammonia, making N available to other organisms. Despite this narrow route via which N is introduced at the biosphere, it is an essential element in proteins, nucleic acids, hormones, signaling compounds, secondary metabolites and vitamins (Krapp, 2015; Battye et al., 2017). In some crops such as cereals, to maximize crop yields farmers use approximately 80 million metric tons of N fertilizers. However, plants use only about 40% of the N applied to the soil, while the remainder is lost to the environment (Tei and Nicola, 2017; Plett et al., 2018). This inefficient use of nitrogen damages the environment and threatens the health of humans and ecosystems inducing soil acidification, air pollution, water eutrophication and may increase the risk of diseases as methemoglobinemia and cancer (Wu et al., 2016; Zhang, 2017).

In 1790, Jean Claude Chaptal was the first scientist to name the N as "nitrogene". Two years later, G. K. Rutherford, a Chemist from Scotland, established the N function for plants growth. In the end of the 19th century, Hellriegel and Wilfarth discovered that microbial communities could fix N_2. All these studies contributed to our understanding of the relation between soil-plant systems and the natural movement of N inside plants (Yadav et al., 2017).

Nitrogen is an essential compound required for successful plant growth and key agricultural input into soils (Zheng et al., 2013). Soil N is present in four major forms: (1) organic matter such as plant fragments, fungi and humus; (2) soil organisms and microorganisms; (3) ammonium ions (NH_4^+) held by clay minerals and organic matter and; (4) mineral-N forms in soil solution including NH_4^+, nitrate (NO_3^-) and low concentrations of nitrite (NO_2^-). The inorganic or mineralized N are the major forms of the element absorbed by most plants (Cameron et al., 2013; Liu et al., 2014).

Nitrogen is a conspicuous building block of many central biomolecules, such as nucleotides, cofactors, alkaloids and amino acids. Amino acids provide the building blocks for synthesizing enzymes, structural and regulatory proteins required for constitutive and stress-responsive metabolism. All vital processes in plants and other organism are associated with enzymes as well as with nucleic acids in which N is an essential constituent. Besides, many signaling compounds, phytormones, defense molecules and allelochemicals contains N. In a macroscopic perspective, N improves

the root system, which is responsible for absorption of water and nutrients (Frungillo et al., 2014; Liu et al., 2015); it enhances fruit quality, growth of leafy vegetables, increases protein content in fodder crops, and utilization of other nutrient as potassium (Yadav et al., 2017).

Plants cannot complete their life cycles and accomplish their physiological functions in the absence of N (Kalaji et al., 2014). Some studies demonstrate that N deficiency decreases plant growth and photosynthesis (Zhang et al., 2014); increases the root-to-shoot ratio and starch content, impairing gene expression and plant metabolism; reduces biomass production, leaf area, chlorophyll content, and photosynthetic capacity (Yin et al., 2017). It is possible to diagnose nutritional disorders in plants by visual symptoms. Appearances of chlorosis (yellow leaves), red, and purple spots on the leaves restricting lateral bud growth, reduced tillering, and reduced pods (Leghari et al., 2016). Nitrogen is a highly mobile nutrient in the soil as well as in plants; consequently, its deficiency signs are unique and first visible on the lower leaves. If the N deficiency persists, older leaves may senescence, especially in legumes (Yadav et al., 2017). There is some evidence that N deficiency induces ethylene evolution. Iqbal et al., 2013 found in their assays with maize seedlings that root sensitivity to ethylene and subsequent aerenchyma formation increased 100-fold during periods of N deficiency. Ethylene also antagonized the effect of NH_4^+ by dramatically inhibiting NH_4^+-stimulated root hair branching.

Thanks to its importance to plants, application of N fertilizers has been crucial to achieve high yields in modern agricultural production. Addition of N to the soil-plant system can be through application of small quantities in organic sources or in larger quantities through inorganic sources. Organic fertilizers like crop residues, manure, and biological N fixation are known to improve soil quality and structure, as well as stimulating enzyme activities, soil microbial biomass and functional diversity and abundance in soil community structure. The main detraction for organic fertilizers is their slow and variable effect on crop yield. Consequently, farmers prefer to use inorganic fertilizers to preserve crop yield, because of their fast availability to plants and homogeneity (Zhao et al., 2016; Yadav et al., 2017), especially in intensive agricultural systems under fluctuating environmental conditions. However, due to negative impacts to the environment, emphasis on selecting plants that use fertilizers more efficiently, increasing yields while maintaining or preferably *decreasing* applied N has been suggested (Han et al., 2015).

The biogeochemical cycle of N in agricultural systems

The N cycle is a complex process involving: 1) fixation of atmospheric N into ammonium; 2) mineralization of N into plant-available inorganic sources, which includes ammonium, nitrite and nitrate; 3) immobilization (or assimilation) into organic matter by microbes and plants and; 4) denitrification, whereby nitrates are reduced back into gaseous N. When organic fertilizer is applied to soils, the mineralization process starts. These processes occur in two steps: the first involves enzymatic reactions that mediate the hydrolysis of organic N compounds into ammonia, called ammonification; the second step is ammonia oxidation to nitrite and nitrate with the help of soil microbes, a process called nitrification. The balance between the two steps determines the flow direction and the availability of soil nitrogen (Wen et al., 2016). In the soil, the net nitrogen generated by the processes of mineralization and immobilization determines the amount of nitrogen that can be assimilated by plants (Liu et al., 2014; Yadav et al., 2017).

Plants and fungi are the only eukaryotic organisms able to assimilate inorganic N. Plants take up mineralized N through their roots in three steps. First, the absorbed nitrate is reduced to nitrite by the enzyme nitrate reductase. Second, nitrite is reduced to ammonia via nitrite reductase. Finally, ammonia is incorporated into amino acids and other organic compounds. Ammonia and nitrates can be stored in the roots. However, whereas part of the nitrate is assimilated into amino acids in the roots, a portion is also translocated to the leaves in which it can be stored and assimilated using the redox potential provided by photosynthesis (Bloom, 2015; Krapp, 2015; Yadav et al., 2017).

Losses of N

A significant amount of applied N can be lost by the soil-plant system. The main mechanisms of N loss from the soil-plant system are ammonia volatilization, nitrate leaching, and denitrification.

Ammonia volatilization is the major pathway of nitrogen loss in agricultural systems worldwide (Pan et al., 2016). In alkaline soil and warm sunny conditions ammonia production is favored; it is estimated that within one week about 20% of this N may volatilize and be lost to atmosphere. In neutral or acid pH soils, especially when urea is applied on soil, significant amounts of ammonia can be lost by volatilization (Cameron et al., 2013; Ni et al., 2014; Yadav et al., 2017). Raymond et al., 2016 list some other factors that influence ammonia volatilization: soil moisture, mineral soil substrate,

relative humidity, soil temperature, surficial wind speed, precipitation, and air temperature. Most of volatilized ammonia is returned to the earth surface through wet or dry deposition causing acidification and eutrophication of natural ecosystems (Mandal et al., 2016). There are some methods to reduce this process (Cameron et al., 2013): a) application of N fertilizers 3-5 cm below of soil surface; b) application of urea before the onset of rain as this washes the urea and ammonium below the soil surface; c) coating urea with polymers to slow down the rate of dissolution of the urea and; d) using N fertilizers with a urease inhibitor coating.

Nitrate leaching is the main source of nitrate in the hydrosphere (Zhou and Butterbach-Bahl, 2014). Loss by leaching occurs when soil is sandy in texture and has sufficient water to cause movement of nitrate through the soil profile. This process occurs easily because the nitrate is mobile in nature and does not strongly adsorb into soil particles (Malcolm et al., 2014; Yadav et al., 2017). According to Quemada et al., 2013, nitrate leaching in irrigated agriculture imposes costs on the farmer and environment; they explain that this process is more intense in irrigated agriculture because crops are abundantly fertilized to achieve high yield potentials. Some factors can contribute to this process, like season and climate, soil properties, fertilizer rate, intense horticultural crop production, and flood irrigation (Cameron et al., 2013). This process represents a threat to the wider environment and to human health. In the environment, high amounts of nitrate entering aquatic systems can result in acidification of streams and lakes and eutrophication of estuaries and coastal waters resulting in algae blooms and death of fish (Crowley and Lovett, 2017), while in human health it can cause methemoglobinemia cancer and heart disease (Cameron et al., 2013). Methods to reduce this process include: a) applying the correct amount of N fertilizer to meet plant demand and reducing excess N input; b) the use of gibberellic acid to stimulate plant growth maximize uptake of N; c) using a nitrification inhibitor to slow down the rate of nitrate produced in the soil from animal urine, fertilizers, and manures; d) using a foliar spray application of nitrification inhibitor in grazed pasture systems and; e) fertilizing in the spring rather than autumn to avoid leaching losses of N mineralized from the soil in autumn (Cameron et al., 2013; Woods et al., 2016).

Denitrification is a well–studied process in bacteria and has more recently also been found in archaea and fungi (Long et al., 2013). This mechanism is most common under waterlogged conditions with lower oxygen level, which increases the population of denitrifying organisms (Cameron et al., 2013). Changes in soil moisture and aeration

can influence the denitrification rate, when soil moisture content is greater than field capacity there is a significant increase in the potential denitrification rate. Other features contributing to this growth include heavy rainfall, irrigation, application of N fertilizer, animal excreta, addition of organic carbon, soil pH, and temperature (Morse et al., 2015). There are a number of practical methods to reduce gas emissions produced by denitrification: a) using lime to increase soil pH; b) using optimum rates of irrigation to avoid creating anaerobic conditions; c) draining the soil to increase aeration; d) improving fertilizer use efficiency to reduce excessive levels of mineral N accumulating in the soil (Cameron et al., 2013).

Nitrogen use efficiency

Nitrogen use efficiency (NUE) can be defined in different ways. The basic definition was proposed in 1981 by Moll and colleagues. They defined NUE as the grain yield produced per unit of available N. Later studies have proposed distinct meanings and methods to measure NUE in different fields, and it has been shown that different cultivars may exhibit varied levels of NUE. Understanding the factors that determine higher or lower NUEs can orient breeding programs or genetic engineering projects towards improving NUE in crops and reducing the demand for N fertilization and the associated problems (Li et al., 2017; Snyder, 2017).

Although the definition of NUE well-established its estimation, on the other hand, is complex, since it can be approached in different ways. One approach frequently used in agronomic research is the *apparent recovery efficiency* (RE), which measures how nutrient uptake increases as a function of the nutrient applied. In turn, the *agronomic efficiency* (AE), and *partial factor productivity* (PFP) consider yield increases in function of the amount of nutrient applied (Xu et al., 2015; Bouchet et al., 2016). Therefore, NUE can be divided into two key-components as follow: *N uptake efficiency* (NUpE) (Xu et al., 2015; Bouchet et al., 2016) and N *utilization efficiency* (Table 1) (NUtE) (Bouchet et al., 2016). Pires et al., 2015 explained another type of agronomic NUE called *economic efficiency* (EE) which aims the maximization of the income per unit of N applied (Fig 1a).

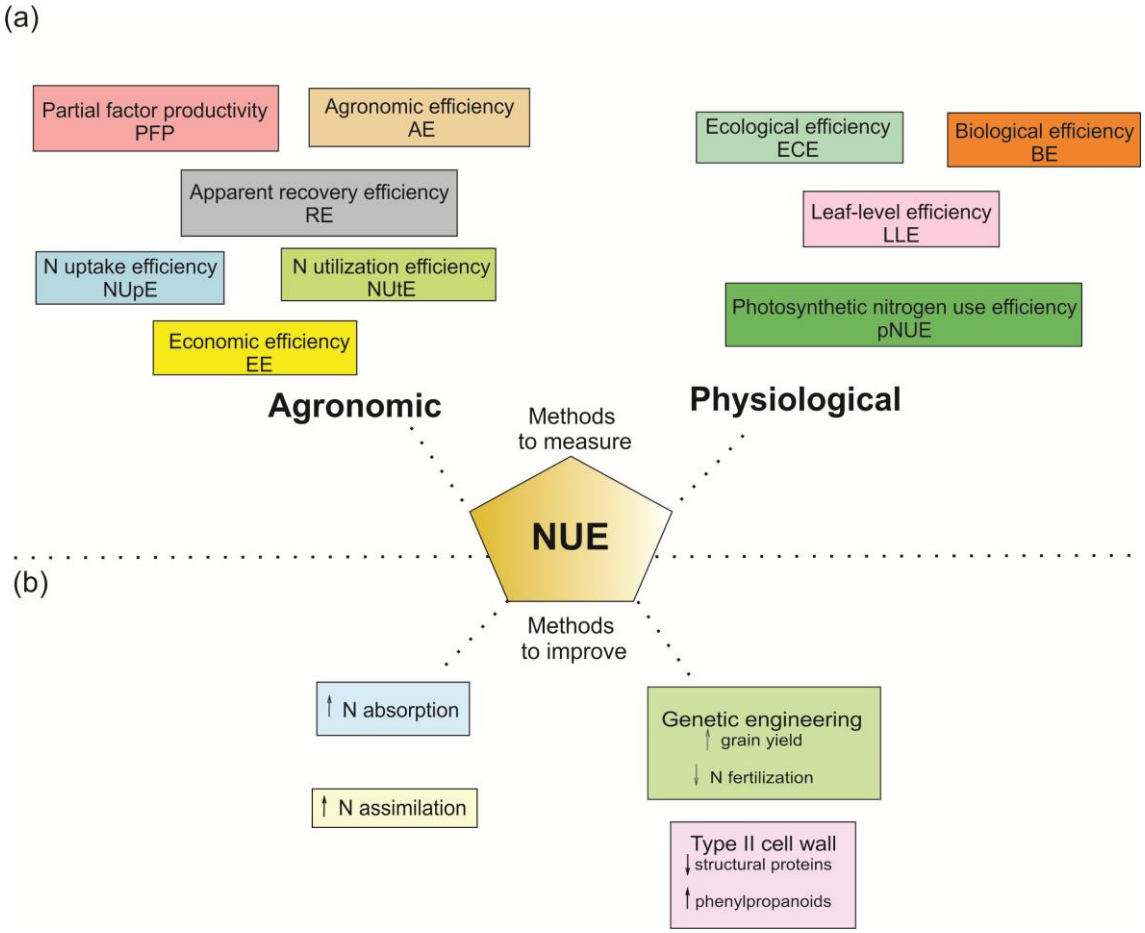

Fig. 1 Scheme showing the major methods to measure and improve nitrogen use efficiency (NUE). (a) The methods to measure NUE are divided in two general groups. Agronomic methods aim to analyze the plant yield by amount of N applied into soil. Physiological methods, on the other hand are interested to evaluate the N inside of plant (e.g. in dry mass, in leaves, in photosynthetic apparatus) by unit of N taken up from the soil. (b) In general, methods to improve NUE are related with raise N assimilation or N absorption. However, recent studies using genetic engineering have maintaining or increasing the grain yield while reducing N applied. In grasses, the primary type II cell wall also can improve NUE. Type II cell wall contains low amount of nitrogenous compounds such as structural proteins, in contrast, these plants have high content of phenylpropanoids that do not demand N in their chemical structure, but they perform similar function by structural proteins in the cell wall.

Table 1. Brief explanation of NUE components

Formula	Definition
NUE = NUpE X NUtE	NUpE=Efficiency of absorption/uptake of supplied N. NUtE= Efficiency of assimilation and remobilization of plant N to ultimately produce grain.

On the other hand, an ecological (ECE) measure of NUE considers flux ratio of dry mass productivity by unit of N taken up from the soil (Cormier et al., 2016). In perennial species this is achieved when annual biomass production = annual biomass

loss and annual N uptake = annual N loss. In 1987, Berendse and Aerts proposed one interesting biological quantification of NUE. They considered two parameters to measure the efficiency of nitrogen use; first, the mean residence time of N or MRT in the plant, and second, the instantaneous rate of carbon fixation per unit of N in the plant. Hirose, 2012, in turn evaluated the leaf-level NUE (LLE); which is expressed as leaf net production per unit N allocated in the same leaf. Finally, NUE can also be expressed as the CO_2 assimilation rate in function of the nitrogen content per leaf area, known as photosynthetic nitrogen use efficiency (pNUE). In comparison with the other approaches to determine NUE, pNUE presents the advantage of allowing comparisons among different plant species (Fig 1b) (Vogan and Sage, 2011; Guo et al., 2016).

In general, any approach to measuring NUE performs a ratio between an output (grain, biomass, CO_2 fixation, N assimilation) and the necessary input of N (applied, taken up or present in a specific plant structure – organ, tissue or ultrastructure). Table 2 presents the major NUE and their calculation (Dobermann, 2005; Yadav et al., 2017).

Table 2. Calculation and interpretation of NUE

Index	Calculation	Interpretation
Agronomic use efficiency or AE (Kg of yield increase per Kg nutrient applied)	$AE = (Y - Y_0)/F$ or $AE = RE \times PE$ **Y** – crop yield with applied nutrients (kg/ha); **Y_o** – crop yield (kg/ha) in a control treatment with no N; **F** – amount of (fertilizer) nutrient applied (kg/ha).	Product of nutrient recovery from mineral or organic fertilizer (RE) and efficiency with which the plant uses each additional unit of nutrient (PE).
Apparent recovery efficiency or RE (Kg increase in N uptake per Kg N applied)	$RE = (U - U_o)/F$ **U** – total plant nutrient uptake in aboveground biomass at maturity (kg/ha) in a plot that received fertilizer; **U_o** – total nutrient uptake in aboveground biomass at maturity (kg/ha) in a plot that received no fertilizer; **F** – amount of (fertilizer) nutrient applied (kg/ha).	RE depends on the congruence between plant demand and nutrient release from fertilizer. RE is affected by the application method (amount, timing, placement, N form) and factors that determine the size of the crop nutrient sink (genotype, climate, plant density, abiotic/biotic stresses).
Physiological efficiency of nitrogen or PE (kg increase yield per kg increase in N uptake from fertilizer)	$PE = (Y - Yo)/(U - Uo)$ **Y** – crop yield with applied nutrients (kg/ha); **Y_o** – crop yield (kg/ha) in a control treatment with	Ability of a plant to transform nutrients acquired from fertilizer into economic yield (grain). Depends on genotype, environment and management. Low PE suggests sub-optimal growth (nutrient deficiency, drought stress, heat stress, mineral toxicities, pests).

	no N; **U** – total plant nutrient uptake in aboveground biomass at maturity (kg/ha) in a plot that received fertilizer; $\mathbf{U_o}$ – total nutrient uptake in aboveground biomass at maturity (kg/ha) in a plot that received no fertilizer.	
Internal utilization efficiency or IE (kg yield per kg nutrient uptake)	IE=Y/U **Y** – crop yield with applied nutrients (kg/ha); **U** – total plant nutrient uptake in aboveground biomass at maturity (kg/ha) in a plot that received fertilizer.	Ability of a plant to transform nutrients acquired from all sources (soil, fertilizer) into economic yield (grain). Depends on genotype, environment and management. A very high IE suggests deficiency of that nutrient. Low IE suggests poor internal nutrient conversion due to other stresses (nutrient deficiencies, drought stress, heat stress, mineral toxicities, pests).
Partial factor productivity or PFP (kg harvested product per kg nutrient applied)	PFP=Y/F or PFP=(Yo/F) + AE **Y** – crop yield with applied nutrients (kg/ha); **F** – amount of (fertilizer) nutrient applied (kg/ha); $\mathbf{Y_o}$ – crop yield (kg/ha) in a control treatment with no N.	Most important for farmers because it integrates the use efficiency of both indigenous and applied nutrients. High indigenous soil nutrient supply (Yo) and high AE are equally important for PFP.
Biologically meaningful of NUE (g dry weight per g of N taken up)	$NUE = A/L_n$ **A** - N productivity; $\mathbf{L_n}$- Mean residence time.	It is important to analyze the trade-off between a high nitrogen productivity and a long mean residence time of nitrogen in the plant (i.e. the period during which the absorbed nitrogen can be used for carbon fixation).
Leaf-level NUE (g leaf net production per g N allocated to the leaf)	$NUE_L = \Delta W_L/\Delta N_L$ $\mathbf{\Delta W_L}$ – leaf net production; $\mathbf{\Delta N_L}$ - amount of N (gN) allocated to the leaf in that period.	As the leaf is a photosynthetic organ, leaf net production is equal to gross production minus leaf day- and night-respiration.
Photosynthetic rate per N content in leaves or pNUE (µmol CO_2 per mmol N)	pNUE = *A*/N ***A*** – photosynthetic rate; **N** – nitrogen content in leaves.	pNUE approach is more suitable for comparing plants from different species.

Nitrogen absorption and assimilation in plants

Nitrate and Nitrite

Nitrate is the main form of N available for plants in aerobic soils; nevertheless, in hydroponic cultures, and agricultural plants, ammonium and amino acids are the principal form of N absorbed (Mokhele et al., 2012). Plants show different responses in physiology and growth to different N sources. The preference for ammonium or nitrate is usually related to physiological adaptations of plants to natural ecosystems (Yang et al., 2013).

Nitrate uptake capacity by roots is determined by three independent factors related to nitrate availability. First, the functional properties of the transporters in the roots that provide the acquisition of nitrate. Second, the density of functional transporters at the plasma membrane of root cells, and third, the surface and architecture of the root system. In addition, effective absorption and assimilation of nitrate depends on the age and non-limiting growth conditions (Léran et al., 2015).

After being taken up, nitrate is reduced into the nitrite by nitrate reductase (NR, EC 1.7.1.1), a cytosolic enzyme present in both roots and leaves. This enzyme is a homodimer containing three prosthetic groups – FAD; molybdenum cofactor (MoCo) and a heme group – associated with each monomer (Masclaux-Daubresse et al., 2010; Limami et al., 2014). Nitrate reduction is NADH-dependent and occurs when two electrons are transferred from NAD(P)H to nitrate producing nitrite. Due to high NO^-_2 reactivity, plant cells transport NO^-_2 from the cytosol plastids of leaves and roots where nitrite is reduced to ammonium by the enzyme nitrite reductase (NiR, EC 1.7.2.1) (Rosales et al., 2011; Shah et al., 2017).

Studies with *Arabdopsis thaliana* showed nitrate uptake can affect cell wall synthesis and modeling. The results identified genes co-expressed with nitrate transporters than ammonium. Below are the major cell wall remodeling genes related to nitrate uptake (Landi and Esposito, 2017):

i) Genes involved in pectin degradation;
ii) Genes implicated in xyloglucan modification, and
iii) Genes related with embryogenesis.

Ammonium

The assimilation of ammonium comprises three main processes: 1) primary nitrogen assimilation, 2) ammonia photo-respiratory re-assimilation, and re-assimilation of

"recycled" nitrogen. The ammonium produced is further assimilated into glutamine and glutamate; the nitrogen donors in the biosynthesis of all essential amino acids. Glutamine and glutamate are used to synthetize aspartate and asparagines; these four amino acids are used to translocate organic nitrogen from source to sink tissues (Shah et al., 2017).

The enzymes responsible for all these processes are glutamine synthetase (GS, EC 6.3.1.2), glutamine-oxoglutarate aminotransferase (Fd-GOGAT, EC 1.4.7.1 and NADH-GOGAT EC 1.4.1.14), aspartate amino transferase (ATT, EC 2.6.1.1), asparagine synthetase (AS, EC 6.3.5.4) and glutamate dehydrogenase (GDH, EC 1.4.1.2) (Jozefowicz et al., 2017).

Plants possess two variants of the GS enzyme; the first (GS1) is find in the cytosol and responsible for assimilation of ammonium generated by protein turnover in leaves. The second (GS2) is responsible for assimilation of the products of nitrate reduction in plastids and of ammonium formed by photorespiration (Jozefowicz et al., 2017). The GOGAT enzymes also present two types: Fd-GOGAT, manages primary ammonium assimilation in leaves, using electrons from ferredoxin (Fd). NADH-GOGAT assimilates ammonium in roots using NAD(P)H provided by the pentose pathway as an electron donor. GOGAT catalyzes the transfer of the amino (NH_2) group from glutamine to the carboxyl group of an oxoglutarate (α-ketoglutarete) to release two glutamates. In average, one Glu is used as a substrate of the GS enzyme, while the other one is consumed by amino acid metabolism (Kojima et al., 2014).

In high temperatures, production of ammonium by photorespiration by C_3 plants exceeds nitrogen assimilation up to 10-fold. Consequently, photorespiratory ammonium must be re-assimilated into glutamine and glutamate. In high ammonium concentrations, the GDH enzyme located in mitochondria catalyze this re-assimilation process (Lam et al., 1996).

Biological nitrogen fixation

Biological nitrogen fixation is an ancient process which consists in conversion of atmospheric nitrogen (N_2) into N forms utilizable by plants, such as ammonium, by a small group of prokaryotes called diazotrophs. By doing it, diazotrophs replenishes the nitrogen in biosphere lost by denitrification. The diazotrophs group of bacteria include the free-living, those that form specific symbiosis with the plant and those that can colonize the surface and internal tissues of the plant, so called endophytic (Glick, 2012).

The association between plants and nitrogen-fixing microbes increases the uptake of nutrients by roots that, in turn, contribute to more carbohydrates to plants (Kiers et al., 2011). One alternative for N fertilizer is the crop inoculation with nitrogen fixing bacteria which can promote an increase into the productivity. Several plant-growth promoting bacteria (PGPB) have already been described. Field experiments suggested that biological nitrogen fixation can contribute with 30% or even more of the total nitrogen demand of the plant (Boddey and Victoria, 1986; de Morais et al., 2012).

The most widely PGPB used as inoculant are rhizobial bacteria, such as *Rhizobium leguminosarum* and *Bradyrhizobium japonicum*. These bacteria form nodules into the root of the Fabaceae (legumes) plants. Such nodules are unique structures of the plant to host the rhizobial cells, which specialize to form bacteroids (Jones et al., 2007). The nodules are lateral root with peripheral vasculature that improve the nutrient uptake and increase the yield (da Costa Neto et al., 2017). The roots exudates a complex mixture of sugars, fatty acids, growth factors, and amino acids, which attracts the microbial community in the soil (Mus et al., 2016) improving the absorption of nutrients by roots (Neal et al., 2012). The internal environment of the nodule is optimized for nitrogen fixation by these bacteroids, and the nitrogen fixed this way is incorporate into the plant biomass. In turn, plants provide a niche and high energy fixed carbon to bacteria configuring a mutualistic cooperation (Halverson and Stacey, 1986).

Although the crop inoculation with rhizobial bacteria is a widely employed technology, grasses cannot beneficiate from it. That is because rhizobial nodules are formed exclusively on roots of legumes. If the grasses are amongst the main crops in the world, the development of PGPB inoculants has a massive environmental and economic interest. In such perspective, many diazotrophic endophytic bacteria capable to colonizes the internal tissues of grasses have been isolated and characterized. This group includes the bacteria *Herbaspirillum seropedicae*, *Azospirillum brasilense*, *Azoarcus spp.* and *Gluconacetobacter diazotrophicus*, amongst others (Elbeltagy et al., 2001; Sevilla et al., 2001; Gyaneshwar et al., 2002; Hurek et al., 2002; Baldani and Baldani, 2005). Beyond the nitrogen fixation, endophytic diazotrophic bacteria can induce plant growth by other mechanisms. These mechanisms include phosphate solubilization, phytohormones and siderophore production and ACC deaminase activity (Souza et al., 2015).

Plants of rice (*Oryza sativa*) inoculated with two different strains of *Azospirillum* increased growth and the content of flavonoids and hydroxycinnamic derivates, which,

in turn, provide more resistance to plants against pathogenic fungal (Chamam et al., 2013). The inoculation of sugarcane with the endophyte *Acetobacter diazotrophicus* indicated that this bacterium actively fixed nitrogen inside the plant, inducing growth in nitrogen limiting conditions (Sevilla et al., 2001). Furthermore, a recent published manuscript used ^{13}N tracer analysis to provide direct evidence that the nitrogen fixed by *Azospirillum brasilense* is incorporated into the plant host biomass (Pankievicz et al., 2015).

Metabolic aspects contributing with NUE

Alkaloids are secondary compounds that contain N in their molecular structure. There are 12,000 known types of alkaloids, of which 25% are produced by higher plants (Jing et al., 2014). The major function of plant alkaloids is protection against herbivores (Ziegler and Facchini, 2008). Similar functions are also performed by phenylpropanoids, the main secondary compounds in grasses (dos Santos et al., 2008) in addition to other structural functions (de Oliveira et al., 2015). As alkaloids biosynthesis consumes N, there is evidence that plants grown in low N concentration have reduced synthesis of alkaloids (Xi et al., 2008) and enhanced amounts of phenylpropanoid (Gazola et al., 2018) (Fig. 2b).

The synthesis of proteins is also reduced by N deficit reducing the amount of structural proteins and photosynthetic rate (Makino, 2003). Rubisco (Ribulose-1,5-bisphosfate carboxylase/oxygenase; EC 4.1.1.39) is the enzyme responsible for photosynthetic fixation of CO_2 into organic compounds in plants (Carmo-Silva et al., 2015). C_3 plants employ typically 20 to 30% of total leaf N in Rubisco. In C_4 plants, only 5 to 9% of leaf N are present in Rubisco (Sage et al., 1987). Because they require less N, C_4 plants have higher photosynthetic rates and biomass production than C_3 plants (Ghannoum, 2005). C_4 grasses have higher pNUE than C_3 grasses. Nitrogen economy of Rubisco in C_4 plants allows a greater nitrogen investment to other plant cell compartments, contributing to high NUE in these plants (Makino, 2003). In addition, the high pNUE of grasses is also attributed to the fact that C_4 metabolism suppresses photorespiration, increasing the efficiency of photosynthesis, especially at higher temperatures. Besides the N economy provided by C_4 metabolism, grasses also present a different kind of cell wall (CW). The Type II CW of grasses presents a distinct kind of hemicellulose (glucuronoarabinoxylan, or GAX) cross-linked by ferulic acid (FA), and a reduced

content of structural proteins when compared with eudicot (Type I) CW (Vogel, 2008; Lamport et al., 2011; Albenne et al., 2014; Hatfield et al., 2017). As the main role of structural proteins in eudicots are similar to that attributed to FA-GAX in grasses, and FA-GAX does not contains N in their composition, the evolution of Type II CW may be associated with improved N economy in grasses, particularly in C_4 species. When CO_2 is abundant, nutrients like N become the main growth limiting factor. In higher temperatures, C_4 species could benefit more than C_3 species from adaptations which reduce the demand for N. In this sense, the higher potential of Type II CW to tolerate depletion of structural proteins (thanks to FA-GAX cross-linkages), could have reduced N dependence, favoring C_4 species (Fig. 2a).

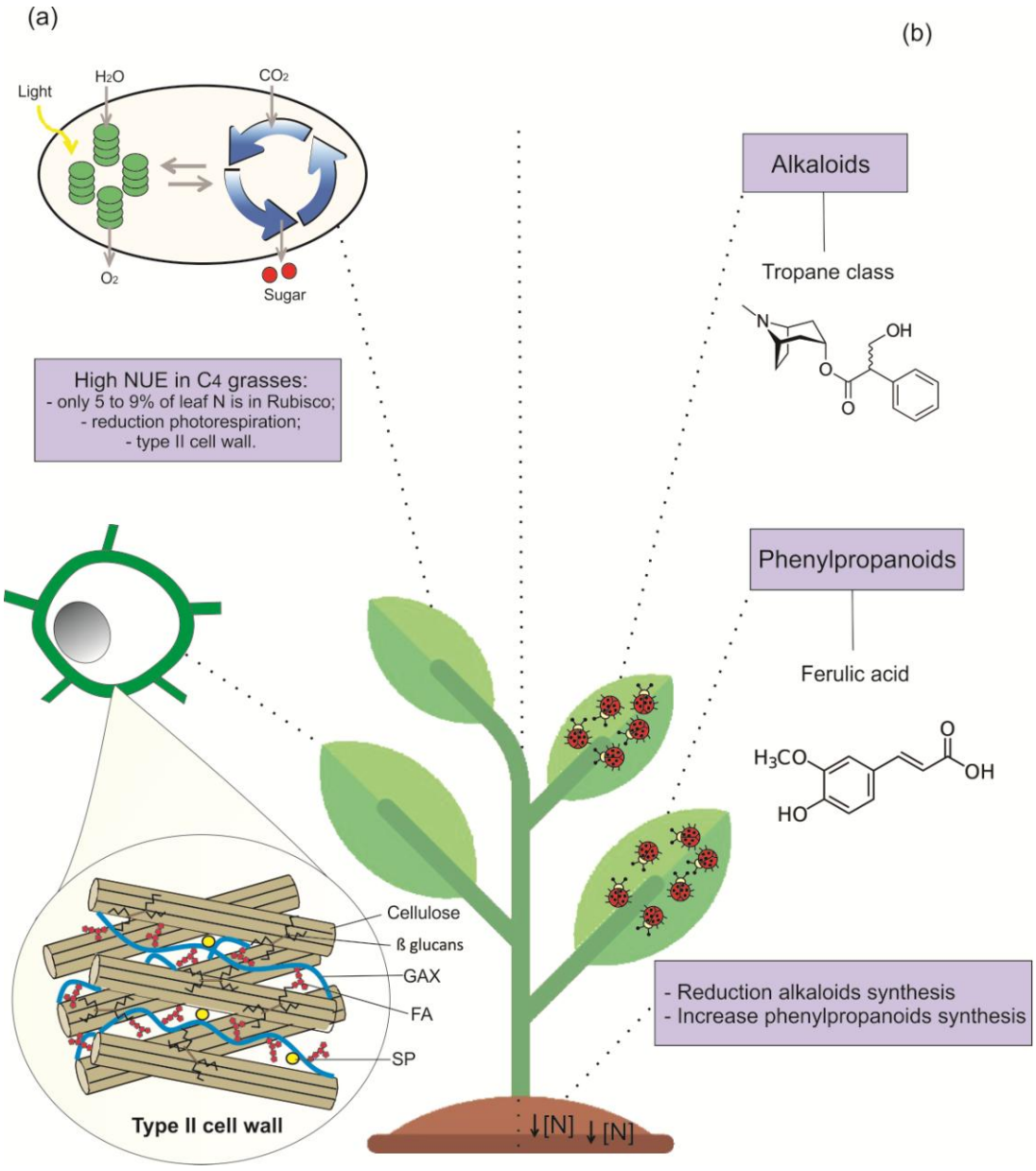

Fig. 2 Metabolic aspects in plant contributing with nitrogen use efficiency (NUE). (a) Healthy C_4 plants use roughly 5 to 9% of leaf N to produce Rubisco. The lower N requirement, together with the reduction of photorespiration process cooperates to high NUE in these plants. But, C_4 grasses can present higher NUE than C_4 eudicotyledons, indicating that an extra characteristic present in C_4 grasses influences their higher NUE. C_4 grasses possess primary type II cell wall with reduced content of SPs and greater amount of FA ester linked with GAXs when compared with eudicotyledons. FA-GAXs contain similar role performed by SPs in eudicotyledons, but FA-GAXs do not demand N in their composition. (b) Plants attacked by herbivores produce alkaloids such as atropine that consumes N. Phenylpropanoids like ferulic acid also are produced against herbivores attack, however, these compounds do not consume N into soil. So, plants in N deficiency tend to reduce alkaloids production and increase the phenylpropanoids synthesis to save N and contribute to NUE. GAXs, glucuronoarabinoxylans. FA, ferulic acid esterified. SPs, structural proteins.

Nitrogen remobilization efficiency

Nitrogen remobilization efficiency can vary by species and genotype. Studies developed with oilseed rape (*Brassica napus* L.) showed low NUE. This inefficiency is a consequence of poor capacity of nitrogen remobilization during the senescence period and during the vegetative stage. N from dead leaves in this species are poorly remobilized from soil to plant (Avice and Etienne, 2014), demanding high N fertilizer application (Gombert et al., 2006). In contrast, grasses such as wheat and rice can mobilize up about 90% of the nitrogen from vegetative organs into the plant (Yang and Udvardi, 2018). The high nitrogen remobilization efficiency in these plants contributes to increased proteins and micronutrients content from senescent to developing organs increasing NUE (Liang et al., 2014). During senescence, plants remobilize N from senescent organs to growing and developing organs (Hollmann et al., 2014). Natural senescence is the age-dependent deterioration of plant cells, ultimately leading to cell death and completion of the plant life cycle. Biotic and abiotic factors are known to activate natural senescence pathways.

During leaf degeneration the photosynthetic rate and chlorophyll content decrease with subsequent leaf yellowing. In leaves, the earliest and most important event during senescence is the degradation of chloroplasts, which contains 70% of leaf N in the form of ammonium, nitrate, amino acids, and peptides. This N is remobilized to other developing tissues, e.g. young leaves, fruits, and grains. The senescence is a controlled process and, as a result, there are many senescence-associated genes which are up or down regulated during this process (Martins et al., 2016). Assays with sugarcane species (*Saccharum* spp.) in three different stages of leaf senescence – nonsenescent,

intermediate, and advanced senescence – have identified differentially regulated genes. Genes up regulated were associated with cell wall modification, signaling proteins, transporters, and proteins involved in oxidoreductase activity. In contrast, down regulated genes were involved in protein folding, and signaling. Transcriptomic data in *Arabdopsis thaliana* shows a reduction of anabolic processes that involve assimilation of N and carbon during senescence. During senescence catabolic processes involving protein degradation increase, along with remobilization of N (Havé et al., 2016); a small number of cell wall related genes have also been reported to be associated to senescence (Martins et al., 2016).

Improving NUE based on genetic diversity

An interesting agricultural challenge is creating sustainable crops with high productivity using less nitrogen fertilizer (Han et al., 2015). It is estimated that about 50-75% of fertilizers applied in agriculture are lost due inefficient uptake by plants and soil leaching (Dresbøll and Thorup-Kristensen, 2014). Moreover, these factors result in higher production costs and damages on the environment (Han et al., 2015). Kant et al., 2011 estimated that only a 1% increase in NUE of plants can save $ 1.1 billion annually. Genetic improvement is thus a potential strategy to increase NUE. This strategy is achieved when morpho-physiological components in plants are changed, and most of these changes are related to the elevation in N uptake, and utilization of applied N (Anbessa and Juskiw, 2012). According to Yadav et al., 2017, the greatest challenge in genetic improvement is to maintain grain yield while reducing the N applied.

Poplar trees transformed with the gene GS1a that codes for a GS isolated from conifers resulted in increased NUE (Lea and Azevedo, 2007). Rice plants overexpressing the barley gene *btg26:alaAT* for an alanine aminotransferase increased biomass and grain yield when compared with control plants when well supplied with N. Similarly, canola plants overexpressing the same barley gene used 40% less N applied on soil, achieving comparable yield to control plants (Good et al., 2007). These examples show the potential of genetic engineering to increase and improve N assimilation efficiency (Shrawat et al., 2008).

To improve nitrogen assimilation and NUE, Yanagisawa et al., 2004, have grown *Arabdopsis* with a Dof1 transcription gene under low-N conditions (for a detailed description of Dof1 gene, please consult the original paper). These transgenic plants

517 presented 30% more N content and no severe growth defects were observed. The
518 *Arabdopsis* study concludes that the Dof1 gene helped to improve NUE.
519 Root length, density, surface area, and number of hairs are associated with higher NUE
520 (Wang et al., 2016). However, there are few studies with genetic modification in roots.
521 As a result, it is difficult to understand the complete relationship between genetic
522 engineering of root traits and NUE. Part of the difficult in studying roots is due to
523 difficulty in extracting them from soil, and the fact that most of methods of analysis are
524 destructive (Wu and Cheng, 2014).
525

526 **CONCLUSION**

527 The intense use of N fertilizers has been associated with high crop yield by farmers but
528 also with higher production costs and ecological damages. Enhancing NUE emerges as
529 a potentially powerful route for improving crop yield, reducing costs with fertilizers and
530 environmental harm. However, NUE comprises a wide diversity of plant physiological
531 traits and improving NUE is an ongoing challenge. Promising studies and technological
532 approaches performed in different crops have revealed interesting phenomena that are
533 improving our understanding of plant features related with NUE that may help
534 overcome the barriers to increasing NUE in crops.
535

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540 **COMPETING INTEREST**

541 The authors declare that they have no competing interests.
542

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Nitrogen saving on the type II cell wall has contributed to the ecological success of C_4 grasses throughout evolution

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Summary

- Both structural proteins in type I cell wall, and ferulic acid (FA) in type II cell wall account for the cross-linking of cell wall polymers which respond for cell wall integrity, cessation of cell growth, and defence against pathogens. As FA requires no N, type II cell wall could contribute to N use efficiency (NUE).

- To investigate the influence of cell wall architecture in N economy we compared photosynthetic NUE (PNUE), structural N content, and structural FA content between C_3 grasses and C_3 eudicotyledons, as well as between C_4 grasses and C_4 eudicotyledons.

- In C_3 plants, the type of cell wall did not influence PNUE. Grasses with C_4 photosynthesis presented lower amount total N and therefore a greater PNUE when compared with C_4 eudicotyledons. In deficit of N, C_4 grasses also present less structural N and higher ester-linked FA, suggesting a contribution of type II cell wall architecture to N economy.

- When carbon is not a restraining factor to plant growth, N becomes the main limiting nutrient. Our data suggest that during evolution, C_4 grasses partially circumvented the limitation of N by transferring the function of structural proteins to FA, contributing to the amaizing ecological success of grass family.

Key words: ecological success, ferulic acid, grasses, N economy, PNUE, structural proteins.

Introduction

Grasses belong to Poaceae family, the most widespread family of plants in agriculture and natural ecosystems (O'Mara, 2012; Tomaškin & Tomaškinová, 2012) being found in tropical, temperate and Artic zones (Saarela *et al.*, 2018). In the angiosperm evolution, grasses (consisting in about 12.000 species) become the fifth most species-rich of flowering plants (Bouchenak-Khelladi *et al.*, 2010), only behind the Fabaceae (beans, 33.000 species), Orchidaceae (orchids, 28.000 species), Asteraceae (daisies, 23.000 species), and Rubiaceae (coffee family, 13.500 species) (Hodkinson, 2018). Although less diverse than these other families, grasses comprises 25% of the green cover of the planet (Sage & Stata, 2015) reflecting a great ability of these plants to adapt to different environments.

Plants require nutrients to growth, in this way, nitrogen (N) is an important macronutrient utilized by plants in the form of NO_3^- and NH_4^+ (Leghari *et al.*, 2016) which influences their productivity and yield (Makino & Ueno, 2018). Nitrogen occurs in soluble compounds such as nitrates, amino acids and proteins, and, the insoluble proteins present in the cell wall and membranes (Onoda et al., 2004; Feng et al., 2009; Hikosaka and Shigeno, 2009; Mu et al., 2016; Onoda et al., 2017). In some crops (not applied to wild plants), 70-80% of N is allocated to chloroplasts. Rubisco, a key enzyme of photosynthesis accounts to 30-40% of that (Onoda *et al.*, 2017). As a consequence, the N limitation into soil affects structural, biochemical, and physiological traits (Sage *et al.*, 1987; Makino & Ueno, 2018), for instance, the size and number of chloroplasts (Makino & Ueno, 2018), the pigments content (Zhao *et al.*, 2017) and the photosynthetic rate (Evans, 1989).

Terrestrial plants present three types of photosynthesis: C_3, C_4 and CAM. For this manuscript, we will focus in C_3 and C_4 photosynthesis. The ancient C_3 photosynthesis responds to 90% of land plants, including important crops responsible for producing food worldwide as wheat, rice, cotton and soybeans (Sage *et al.*, 2013). In the chloroplast stroma, the enzyme Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) combines ribulose bisphosphate (RuBP) and CO_2 creating the three-carbon molecule (C_3) phosphoglycerate (PGA) in the series of reactions named photosynthetic carbon reduction or PCR cycle. The PGA is converted in triose phosphates, by which most of them are used to regenerate RuBP and keep the carboxylation cycle (Sage *et al.*, 2013). In low CO_2 levels and elevated temperatures, the Rubisco oxygenase activity is

stimulated (Sage & Kubien, 2007; Taylor *et al.*, 2010; Sage *et al.*, 2012) because of higher enzyme O_2 affinity and lower atmospheric CO_2 solubility (Sage *et al.*, 2013). The oxygenation process, commonly termed photosynthetic carbon oxidative or PCO cycle, produces one PGA and a two-carbon toxic molecule of phosphoglycolate (PG). The PG is metabolized back to PGA in an expense reaction that consumes ATP and liberates previously fixed CO_2 reducing the photosynthetic capacity and efficiency in 30% or more (Fig. 1a) (Sage, 2016).

In the ancient atmosphere, the appearing of photosynthetic organisms increased the atmospheric O_2, consequently, the high photorespiration created a harmful environment to plant survival (Mallmann *et al.*, 2014; Sage, 2016; Sage *et al.*, 2018). Sage, 2017 suggests that the reduction in CO_2, water availability and raise photorespiration provided the selection pressure to C_4 photosynthesis evolution (Fig.1a). So, this photosynthesis appears as a carbon concentrating mechanism occurring in two different cellular compartments, mesophyll and bundle sheath cells (Kranz anatomy) that minimizes photorespiration and loss of carbon (Sage, 2002; Mallmann *et al.*, 2014; Sage & Khoshravesh, 2016). Recent studies with C_4 plants have demonstrated an essential function of carbonic anhydrase to net CO_2 concentrating. This enzyme helps to maintain the chemical equilibrium of CO_2 and HCO_3^-, collaborating for high CO_2 assimilation rates (Caemmerer *et al.*, 2004; Boyd *et al.*, 2015; Zhou *et al.*, 2019). In C_4 photosynthesis, the first enzyme, phospho*enol*pyruvate carboxylase (PEPC) assimilates bicarbonate (HCO_3^-) and phospho*enol*pyruvate (PEP) into oxaloacetate in mesophyll cells. The four-carbon organic acid (C_4) is carried to bundle sheath cells where Rubisco is localized. In this inner compartment, CO_2 is released via decarboxylation reaction and fixed by Rubisco. Sage et al., 2013 explain the CO_2 accumulates 10 to 20 times greater in C_4 bundle sheath than in C_3. The other product - three-carbon organic acid (pyruvate) - is shuttled back to mesophyll cells where ATP is used to regenerate PEP (Fig. 1b) (Sage *et al.*, 2013, 2014).

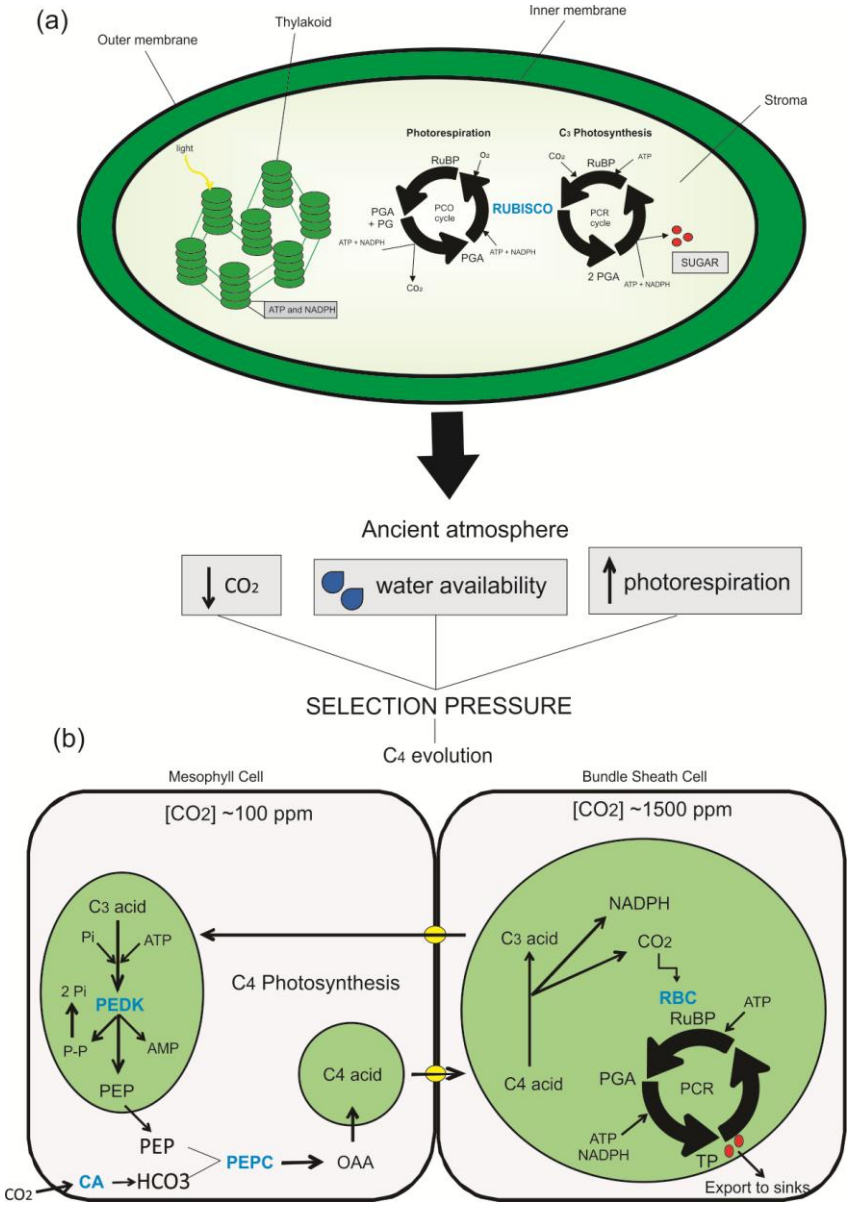


Fig. 1 Schematic of the general plan of the C_3 photosynthetic assimilation cycle, factors that contributed with C_4 photosynthesis evolution, and C_4 photosynthetic assimilation cycle. (a) Chloroplast illustration showing the light reactions in thylakoid to produce ATP and NADPH. In stroma, ATP and NADPH are used to convert CO_2 to sugars in a series of reactions started by the enzyme Rubisco (PCR cycle). Rubisco, however, is a flawed enzyme because it can also fix O_2 to RuBP forming no value molecule (PG) in the photosynthetic carbon oxidative cycle (PCO cycle). With the appearing photosynthetic organisms, atmospheric CO_2 decreased, the water availability and high photorespiration provided the selection pressure to C_4 evolution. (b) In C_4 photosynthesis, a carbon-concentrating mechanism in MSC and BSC reduces the photorespiration and carbon loss. PGA, phosphoglycerate; PG, phosphoglycolate; RuBP, ribulose 1,5-bisphosphate; CA, carbonic anhydrase; PEP, phosphoenolpyruvate; PEPC, PEP carboxylase; OAA, oxaloacetate; RBC, Rubisco; TP, triose phosphates; PPDK, pyruvate Pi-dikinase; MSC, mesophyll cell; BSC, bundle sheath cell. The green ovals/circles represent chloroplasts; the light gray area is stroma or cytoplasm; the yellow small circles are plasmodesmata.

The photosynthetic capacity vary among species (Hikosaka & Shigeno, 2009), and, the principal factor responsible for this variation is the Photosynthetic Nitrogen Use Efficiency (PNUE) (Takashima *et al.*, 2004) normally defined as the amount of carbon fixed per unit of N invested by the plant (Vogan & Sage, 2011; Li *et al.*, 2013). Hikosaka and Shigeno, 2009 describe that higher PNUE in plants is associated with the leaf N content allocated to Rubisco, and its efficiency for carbon fixation. However, exceptions for that can be observed. Analyses of species with the same photosynthetic metabolism show variation in PNUE. The C_4 grass *sorghum bicolor* presents higher PNUE (0.42 μmol mmol s^{-1}) than C_4 eudicotyledon *Amaranthus retroflexus* (0.28 μmol mmol s^{-1}) (Sage *et al.*, 1987; Makino & Ueno, 2018) suggesting one extra feature in grasses that may be contributing with their higher PNUE.

Differently of noncommelinid monocots (e.g. liliales, orchidales and asperagales orders) and all eudicotyledons orders that contain primary type I cell wall, grasses and related commelinids possess a primary type II cell wall with two important changes in their architectures and composition (Tiné *et al.*, 2004). First, a substantial reduction in the content of xyloglucan (1-5% dry weight), and structural proteins (1% dry weight). Second, an increase in glucuronoarabinoxylan (GAX) as the main hemicellulose (20-30% dry weight) and a complex network of cross-linked hydroxycinnamic acids (1-5% dry weight) such as ferulic acid esterified to GAX (FA-GAX). Another conspicuous characteristic present exclusively in Poales order is the presence of β(1→3), β(1→4) mixed-linkage glucans, also dubbed β-glucans (10-30% dry weight), for short (Tiné *et al.*, 2004; Vogel, 2008; de O. Buanafina, 2009; Keegstra, 2010; de Oliveira *et al.*, 2015; Hatfield *et al.*, 2017). In case of type I cell wall, the principal hemicellulose is the xyloglucan (20-25% dry weight), and this has high amount of pectin (20-35% dry weight) and structural proteins (10% dry weight), but low content of phenolic compounds (minor, except order Caryophyllales) (de Oliveira, Buanafina and Cosgrove, 2013; de Oliveira et al., 2015). Because of influence of cell wall composition in NUE, the aim of the current work was to investigate the N saving into structural proteins by replacement to ferulic acid ester linked in the primary type II cell wall. For this proposal, species of grasses and eudicotyledons (C_3 and C_4) were grown in different N concentration, photosynthesis (gas exchange and PNUE), total nitrogen, structural nitrogen and ferulic acid ester-linked in type I and II cell wall were evaluated.

Materials and Methods

Plant growth conditions

The species in this study were selected according to their phylogenetic distribution, ecological growth habitat and seeds availability. Seedlings of *Triticum aestivum* L., *Phalaris, Dicanthelium oligosanthes* (C_3 grasses), *Flaveria pringlei*, *Abelmoschus esculentus* L., *Atriplex lentiformis* (C_3 eudicotyledons), *Saccharum officinarum* L., *Zea mays*, *Setaria viridis* (C_4 grasses), *Blepharis ciliares*, *Amaranthus edulis* and *Gomphrena globosa* (C_4 eudicotyledons) were transplanted into 5-15L pots (1 plant per pot) containing 50% sand, 25% vermiculite and 25% perlite. Plants were grown in an artificially illuminated glasshouse (University of Toronto, Ontario, Canada) at 21/24ºC day and 18/22ºC night, PAR 1200 to 2000 μmol m^{-2} s^{-1} and 60% relative humidity. Plants with different leaf N contents were obtained by watering three times on week those with Johnson-Hoagland´s solution modified contain 0 mM (deficit condition), 1 or 0.5 mM (low condition), 3 or 2 mM (medium condition), and 6 or 4 mM (normal condition) of N (as ammonium nitrate) for 4 weeks. The concentration of K, P, Ca, Mg and the micronutrients were identical in all treatment solutions, including in normal condition. In deficit, low and medium N treatments, SO_4^{-2}, and Cl^- were used to replace NO_3^- (Adapted from Sage et al., 1987). For all N conditions *n*= 4-6 was assumed.

Physiological measurements

Gas exchange measurements were performed on 30-d-old plants on young fully expanded leaf. Measurements were taken on a LiCOR 6400 infrared gas analyzer (Lincoln, NE, USA) as described to Sage et al., 1987 and Onoda et al., 2004. The leaf chamber conditions were: light intensity 1200 μmol m^{-2} s^{-1} PAR to C_3 plants and 2000 μmol m^{-2} s^{-1} PAR to C_4 plants, humidity 60%, leaf temperature 25ºC to C_3 plants and 30ºC to C_4 plants, flow 300 μmol s^{-1} and CO_2 concentration 1000 ppm (Evans & Santiago, 2014).

Leaf area analysis

Leaf area was measured from each plant by excising the young fully expanded leaves, flattening and imaging. Areas were calculated in IMAGEJ (FIJI v.1.51u) using thresholding and the magic wand tool.

Photosynthetic nitrogen use efficiency (PNUE) calculation

PNUE was calculated using photosynthetic rate per unit leaf area divided by leaf N content per unit area (Onoda *et al.*, 2004; Makino & Ueno, 2018).

$$PNUE = A/TN$$

A= photosynthetic rate

TN = total leaf nitrogen

Biochemical assays

Total leaf nitrogen

Leaf discs of 1-7 cm diameter were punched out from each species, if a leaf was too small; the next lower leaf was added. The leaf samples were air-dried at 60ºC for 48 hours, and ground separately in a mill. The total N concentration was quantified using 6 mg of dry leaf in an elemental combustion system (ECS 4010) with a typical machine precision of ±0.01% N (Onoda *et al.*, 2004; Harrison *et al.*, 2009; Hikosaka & Shigeno, 2009).

Cell wall nitrogen

Structural N concentration was obtained using a method adapted from (Onoda *et al.*, 2004; Takashima *et al.*, 2004; Harrison *et al.*, 2009; Gérant *et al.*, 2017). Approximately 50 mg of leaf dried power was mixed in 3 ml of methanol/chloroform/water (12/5/3, v/v/v) in tissue homogenizer to solubilize the non-structural N fraction. After centrifugation (2000*g*, 10 min) the supernatant was discarded. To complete wash pellet, homogenization and centrifugation were repeated twice. The washed pellet was dried overnight at room temperature and re-suspended in 1.5 ml of 62.5 mM citrate buffer at pH 6.8 containing 1% (v/v) sodium dodecyl sulphate (SDS). The samples were agitated, centrifuged (9000*g*, 5 min), and the supernatant with soluble proteins and organelle was removed. This step was repeated twice. The excess of SDS was removed adding 1.5 ml of methanol/chloroform/water (12/5/3, v/v/v) three times. Then, the pellet was air-dried at 60ºC for 24 hours and the dry mass of pellet was assumed to represent the leaf structural biomass. 2-7 mg of structural biomass was used to determine the structural N content in an elemental combustion system (ECS 4010) with a typical machine precision of ±0.01% N.

Alkaline extraction of ferulic acid (FA)

Ester-bound FA was extracted after mild alkaline hydrolysis. In brief, 100 mg of biomass was homogenized with 4 ml of methanol (50%, v/v) and incubated at 80°C for 90 min. After centrifugation (2.180*g*, 4ºC, 15 min), the supernatant was discarded, and the pellet was washed twice as above. The pellet was dried at 60°C for 24 hours. The dry cell wall was resuspended in 5 ml of 0.5 M NaOH and incubated at 96°C for 2 hours. The supernatant was acidified to pH 2.0 with 6 M HCl, centrifuged at 2.180*g*, 4ºC for 15 min and then extracted twice with anhydrous ethyl ether. The ethyl ether extracts were combined and dried at 40°C. The samples were resuspended in methanol/acetic acid 4% (30/70, v/v) and analyzed with a Shimadzu® Liquid Chromatograph (HPLC) equipped with a LC-10AD pump, a CBM-101 Communications Bus Module, a Rheodyne® injector, and a SPD-10A UV-VIS detector. Ferulic acid was separated on C18 column (250 mm × 4.6 mm, 5 µm; Shimpack CLC-ODS (M); Shimadzu®) with equivalent pre-column (10 × 4.6 mm). The mobile phase was methanol/acetic acid 4% (30/70, v/v) with a flow rate of 0.8 ml/min in isocratic mode. Absorption of FA was detected at 322 nm and quantified according to standard values (de Ascensao & Dubery, 2003; M. de Oliveira *et al.*, 2016).

Graphs and statistical analysis

The experimental design was completely randomized, with each plot represented by a plastic pot containing a plant. Data were expressed as the mean of independent experiments $\pm$ SEM. Graphs were produced, and statistical analysis conducted using GraphPad Prism® software package (version 5.01 GraphPad software Inc., USA) where unpaired two-side *t*-test was performed. A significance level of $p \leq 0.05$ was considered for all analysis.

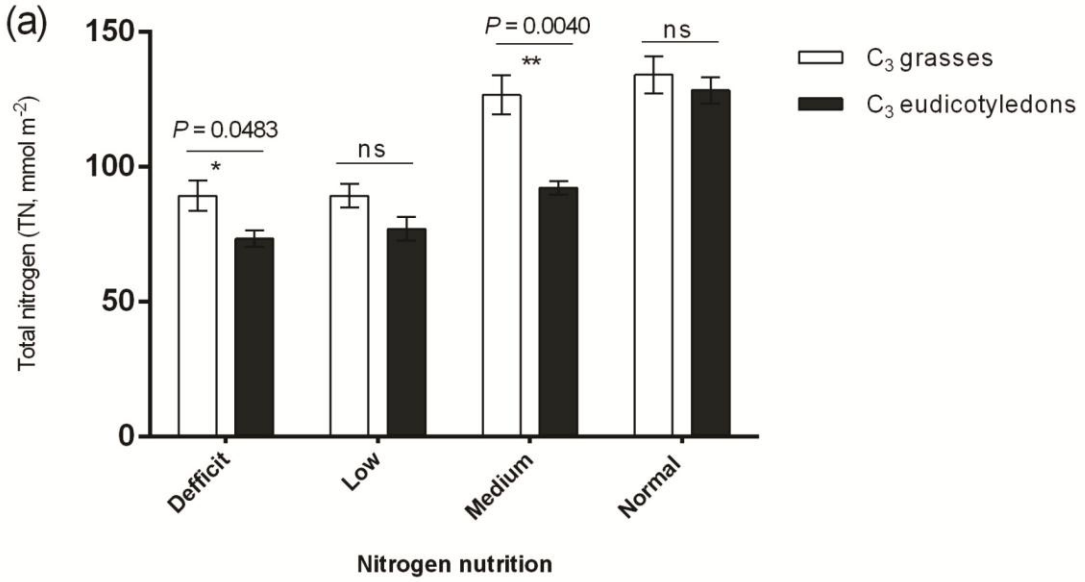
Results

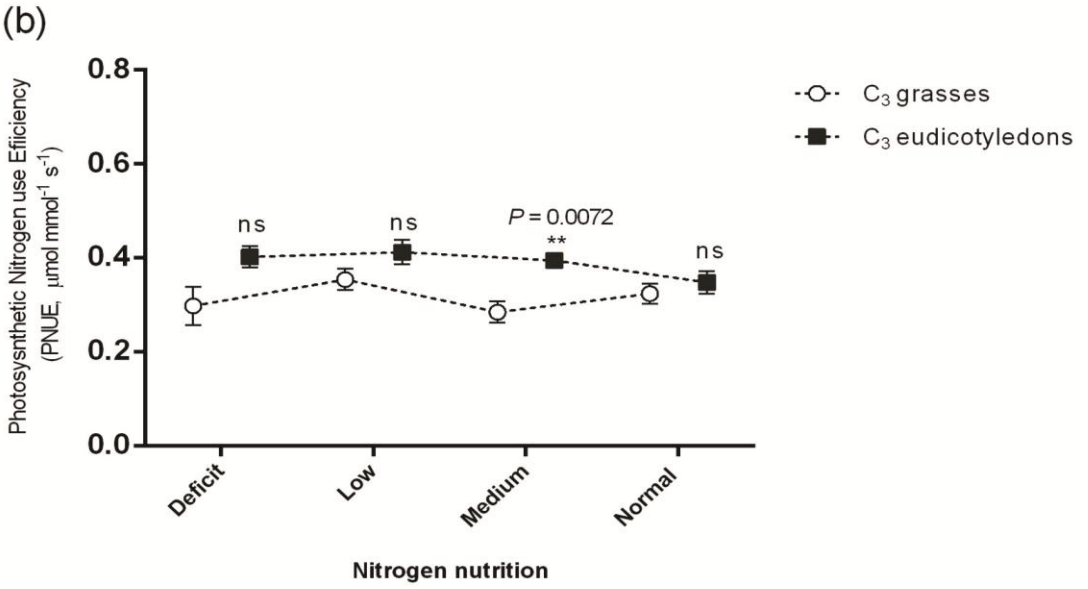
High total N content decreased the PNUE in C_3 grasses

C_3 grasses presented greater total N content in deficit (by 21%) and medium N condition (by 37%), while the total N in these plants did not differ in low and normal N condition when compared with C_3 eudicotyledons (Fig. 2a). The higher total N amount in C_3 grasses in medium N condition reflected directly in their lower PNUE. These plants had 28% lesser PNUE than C_3 eudicotyledons in medium N condition (Fig. 2b).

The type II cell wall in C_3 grasses has not affected their N economy

In both, C_3 grasses and C_3 eudicotyledons in all N condition, the structural N amount in the cell wall was not significantly different. The results showed that the structural N content in both species has increased when the N availability to plant was higher (Fig. 2c). In all N conditions, C_3 grasses presented higher concentration of ferulic acid ester-linked in their cell wall than C_3 eudicotyledons, a characteristic of type II cell wall. However, the content of ferulic acid in C_3 grasses increased 36% in N deficit, when compared with the same plant grown in normal N. C_3 eudicotyledons also presented higher ferulic acid content (by 420%) in N deficit than in normal conditions. But, when compared C_3 eudicotyledons with C_3 grasses in different N condition, the ferulic acid ester-linked was extremely higher in C_3 grasses as follow, 446% in deficit, 467% in low, 1546% in medium and 1990% in normal N condition (Fig. 2d).


(a)
Total nitrogen (TN, mmol m-2)
150
100
50
0
P = 0.0483
*
ns
P = 0.0040
**
ns
Defficit
Low
Medium
Normal
Nitrogen nutrition
C3 grasses
C3 eudicotyledons


(b)
Photosysnthetic Nitrogen use Eficiency (PNUE, µmol mmol-1 s-1)
0.8
0.6
0.4
0.2
0.0
ns
ns
P = 0.0072
**
ns
Deficit
Low
Medium
Normal
Nitrogen nutrition
C3 grasses
C3 eudicotyledons

1154

1155

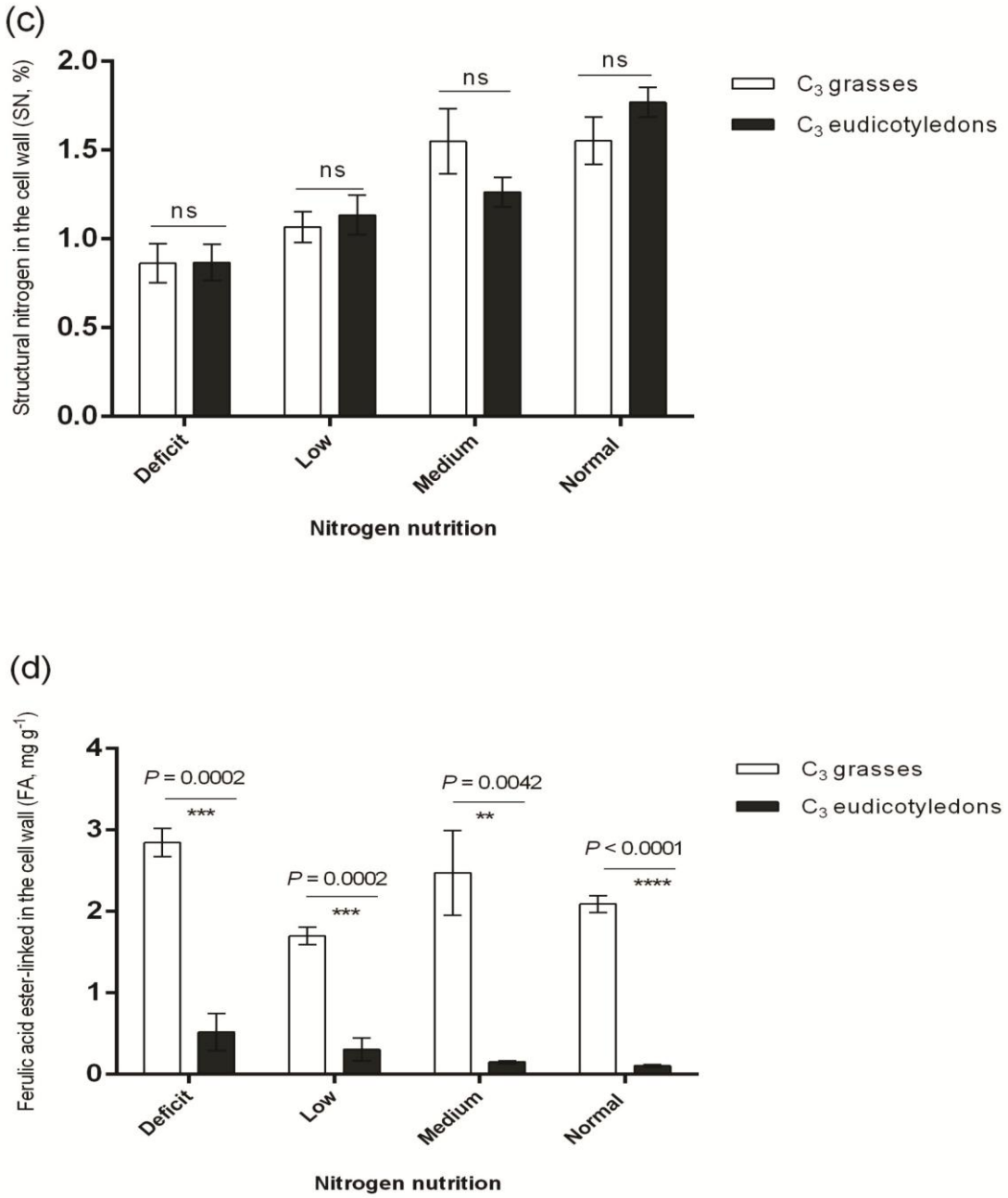


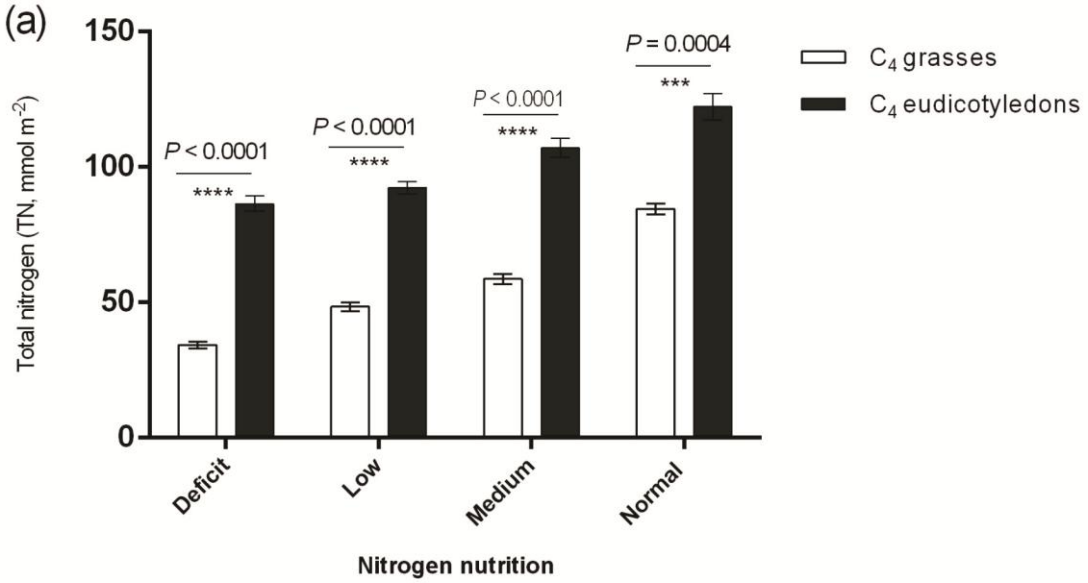
Fig. 2 Physiological and structural traits in C_3 grasses and C_3 eudicotyledons cultivated in different N nutrition. (a) Total N in leaves. (b) photosynthetic nitrogen use efficiency (PNUE). (c) structural N in the cell wall and (d) ferulic acid ester-linked in the cell wall. *Mean values differ statistically between species in the same N nutrition ($p \leq 0.05$). **Mean values differ statistically between species in the same N nutrition ($p \leq 0.01$). ***Mean values differ statistically between species in the same N nutrition ($p \leq 0.001$). ****Mean values differ statistically between species in the same N nutrition ($p \leq 0.0001$). n = 4-6 biological replicates, ±SEM, unpaired two-side t-test. ns, not significant.

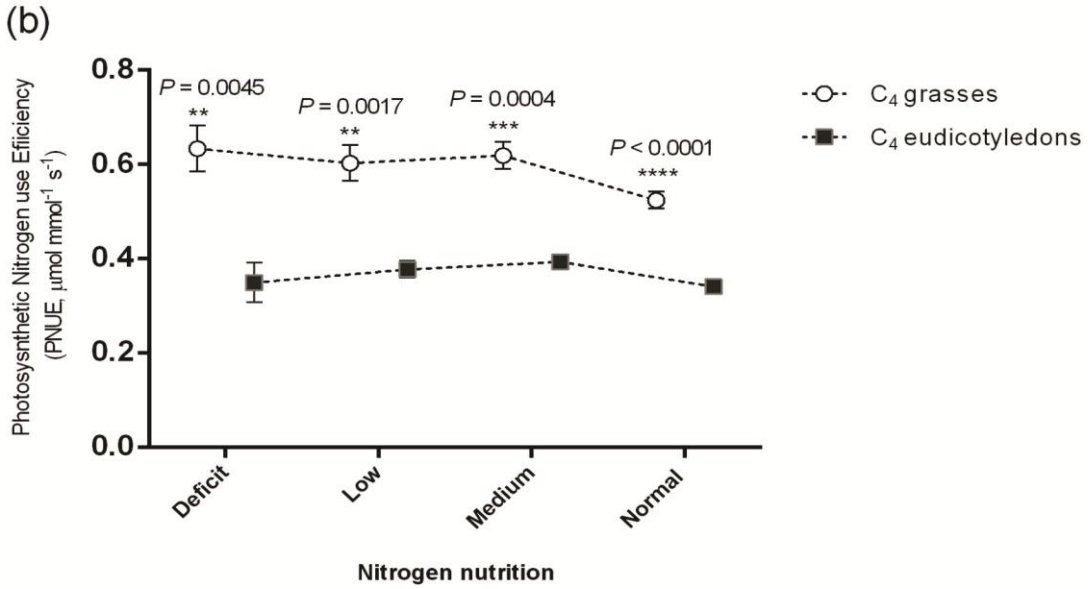
Reduced total N content influenced the greater PNUE in C_4 grasses

C_4 grasses presented lower total N amount in their leaves than C_4 eudicotyledons in all N condition. In N deficit, the total N content in C_4 grasses was 60% lower than in C_4 eudicotyledons. This difference decreased in low and medium N until it arrives 31% in normal N condition (Fig. 3a). The reduced total N content in C_4 grasses reflects in their higher PNUE when compared to C_4 eudicotyledons. When the N was limiting, C_4 grasses increased 81% PNUE. In low, medium and normal N condition, the PNUE in C_4 grasses continued higher than PNUE observed in C_4 eudicotyledons, arriving 60%, 57% and 54% respectively (Fig. 3b).

The type II cell wall architecture in C_4 grasses have contributed to their N economy

In general, C_4 grasses contained lower amount of structural N in their cell wall than C_4 eudicotyledons, except in medium N condition. The structural N content decreased significantly in C_4 grasses in N scarcity, 58% in deficit and 42% in low N condition in comparison to corresponding C_4 eudicotyledons. In normal N condition, C_4 grasses still possess lesser structural N content than C_4 eudicotyledons, arriving in 26%. In both species, the structural N in the cell wall has increased according to availability of N into soil (Fig. 3c). In other hand, the ferulic acid ester-linked in the cell wall was higher in C_4 grasses than in C_4 eudicotyledons in all N conditions as follow, 177% in deficit, 362% in low, 307% in medium and 267% in normal N condition. When compared C_4 grasses in deficit with C_4 grasses in normal N condition, plants in deficit presented 113% greater ferulic acid ester-linked than plants in normal condition. This behavior also was observed in C_4 eudicotyledons, where, plants in N deficit had 181% more ferulic acid ester-linked than plants in normal condition (Fig. 3d).


(a)
Total nitrogen (TN, mmol m-2)
150
100
50
0
P < 0.0001
****
P < 0.0001
****
P < 0.0001
****
P = 0.0004
***
Deficit
Low
Medium
Normal
Nitrogen nutrition
C4 grasses
C4 eudicotyledons


(b)
Photosysnthetic Nitrogen use Efiiciency (PNUE, µmol mmol-1 s-1)
0.8
0.6
0.4
0.2
0.0
P = 0.0045
**
P = 0.0017
**
P = 0.0004
***
P < 0.0001
****
Deficit
Low
Medium
Normal
Nitrogen nutrition
C4 grasses
C4 eudicotyledons

1193

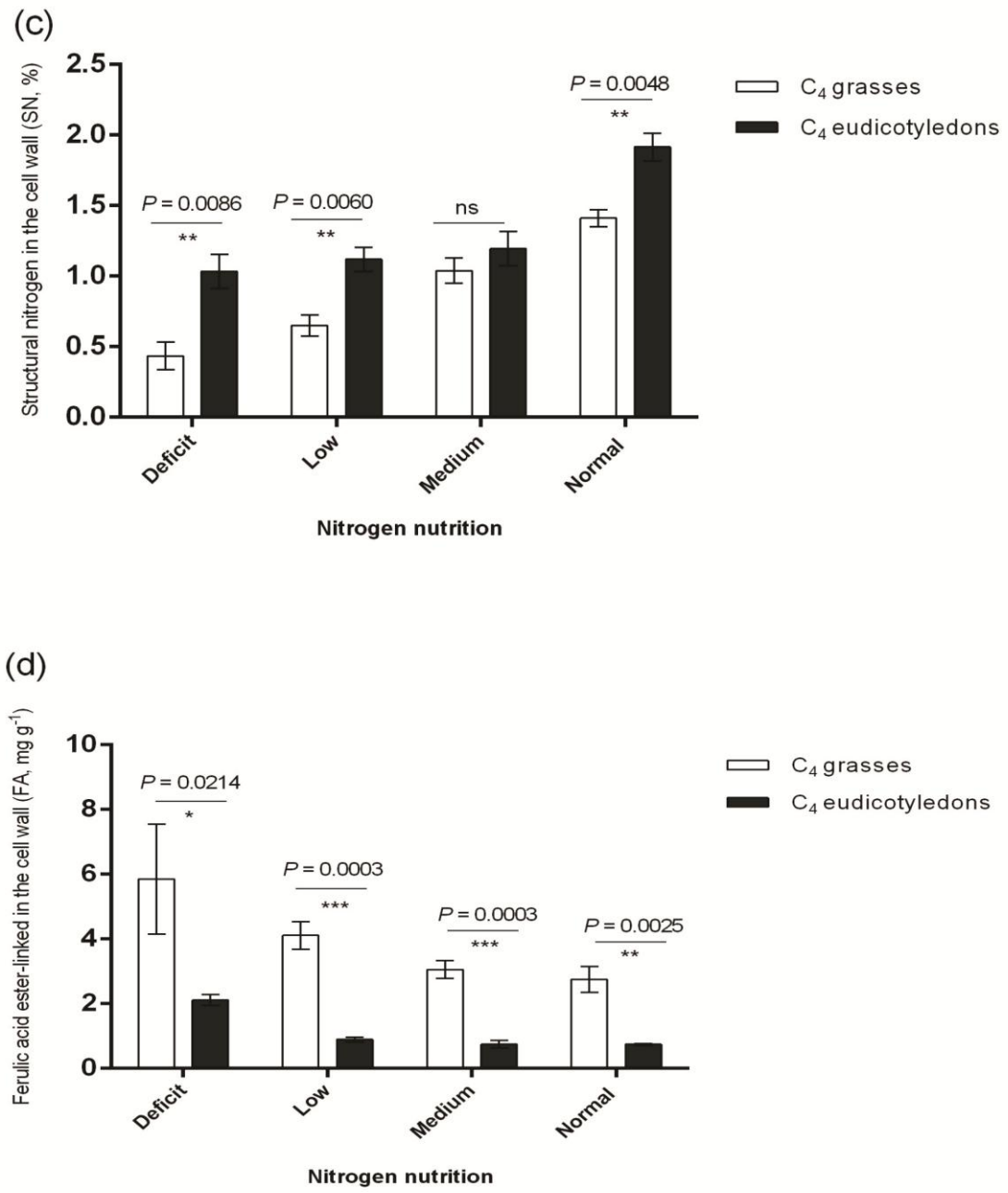


Fig. 3 Physiological and structural traits in C_4 grasses and C_4 eudicotyledons cultivated in different N nutrition. (a) Total N in leaves. (b) photosynthetic nitrogen use efficiency (PNUE). (c) structural N in the cell wall and (d) ferulic acid ester-linked in the cell wall. *Mean values differ statistically between species in the same N nutrition ($p \leq 0.05$). **Mean values differ statistically between species in the same N nutrition ($p \leq 0.01$). ***Mean values differ statistically between species in the same N nutrition ($p \leq 0.001$). ****Mean values differ statistically between species in the same N nutrition ($p \leq 0.0001$). n = 4-6 biological replicates, ±SEM, unpaired two-side t-test. ns, not significant.

Discussion

Our investigation showed that the total N content in all species, both C_3 and C_4 tend to increase when higher N concentration is available into substrate (Fig. 2a and 3a). In conditions where the N was the major limiting factor to growth, C_4 grasses have increased PNUE when compared with C_4 eudicotyledons (Fig. 3b). Similar results have been reported in *Sorghum bicolor*, which presented lower total N content (28 mmol m^{-2}) and higher PNUE (0.64 μmol $mmol^{-1}$ s^{-1}) in N scarcity. This response might be a common feature in C_4 grasses, suggesting that plants use N more efficiently to preserve photosynthesis efficiency (Ghannoum et al., 2005; Makino and Ueno, 2018). In general, two principal features are attributed to explain the greater PNUE in C_4 plants: i) C_4 metabolism suppresses photorespiration, especially at higher temperatures (Sage *et al.*, 1987, Makino, 2003, Li et al., 2013) what increases photosynthetic rates, and ii) because of that they allocate only 5 to 9% of N into Rubisco (contrasting with up to 40% in C_3 plants). However, our results revealed that C_4 grasses had higher PNUE than C_4 eudicotyledons in all conditions of N nutrition, despite both species have C_4 photosynthetic metabolism (Fig. 3b). Thus, additional features in C_4 grasses must respond for that difference. Our data suggest that the type II cell wall composition in C_4 grasses contributes to the N economy and improved NUE in C_4 plants. The structural nitrogen analysis (Fig. 2c and 3c) and ferulic acid ester-linked (Fig. 2d and 3d) in this study indicated the replacement of structural proteins by ferulic acid in the type II cell wall, might have influenced the N economy. The structural proteins (e.g. extensins) in the cell wall are the major nitrogenous compounds cross-linking the components in the wall, helping to maintain the cell wall integrity and to protect the cell against pathogen and herbivore attack (Lamport *et al.*, 2011). Ferulic acid cross-linkage presents similar functions of those performed by structural proteins; however, they do not demand for N in their chemical structure (Vogel, 2008; Albenne et al., 2014; Hatfield et al., 2017).

Although the percentage of structural proteins only in leaves is not enough to respond for all the difference in total N found in leaves, the N economy in type II cell wall is notable considering the whole plant. For example, an estimate of the content of structural N in whole plant showed that C_4 grasses grown in N poor soils have invested between 2.6 to 4 mg of N to produce structural proteins. In contrast, C_4 eudicotyledons have allocated 4.5 to 7 mg of N to structural proteins. In turn C_4 grasses and C_4 eudicotyledons have used both about 13 mg of N to produce Rubisco (Almeida et al.,

2016). Thus, the amount of N allocated in structural proteins represents between 20-30% of the N allocated in Rubisco in C_4 grasses, and can reach more than 50% of the N allocated in Rubisco in C_4 eudicotyledons.

Therefore, our data suggest that during the evolution of C_4 plants, N limitation might have worked as a selection pressure to enhance N use efficiency. Thanks to their special kind of cell wall architecture, grasses could transfer the function performed by structural proteins to ferulic acid reducing the demand for N to build their cell walls. In C_3 plants, the proportion of structural proteins in the cell wall is similar and not affecting the PNUE in these plants (Fig. 2b, c). The high concentration of ferulic acid ester-liked in C_3 grasses (Fig. 2d) appeared to be exclusively related with the metabolism of the type II cell wall since it did not affect PNUE in these plants. Here, the CO_2 is still the principal limiting factor to plant growth, because of photorespiration, and the type II cell wall has not adapted to contribute to N economy, even in deficit.

Conclusion

Besides the increase in PNUE provided by the reduction avoiding photorespiration and reduction in Rubisco content in C_4 metabolism, other components seem to have been selected to contribute for the N economy in C_4 plants. Our data suggest that in C_4 grasses, cell wall architecture provides an additional adaptation for allow an economy of N. The low content of structural N and the high content of ferulic acid in C_4 grasses when compared with C_4 eudicotyledons suggests that, during evolution, the distinct type II cell wall of C_4 grasses provided an additional way to reduce N demand: the partial replacement of extensins-like structural proteins, by ferulic acid in the function of cross-linking the cell wall components in type II cell walls. The higher PNUE provided by their enhanced photosynthetic metabolism together with the lower content of structural proteins in the type II cell walls may have contributed synergistically to the conspicuous ecological success that grasses obtained in the planet.

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Enhancement of Higher Education Personal (CAPES). G. E. Barreto is recipient of CAPES fellowship.

Author contributions

G.E.B and W.D.S wrote the manuscript, and the last author agrees to serve as the author responsible for contact and ensures communication; M.S and R.F.S supervised the research; O.F.F helped with results discussion. All the authors read and approved the final manuscript.

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Supplementary data

C_3 grasses

Triticum aestivum L.

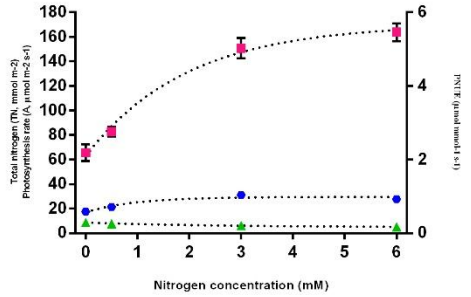

Figure 1. Relationships between some major leaf traits in **Wheat** cultivated in different N concentration. (a) Total nitrogen (TN, ■); (b) Light-saturated photosynthetic rate per unit area (A, ●) and (c) photosynthetic N use efficiency (PNUE, ▲) (=A/TN). (a) no correlation, n=4, R^2 = 0,8774, P<0,0633. (b) no correlation, n=4, R^2 = 0,5874, P<0,2336. (c) correlation, n=4, R^2 = 0,9230 , P<0,0393.

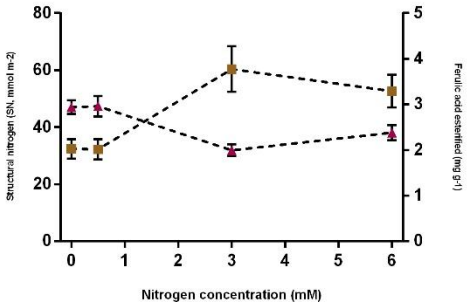

Figure 2. Relationships between stuctural nitrogen and ferulic acid in the cell wall of **Wheat** cultivated in different N concetration. (a) Structural nitrogen (SN, ■); (b) Ferulic acid (FA, ▲). (a) no correlation, n=4, R^2 = 0,5946, P<0,2289. (b) no correlation, n=4, R^2 = 0.4719, P<0,3130.

Dicanthelium oligosanthes

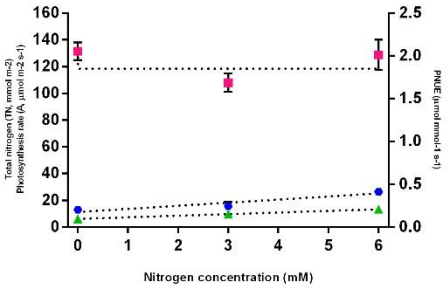

Figure 3. Relationships between some major leaf traits in ***Dicanthelium oligosanthes*** cultivated in different N concentration. (a) Total nitrogen (TN, ■); (b) Light-saturated photosynthetic rate per unit area (A, ●) and (c) photosynthetic N use efficiency (PNUE, ▲) (=A/TN). (a) no correlation, n=4, R^2 = 0,07256, P<0,7306. (b) no correlation, n=4, R^2 = 0,6944, P<0,1667. (c) no correlation, n=4, R^2 = 0,4763, P<0,3098.

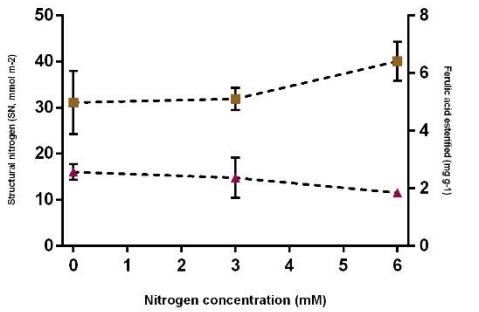

Figure 4. Relationships between stuctural nitrogen and ferulic acid in the cell wall of ***Dicanthelium oligosanthes*** cultivated in different N concetration. (a) Structural nitrogen (SN, ■); (b) Ferulic acid (FA, ▲). (a) no correlation, n=4, R^2 = 0,8157, P<0,2825. (b) no correlation, n=4, R^2 = 0,9388, P<0,1591.

Phalaris

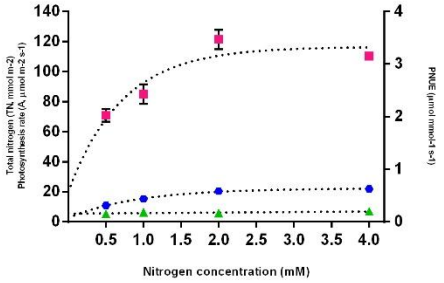

Figure 5. Relationships between some major leaf traits in ***Phalaris*** cultivated in different N concentration. (a) Total nitrogen (TN, ■); (b) Light-saturated photosynthetic rate per unit area (A, ●) and (c) photosynthetic N use efficiency (PNUE, ▲) (=A/TN). (a) no correlation, n=4, R^2=0,5236, P<0,2764. (b) no correlation, n=4, R^2=0,7946, P<0,1086. (c) no correlation, n=4, R^2 = 0,6573, P<0,1892.

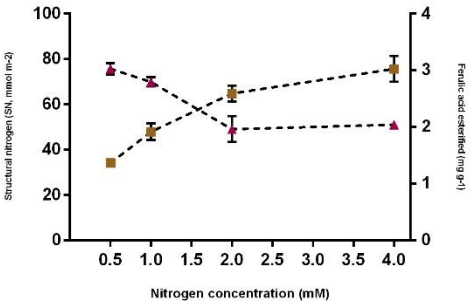

Figure 6. Relationships between stuctural nitrogen and ferulic acid in the cell wall of ***Phalaris*** cultivated in different N concetration. (a) Structural nitrogen (SN, ■); (b) Ferulic acid (FA, ▲). (a) no correlation, n=4, R^2=0,8850, P<0,0593. (b) no correlation, n=4, R^2=0,6651, P<0,1845.

1415

1416 ***C_3 eudicotyledons***

Flaveria pringlei

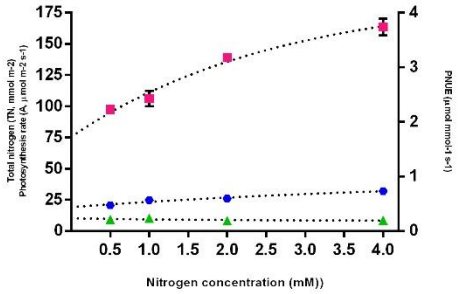

Figure 7. Relationships between some major leaf traits in ***Flaveria pringlei*** cultivated in different N concentration. (a) Total nitrogen (TN, ■); (b) Light-saturated photosynthetic rate per unit area (A, ●) and (c) photosynthetic N use efficiency (PNUE, ▲) (=A/TN). (a) correlation, n=3, R^2=0,9513 , P<0,0247. (b) correlation, n=3, R^2=0,9454, P<0,0277. (c) no correlation, n=3, R^2 = 0,3405, P<0,4165.

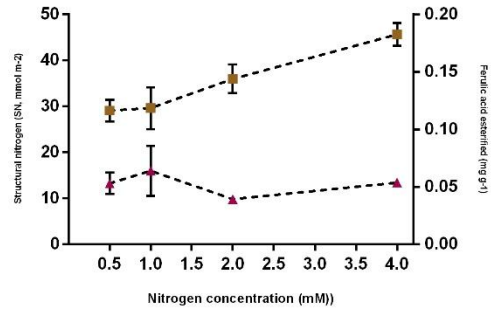

Figure 8. Relationships between stuctural nitrogen and ferulic acid in the cell wall of ***Flaveria pringlei*** cultivated in different N concetration. (a) Structural nitrogen (SN, ■); (b) Ferulic acid (FA, ▲). (a) correlation, n=4, R^2=0,9891, P<0,0055. (b) no correlation, n=4, R^2=0,04727, P<0,7826.

Abelmoschus esculentus

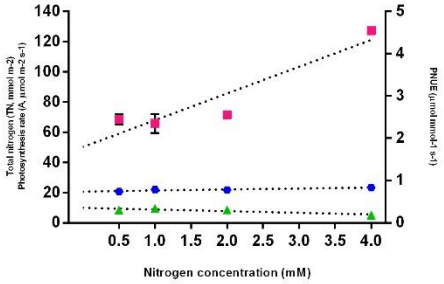

Figure 9. Relationships between some major leaf traits in ***Okra*** cultivated in different N concentration. (a) Total nitrogen (TN, ■); (b) Light-saturated photosynthetic rate per unit area (A, ●) and (c) photosynthetic N use efficiency (PNUE, ▲) (=A/TN). (a) no correlation, n=4, R^2=0,8731, P<0,0656. (b) no correlation, n=4, R^2= 0,8349, P<0,0862. (c) no correlation, n=4, R^2 = 0,8159, P<0,0967.

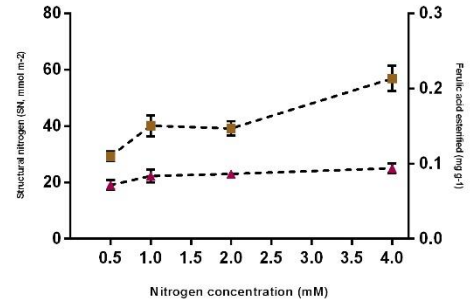

Figure 10. Relationships between stuctural nitrogen and ferulic acid in the cell wall of ***Okra*** cultivated in different N concetration. (a) Structural nitrogen (SN, ■); (b) Ferulic acid (FA, ▲). (a) no correlation, n=4, R^2=0,8985, P<0,0521. (b) no correlation, n=4, R^2=0,8105, P<0,0997.

1417

Atriplex lentiformis

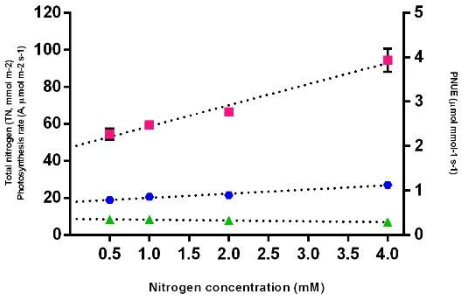

Figure 11. Relationships between some major leaf traitsin ***Atriplex lentiformis*** cultivated in different N concentration. (a) Total nitrogen (TN, ■); (b) Light-saturated photosynthetic rate per unit area (A, ●) and (c) photosynthetic N use efficiency (PNUE, ▲) (=A/TN). (a) correlation, n=4, R^2=0,9808, P<0,0096. (b) correlation, n=4, R^2 = 0,9771, P<0,0115. (c) correlation, n=4, R^2 = 0,9873, P<0,0064.

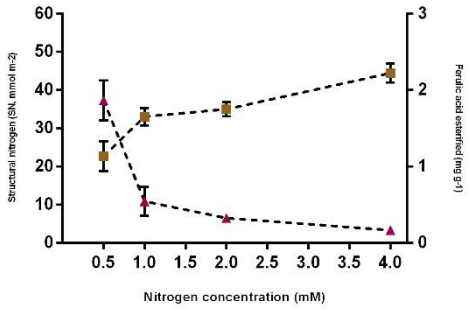

Figure 12. Relationships between stuctural nitrogen and ferulic acid in the cell wall of ***Atriplex lentiformis*** cultivated in different N concetration. (a) Structural nitrogen (SN, ■); (b) Ferulic acid (FA, ▲). (a) no correlation, n=4, R^2 = 0,8730, P<0,0656. (b) no correlation, n=4, R^2 = 0,5405, P<0,2648.

1418

1419 ***C_4 grasses***

Saccharum officinarum

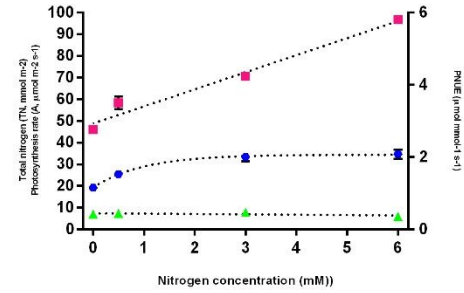

Figure 13. Relationships between some major leaf traits in **Sugar cane** cultivated in different N concentration. (a) Total nitrogen (TN, ■); (b) Light-saturated photosynthetic rate per unit area (A, ●) and (c) photosynthetic N use efficiency (PNUE, ▲) (=A/TN). (a) correlation, n=4, R^2 = 0,9697, P<0,0153. (b) no correlation, n=4, R^2 = 0,7907, P<0,1108. (c) no correlation, n=4, R^2 = 0,3133, P<0,4402.

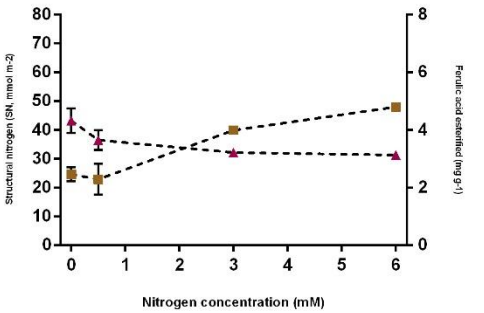

Figure 14. Relationships between stuctural nitrogen and ferulic acid in the cell wall of **Sugar cane** cultivated in different N concetration. (a) Structural nitrogen (SN, ■); (b) Ferulic acid (FA, ▲). (a) correlation, n=4, R^2 = 0,9488, P<0,0259. (b) no correlation, n=4, R^2 = 0,6996, P<0,1636.

Zea mays

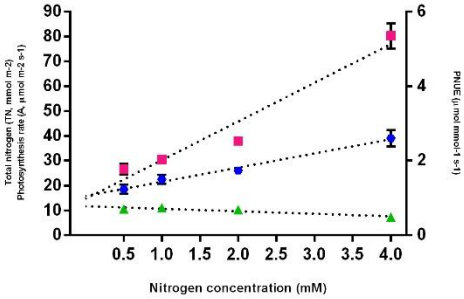

Figure 15. Relationships between some major leaf traits in **Maize** cultivated in different N concetration. (a) Total nitrogen (TN, ■); (b) Light-saturated photosynthetic rate per unit area (A, ●) and (c) photosynthetic N use efficiency (PNUE, ▲) (=A/TN). (a) correlation, n=4, R^2 = 0,9514, P<0,0246. (b) correlation, n=4, R^2 = 0,9892, P<0,0054. (c) no correlation, n=4, R^2 = 0,8683, P<0,0682.

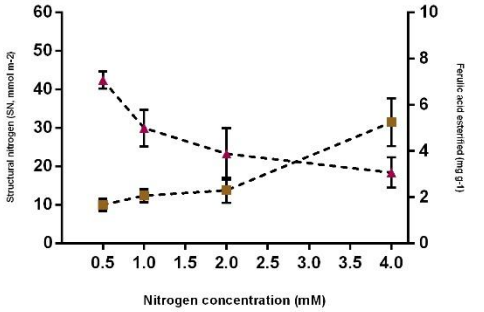

Figure 16. Relationships between stuctural nitrogen and ferulic acid in the cell wall of **Maize** cultivated in different N concetration. (a) Structural nitrogen (SN, ■); (b) Ferulic acid (FA, ▲). (a) correlation, n=4, R^2 = 0,9292, P<0,0360. (b) no correlation, n=4, R^2 = 0,7736, P<0,1205.

1420

Setaria viridis

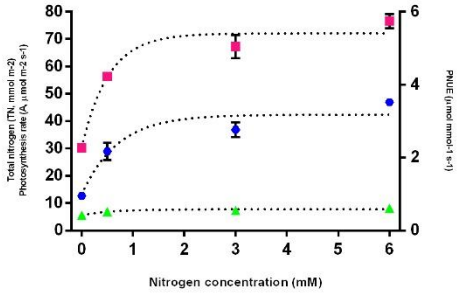

Figure 17. Relationships between some major leaf traits in ***Setaria viridis*** cultivated in different N concentration. (a) Total nitrogen (TN, ■); (b) Light-saturated photosynthetic rate per unit area (A, ●) and (c) photosynthetic N use efficiency (PNUE, ▲) (=A/TN). (a) no correlation, n=4, R^2 = 0,7408, P<0,1393. (b) no correlation, n=4, R^2 = 0,8315, P<0,0881. (c) no correlation, n=4, R^2 = 0,8338, P<0,0869.

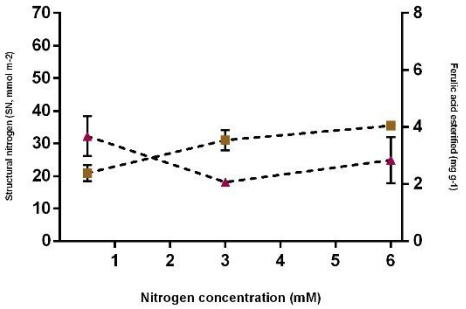

Figure 18. Relationships between stuctural nitrogen and ferulic acid in the cell wall of ***Setaria viridis*** cultivated in different N concetration. (a) Structural nitrogen (SN, ■); (b) Ferulic acid (FA, ▲). (a) no correlation, n=4, R^2 = 0,9280, P<0,1730. (b) no correlation, n=4, R^2 = 0,2233, P<0,6867.

1421

1422 ***C_4 eudicotyledons***

Blepharis ciliares

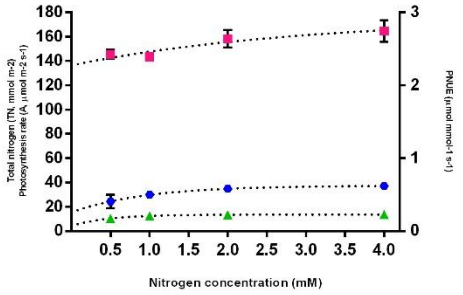

Figure 19. Relationships between some major leaf traits in ***Blepharis ciliares*** cultivated in different N concentration. (a) Total nitrogen (TN, ■); (b) Light-saturated photosynthetic rate per unit area (A, ●) and (c) photosynthetic N use efficiency (PNUE, ▲) (=A/TN). (a) no correlation, n=4, R^2 =0,8722 , P<0,0661. (b) no correlation, n=4, R^2 = 0,8111 , P<0,0994. (c) no correlation, n=4, R^2 = 0,6243, P<0,2098.

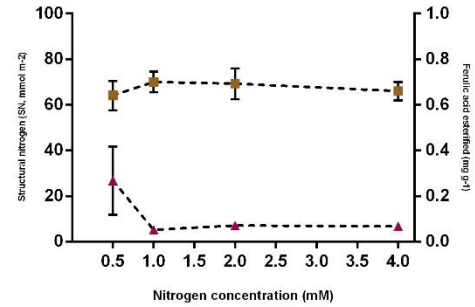

Figure 20. Relationships between stuctural nitrogen and ferulic acid in the cell wall of ***Blepharis ciliares*** cultivated in different N concetration. (a) Structural nitrogen (SN, ■); (b) Ferulic acid (FA, ▲). (a) no correlation, n=4, R^2 = 0,001533, P<0,9609. (b) no correlation, n=4, R^2 = 0,3025, P<0,4500.

Amaranthus edulis

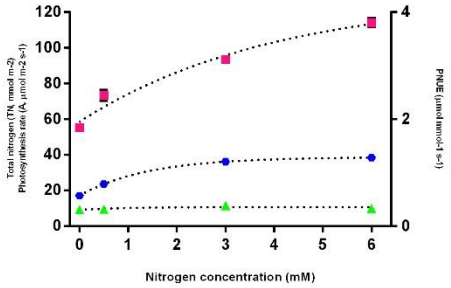

Figure 21. Relationships between some major leaf traits in ***Amaranthus edulis*** cultivated in different N concentration. (a) Total nitrogen (TN, ■); (b) Light-saturated photosynthetic rate per unit area (A, ●) and (c) photosynthetic N use efficiency (PNUE, ▲) (=A/TN). (a) correlation, n=4, R^2 = 0,9410, P<0,0299. (b) no correlation, n=4, R^2 = 0,8398, P<0,0836. (c) no correlation, n=4, R2 = 0,4295, P<0,5705.

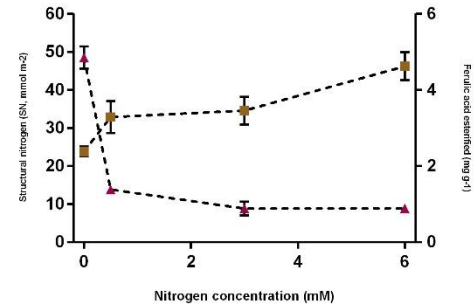

Figure 22. Relationships between stuctural nitrogen and ferulic acid in the cell wall of ***Amaranthus edulis*** cultivated in different N concetration. (a) Structural nitrogen (SN, ■); (b) Ferulic acid (FA, ▲). (a) correlation, n=4, R^2 = 0,9292, P<0,0360. (b) no correlation, n=4, R^2 = 0,7736, P<0,1205.

1423

Gomphrena globosa

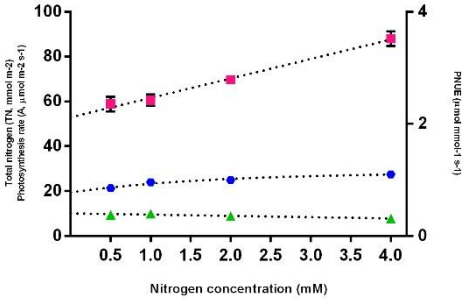

Figure 23. Relationships between some major leaf traits in ***Gomphrena globosa*** cultivated in different N concentration. (a) Total nitrogen (TN, ■); (b) Light-saturated photosynthetic rate per unit area (A, ●) and (c) photosynthetic N use efficiency (PNUE, ▲) (=A/TN). (a) correlation, n=4, R^2=0,9923, P<0,0039. (b) no correlation, n=4, R^2=0,9007, P<0,0509. (c) no correlation, n=4, R2 = 0,8689, P<0,0679.

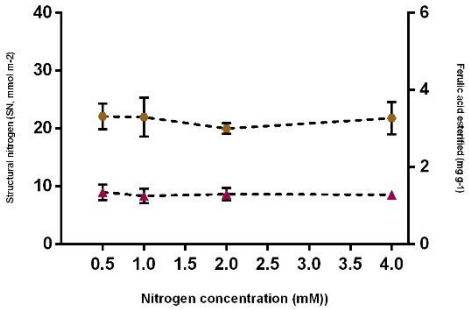

Figure 24. Relationships between stuctural nitrogen and ferulic acid in the cell wall of ***Gomphrena globosa*** cultivated in different N concetration. (a) Structural nitrogen (SN, ■); (b) Ferulic acid (FA, ▲). (a) no correlation, n=4, R^2=0,03172, P<0,8219. (b) no correlation, n=4, R^2=0,1010, P<0,6821.

Ferulic acid exaptation and N economy in grass cell wall

Short title: Grass cell wall evolution

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ABSTRACT

Grass covers ¼ all green cover in the world. The most important agricultural cultures responsible for producing food and bioenergy are grasses. The oldest Poaceae lineages known are from 71 million years ago. The dry, warm habitats and the lower content of atmospheric CO_2 have driven the C_4 plants evolution from C_3 ancestors. The C_4 photosynthesis represents an important example of convergent evolution with 66 known independent origins, both in monocot and eudicot angiosperms. An important distinction between grasses and eudicots is that the first presents a type II cell wall. Poor in nitrogenated components as extensin proteins, this cell wall presents higher amounts of ferulic acid (FA) ester-linked to glucuronoarabinoxylan (GAX). Both, extensins and FA-GAX perform similar roles in cell wall: cross-linkage of cell wall polymers, controlling cell growth and cell wall recalcitrance to pathogens. However, FA-GAX does not demand N in their chemical structure. Since C_4 species are more efficient in CO_2 assimilation, N becomes the most limiting factor for plant growth. Therefore, the decrease in cell wall N by transferring the function of extensins to FA-GAX could have maximized the growth potential of grasses in poor soils, helping them to conquer their high evolutionary success.

Keywords: ferulic acid, grasses cell wall, grasses evolution, structural proteins.

INTRODUCTION

Grasses are the most widespread family of plants in agriculture and natural ecosystems. They perform a fundamental role to our economic development by sequestering the excess of carbon we produce and therefore mitigating the global warming (O'Mara 2012; Tomaškin & Tomaškinová 2012). Grasses have also an immense impact in human development being in the base of cattle raising and agriculture of most of the great civilizations worldwide: for instance, rice in Asia, wheat in Mesopotamia and Egypt, sorghum in Europe and maize in America. Grasses are used for building (bamboo), food (rice, wheat, maize), forage (*Panicum*, *Brachiaria*), and bioenergy industry (maize, sugarcane) (Cotton *et al.* 2015; Hodkinson 2018). The objective of this approach was to clarify the influence of type II cell wall composition, especially the low content of extensins and high amount of FA-GAX in the evolutionary process and success of grasses.

THE CELL WALL

Since Robert Hook's first description of the empty spaces surrounded by "cell walls" in slices of cork reproduced in his 1664 *Micrographia* (Lampugnani, Khan, Somssich & Persson 2018), many sophisticated tools have been improved our knowledge about the cell wall origins, composition, organization and functions. The cell walls determine sizes and shapes to different cells, tissues and organs. Cell walls create a protective barrier against potential pathogen and form an interface among adjacent cells, allowing the intercellular communication (Keegstra 2010). These can also be the site for nodule symbiosis and arbuscular mycorrhiza in *Petunia hybrida* with the fungus belongs to *Glomeromycota* order (Rich et al., 2014). Nonetheless, in some tissues e.g.

xylem, fibers, and the cork observed by Hook, the cell wall is the only remnant of the cells that once built them.

In the same plant development stage, cell walls can be classified in primary and secondary. Primary is capable of sustaining cell growth. In many tissues, the cell can conserve such a feature even after the cell has reached its final expansion as parenchyma cells. In other cells, after the expansion is finished, the cells may start to deposit new layer of cell wall next to the plasma membrane to produce a thickened secondary cell wall (Albersheim et al., 2011). Primary and secondary cell walls may present similar composition or differ in the arrangement, mobility, mechanical properties and structure of polymers. The primary cell wall is thin, hydrated and it is fundamental for plant morphogenesis. It is composed by cellulose cross-linked by hemicelluloses and pectin. On the other hand, the secondary wall provides rigidity and strength to plant. This rigidity is in part due to lignin that is embedded in cellulose and hemicellulose molecules. Lignin is related to recalcitrance of cell wall that make more difficult the chemical and enzymatic degradation (Cosgrove & Jarvis 2012). Studies with *Arabidopsis* expressing transcription factors (AP2/ERFs) that regulate primary cell wall deposition show complete substitution of secondary to primary cell wall. This substitution allows to create plants biomass less recalcitrant to saccharification processes (Sakamoto *et al.* 2018).

Angiosperms present at least two very distinct types of primary cell walls. The type I is found in eudicotyledons and noncommelinid monocots (e.g. liliales, orchidales and asperagales orders). The principal hemicellulose is the xyloglucan (20-25% dry weight) and high content of pectin (20-35% dry weight) and structural proteins (10% dry weight), but low content of phenolic compounds (minor, except order Caryophyllales) (de Oliveira Buanafina & Cosgrove 2013; de Oliveira *et al.* 2015). In

contrast, grasses and related commelinids possess a type II cell wall (Fig. 1), which presents two important changes in their architecture and composition (Tiné, Urbanowicz, Rayon, Buckeridge & Carpita 2004). First, a substantial reduction in the content of xyloglucan, pectin and structural proteins. Second, an increase in glucuronoarabinoxylan (GAX) as the main hemicellulose (20-30% dry weight) and a complex network of cross-linked hydroxycinnamic acids (1-5% dry weight) such as ferulic esterified to GAX (FA-GAX). Another conspicuous characteristic present exclusively in Poales order is the presence of β(1→3), β(1→4) mixed-linkage glucans, also dubbed β-glucans, (10-30% dry weight), for short (Tiné *et al.* 2004; de O. Buanafina 2009; Keegstra 2010; de Oliveira *et al.* 2015; Hatfield, Rancour & Marita 2017).

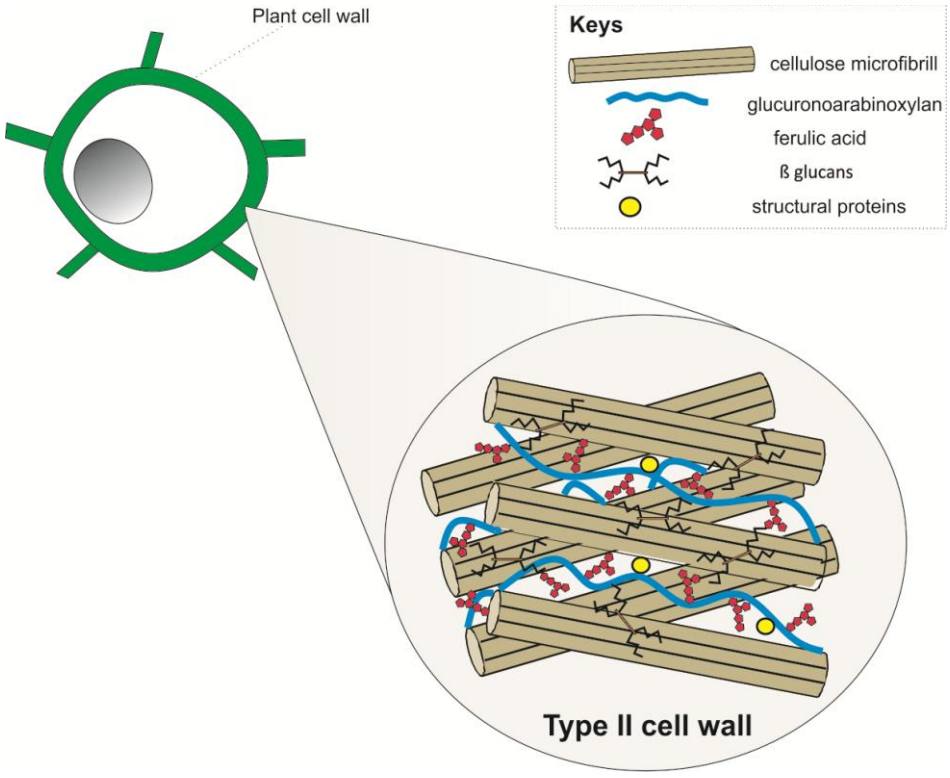

Fig. 1 Illustration of primary type II cell wall presents in grasses. Cellulose microfibril is the major polysaccharides that connected glucuronoarabinoxylan branched with ferulic acid (FA-GAX). Other hemicellulose presents exclusively in Poales order is β-glucan. Low amount of structural proteins is observed in this type cell wall suggesting the replacement of that by FA-GAX, which might have contributed to N economy and success of grasses during their evolutionary process.

GRASSES EVOLUTION

Grasses belong to Poaceae, one of the largest families of flowering plants with a single seed leaf (monocotyledons), corresponding to approximately 11,500-12,000 species, divided in 750-770 genera, which inhabits all continents (Glémin & Bataillon 2009; Bouchenak-Khelladi, Verboom, Savolainen & Hodkinson 2010; Saarela *et al.* 2018). In the evolutionary process of angiosperms, they became the fifth most species-rich family of flowering plants (Hodkinson, 2018). However, the ecological success is also reflected in the fact that grasses respond for 25% of the green cover of the planet (Sage & Stata 2015).

Phylogenetic and fossil data have contributed to presume where and when grasses emerged. The first lineages of Poaceae were discovered by fossils data (phytoliths) in the Late Cretaceous in northern Gondwana in Africa and South America 71 Mya (Soreng *et al.* 2015; Wang *et al.* 2015). Piperno 2005 revealed that dinosaurs as Tyrannosaurus sauropods probably ate the first grass lineages. Studies of Glémin & Bataillon 2009; Strömberg 2011; Hodkinson 2018 showed that phytoliths found on the India continent indicate that five subgroups of grasses achieved a greater distribution, suggesting that the diversification happened in the Eocene 15 Mya (Bouchenak-Khelladi *et al.* 2010).

The grass family is monophyletic, consisting of two major clades (BOP and PACMAD) that includes 12 subfamilies (Soreng *et al.* 2015; Rangan, Furtado & Henry 2016; Hodkinson 2018; Saarela *et al.* 2018). The BOP clade (often quoted as the BEP clade) is composed to three subfamilies: Bambusoideae, Oryzoideae, and, Pooideae. Next, the PACMAD clade contains six subfamilies: Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae, and, Danthonioidae. Three diverging subfamilies (Anomochloideae, Puelioideae and Pharoideae) are considered the

1599 successive sister groups of BOP and PACMAD clades (Cotton *et al.* 2015; Burke *et al.*
1600 2016; Soreng *et al.* 2017).

1601 Within grasses, 45% of species (5044 species) fix the CO_2 using the C_4
1602 photosynthesis (Rangan *et al.* 2016; Haven & Thomas 2017). As a consequence of their
1603 separate compartments in the leaf, bundle sheath and mesophyll cells (Kranz anatomy),
1604 C_4 plants can concentrate CO_2 around the enzyme Ribulose-1,5-bisphosphate-
1605 carboxylase/oxigenase, Rubisco, limiting its oxigenase activity known as
1606 photorespiration (Sage *et al.* 2013) and increasing 1.3 to 4 times the instantaneous water
1607 use efficiency and nitrogen use efficiency when compared to C_3 plants (Sage, Christin
1608 & Edwards 2011; Sage, Sage & Kocacinar 2012). The C_4 photosynthetic metabolism is
1609 the most efficient photosynthesis on the Earth, responding for a quarter of global
1610 primary productivity (Rangan *et al.* 2016; Haven & Thomas 2017). Because of their
1611 high photosynthetic efficiency, C_4 plants demand less amount of Rubisco in the leaf
1612 (50-80%) economizing N allocated to photosynthesis (Sage & Zhu 2011).

1613 This type of photosynthesis represents an important example of convergent
1614 evolution with 66 independent origins, of which, 23 are grasses (Sage & Stata 2015).
1615 Sage (2004, 2017) suggests that C_4 grasses arose from C_3 ancestors, during the
1616 Oligocene between 30-35 Mya. In contrast, C_4 eudicotyledons appeared later, less than
1617 20 Mya (Gowik & Westhoff 2010). The low atmospheric CO_2 content, hot, dry and
1618 salinized habitats have driven the emergence of C_4 photosynthesis (Ehleringer, Sage,
1619 Flanagan & Pearcy 1991; Taylor *et al.* 2012; Griffiths, Weller, Toy & Dennis 2013;
1620 Sage & Stata 2015; Haven & Thomas 2017; Liddy *et al.* 2018). The Figure 2
1621 summarizes the most important events that have happened during the evolution of C_4
1622 plants.

A distinguished characteristic of Poales shared with only a few related orders collectively known as commelinids is their cell wall architecture, known as primary type II cell wall (CWII) (Fig 1). They distinguishes from primary type I cell walls (CWI) of eudicotyledons and noncommelinid monocots mostly for presenting lower content of structural proteins and pectin, as well as by presenting feruloylated-glucuronoarabinoxylan (GAX-FA) as the main hemicellulose, instead of the xyloglucan, found in noncommelinids (Carpita & Gibeaut 1993; Tiné *et al.* 2004; de O. Buanafina 2009; Martins *et al.* 2016). Feruloylated GAX perform in CWII, roles very similar to those of structural proteins like extensins (Lamport, Kieliszewski, Chen & Cannon 2011). When activated by specific apoplastic oxidases, FA moieties from GAX and tyrosine residues from extensins, cross-link among themselves as well as with other cell wall components like lignin (dos Santos *et al.* 2008) reducing the cell extensibility and the accessibility of polysaccharidases from pathogens. Both extensins and FA-GAX are involved in control of cell integrity, cessation of cell growth (Tiné *et al.* 2004) and defense against the attack of pathogens (de Oliveira *et al.* 2015).

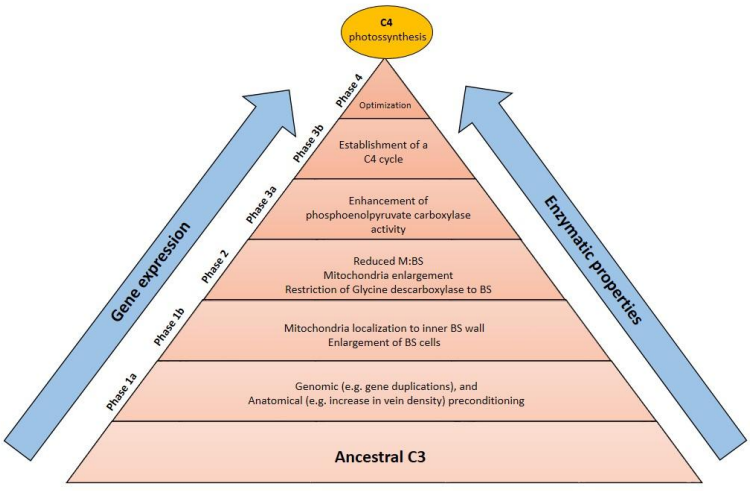

Fig. 2 Stepwise evolution of C_4 photosynthesis. C_3 plants were the ancestors of C_4 plants. During the evolution process, C_3 leaves resembled a version of C_4 photosynthesis including the increase of organelle numbers along the inner bundle sheath cells (BSC), high vein density and enlarged BSC. These features activated the BSC and increased the likelihood that photorespired CO_2 can be refixed in the BSC. The C_4 metabolic cycle can be upregulated, and Rubisco and the C_3 cycle relocated to BSC, creating C_4 photosynthesis. M, mesophyll cells.

THE RELATIONSHIP BETWEEN THE TYPE II CELL WALL AND THE EVOLUTION OF C_4 GRASSES

The increase in temperature rises the oxigenase activity of rubisco, making CO_2 assimilation the most important limiting factor to plant growth (Sage 2004; Sage & Zhu 2011). Changes in the anatomy and biochemistry of C_4 leaves during their evolutionary process allowed these plants to reduce photorespiration (Sage, Khoshravesh & Sage 2014). When the carbon is abundant, N becomes the main limiting factor for plant growth. The limiting availability of N into the soil may have worked as a selection pressure to C_4 grasses to adapt their biochemistry to save N. The higher photosynthetic efficiency of the Hatch-Slack cycle reduces the demand of N to photosynthetic apparatus, with no prejudice to growth process. Indeed, C_4 grasses present 26% lesser structural N in their cell wall than C_4 eudicotyledons (Fig 3c, Chapter 2). Structural proteins (e.g. extensins) are the major nitrogen compounds into the cell wall, as we saw, they cross-link the components in the wall, maintaining the cell wall integrity and protecting the cell against pathogen and herbivore attack (Lamport *et al.* 2011). As FA-GAX does not demand N in their chemical structure and performs similar functions of those performed by structural proteins, transferring the function of extensins to FA-GAX would provide an additional way to C_4 grasses to reduce their dependence of N. A strong element in favor of this hypothesis is that the amount of structural N in C_4 grasses is lower than that found in C_3 and C_4 eudicotyledons, and C_3 grasses (Fig 2c and 3c, Chapter 2), while the amount of ferulic acid ester-linked in C_4 grasses is greater than that found in C_3 grasses, C_3 eudicotyledons and C_4 eudicotyledons (Fig 2d and 3d, Chapter 2). The higher N use efficiency provided by their enhanced photosynthetic and their distinct cell wall may have been an important upgrade to C_4 grasses conquer nutrient poor soils and obtain their extraordinary ecological success.

CONCLUSIONS

The reduction in the nitrogenous compounds in type II cell wall, together with the replacement of these compounds to ferulic acid suggests that the architecture of C_4 grasses cell wall have influenced their nitrogen use efficiency. During their evolutionary pathway, grasses had to compete with other plants by space, light and especially nutrients like N. This change acted as a selection pressure, making that C_4 grasses would find an alternative to reduce their N dependence in their cell wall, while investing the scarce nutrient to growth process obtaining ecological success.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

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