ANDRÉ BALDANSI ANDRADE

PHYSIOLOGICAL AND BIOCHEMICAL MECHANISMS RELATED WITH THE NITROGEN USE EFFICIENCY IN TOBACCO GENOTYPES

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Prof. Dr. Douglas Ramos Guelfi Silva
Orientador

Prof. Dr. Valdemar Faquin
Co-orientador

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MECANISMOS FISIOLÓGICOS E BIOQUÍMICOS RELACIONADOS COM A EFICIÊNCIA DE USO DE NITROGENIO EM GENÓTIPOS DE TABACO

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciência do Solo, área de concentração em Fertilidade do Solo e Nutrição de Plantas, para a obtenção do título de Doutor.

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LAVRAS – MG
2021

Ao meu tio Pedro D. Moreira (in memoriam)

Dedico

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RESUMO GERAL

Conhecer a eficiência de uso do nitrogênio (EUN) do tabaco é crucial para minimizar a poluição ambiental, embora a EUN raramente seja conhecida para vários genótipos desta cultura. No primeiro experimento, por meio do crescimento de diferentes genótipos em soluções nutritivas contrastantes quanto à disponibilidade de nitrogênio (N), os objetivos foram caracterizar cinco componentes da EUN de 28 genótipos e classificá-los de acordo com sua eficiência e responsividade à disponibilidade de N. Em média, a eficiência fisiológica de uso do N (razão entre massa seca e concentração de N da planta), o índice de colheita de N (razão entre o acúmulo de N nas folhas pelo acúmulo de N na planta) e a eficiência de absorção de N (razão entre acúmulo de N na planta pela massa seca de raízes) diminuíram em 16%, 4% e 57%, respectivamente, sob condições de deficiência de N, enquanto a eficiência de utilização de N (razão entre massa seca e acúmulo de N da planta) diminuiu em 43% com o fornecimento de N adequado. A eficiência relativa de uso do N (razão entre massa seca produzida sob deficiência e nível adequado de N) variou de 35% a 59% entre os genótipos. Todos os genótipos dos grupos varietais Virginia e Maryland foram eficientes e os dos grupos Burley, Comum e Dark foram ineficientes. A responsividade variou entre os genótipos dentro dos grupos varietais, exceto para os genótipos Maryland, que foram não responsivos. No segundo experimento, foram estudados quatro genótipos eficientes e responsivos (ER) e quatro não eficientes e não responsivos (NENR) selecionados do primeiro experimento. O objetivo foi avaliar as respostas em trocas gasosas e das enzimas do metabolismo do N de genótipos de tabaco sob diferentes disponibilidades de N. A análise de componentes principais (PCA) revelou que a concentração de CO_2 intercelular (Ci), teores de flavonoides, de antocianinas e atividade da glutamina sintase (GS) foram parâmetros relacionados à deficiência de N, enquanto a taxa fotossintética líquida (Pn), atividades da nitrato redutase (NR), glutamato sintase (GOGAT), glutamato desidrogenase (GDH), massa seca total (DM), acúmulo de N total (NA), eficiência instantânea de carboxilação (k), conteúdo de clorofila total, balanço de N (razão entre os teores de clorofila e flavonoides), eficiência no uso da água (WUE), transpiração (E) e condutância estomática (gs) foram associados ao fornecimento adequado de N. De modo geral, os genótipos ER apresentaram menor teor de antocianinas, maior teor de clorofila e flavonoides, maiores k, eficiência fotossintética de uso do N(PNUE), atividades da NR, GDH e da GOGAT em comparação com os genótipos NENR. Sob deficiência de N, os genótipos ER revelaram geralmente maior conteúdo de flavonoides, PNUE, atividades da NR e GDH quando comparados aos genótipos NENR nesta mesma condição. A maior DM e NA dos genótipos ER se deve ao maior pareamento de diversos parâmetros dos metabolismos de carbono (C) e N aqui investigados. As descobertas aqui relatadas são úteis na indicação de genótipos com eficiência e responsividade distintas ao fornecimento de N, que podem ser escolhidos de acordo com o nível de N no solo ou da disponibilidade de fertilizantes nitrogenados em lavouras de tabaco de todo o mundo. De forma geral, os dados obtidos nesta tese podem levar a um uso mais sustentável do nitrogênio e podem apoiar programas de melhoramento de tabaco para a EUN.

Palavras-chave: Seleção. *Nicotiana tabacum* L. Atividade enzimática. Trocas gasosas. Disponibilidade de nitrogênio. Solução nutritiva. Dualex.

GENERAL ABSTRACT

Knowing the nitrogen use efficiency (NUE) of tobacco is crucial to minimize environmental pollution, although NUE is rarely known for numerous genotypes for this crop. In the first experiment, through the growth of different genotypes in nutritive solutions contrasted in nitrogen (N) availability, the aims were to characterize five components of the NUE of 28 genotypes and to classify them according to their efficiency and responsiveness to N availability. On average, the physiological N use efficiency (ratio between dry mass and N concentration of the plant), N harvest index (ratio between leaves N accumulation by plant N accumulation), and N uptake efficiency (ratio between plant N accumulation by root dry mass) decreased by 16%, 4%, and 57%, respectively, under N-deficient conditions, while N utilization efficiency (ratio between dry mass and plant N accumulation) decreased by 43% at adequate N supply. The relative efficiency of N use (ratio between dry mass produced under deficiency and adequate N supply) varied from 35% to 59% among genotypes. All genotypes of the Virginia and Maryland varietal groups were efficient, and those of the Burley, Comum, and Dark groups were inefficient. The responsiveness varied among genotypes within the varietal groups, except for Maryland genotypes, being non-responsive. In the second experiment, we studied four efficient and responsive (ER) genotypes and four non-efficient and non-responsive (NENR) genotypes selected of the first experiment. The aim was to evaluate the responses in gas exchanges and of the N metabolism enzymes of tobacco genotypes under different N availabilities. Principal component analysis (PCA) revealed that the intercellular CO_2 concentration (Ci), flavonoids contents and anthocyanins and glutamine synthase (GS) activity were parameters related to N deficiency, whereas net photosynthetic rate (Pn), activities of nitrate reductase (NR), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH), total dry mass (DM), total N accumulation (NA), instantaneous carboxylation efficiency (k), total chlorophyll, N balance (ratio between the chlorophyll and flavonoids contents), water use-efficiency (WUE), transpiration (E) and stomatal conductance (gs) were associated with adequate N supply. Generally, genotypes ER showed lower anthocyanins content, higher chlorophyll and flavonoids content, higher k, photosynthetic N use efficiency (PNUE), NR, GDH and GOGAT activities compared to genotypes NENR. Under N deficiency, ER genotypes revealed generally higher flavonoids content, PNUE, NR and GDH activities when compared to genotypes NENR under this same condition. The greater total DM and total NA of the genotypes ER is due to the greater pairing of diverse parameters of both C and N metabolisms here investigated. The findings reported here are useful in indicating genotypes with different efficiency and responsiveness to the N supply, which can be chosen according to the level of N in the soil or the availability of nitrogen fertilizers in tobacco crops worldwide. In general, the data obtained in this thesis can lead to a more sustainable use of nitrogen and can support tobacco breeding programs for NUE.

Keywords: Screening. *Nicotiana tabacum* L. Enzymatic activity. Gas exchanges. N availability. Nutritive solution. Dualex

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO GERAL

O tabaco (*Nicotiana tabacum* L.) é uma cultura não alimentícia muito importante para a economia de vários países. Em 2019, esta cultura foi cultivada em aproximadamente 3,6 milhões de ha em todo o mundo, resultando em uma produção aproximada de 6,7 milhões de toneladas (FAOSTAT, 2020).

O nitrogênio (N) é um importante nutriente para a cultura do tabaco, havendo aumento das produtividades através das adubações nitrogenadas (CHAOQIANG JIANG; CHAOLONG ZU; HUOYAN WANG, 2015; ISABELLA; GIAMPAOLO; ALBINO, 2017; ZOU et al., 2017; CHEN et al., 2020; SOARES et al., 2020). Doses de N aquém da necessidade da cultura podem diminuir a produtividade, enquanto doses acima representam desperdício de recursos financeiros, além de haver riscos ao meio ambiente. Entretanto, a tendência é que se ultrapasse as doses de N nas adubações devido ao receio dos agricultores quanto à ocorrência de deficiências (MARCHETTI; CASTELLI; CONTILLO, 2006) e consequente queda na produtividade.

Em 2050, estima-se que serão gastos $227,4 bilhões com fertilizantes nitrogenados (GOOD; BEATTY, 2011). Dado o aumento da população mundial estimado nas próximas décadas, o aumento da produtividade das culturas será necessário, já que em muitos países não é possível o aumento de áreas. Assim, o aumento da eficiência do uso de N (EUN) deve ser constantemente incentivado para todas as culturas. Entretanto, a EUN tem sido secundária no melhoramento de plantas quanto a estresses abióticos, sendo a prioridade os trabalhos relacionados à seca (HIREL et al., 2011). A EUN é função das interações entre parâmetros de desenvolvimento, fisiológicos e de ambiente, sendo característica de um determinado genótipo de uma espécie (HIREL et al., 2011). Segundo estes autores, é necessário fazer levantamentos de uma ampla faixa de genótipos que abrangem ampla diversidade genética dentro de uma dada cultura. Na cultura do tabaco, o requerimento das adubações nitrogenadas pode ser bastante diferente entre genótipos. Tabacos pertencentes ao grupo varietal Burley requerem maiores doses de N, entretanto possuem produtividades similares em relação a outros grupos varietais (LEWIS et al., 2012). Ainda, doses de N em excesso levam à redução na qualidade do tabaco, seu valor comercial, além de dificultar a colheita mecanizada e a cura das folhas (MARCHETTI; CASTELLI; CONTILLO, 2006).

Segundo Ruiz et al. (2006), a seleção e o melhoramento de cultivares que possuem alta EUN tem se tornado indispensável dada a importância deste nutriente para a cultura. Isto porque a eficiência de uso de nutrientes é função de vários fatores, mas o mais provável é a existência de diferenças genéticas entre os vários tipos de tabaco (LEWIS et al., 2012). O aproveitamento da variabilidade genética já existente ou aquela que possa ser criada irá contribuir valorosamente para o estudo cuidadoso da genética e fisiologia da EUN e avaliar o grupo de genes ou genes que estão envolvidos (HIREL et al., 2011). Segundo Amirhandeh, Norouzi e Nosratabad (2013), a EUN deve ser incrementada pelo uso de engenharia biotecnológica ou seleção de cultivares desejáveis.

A forma nitrogenada predominantemente absorvida pelo tabaco é o nitrato (NO_3^-). Quando absorvido pelas raízes, o N-NO_3^- é reduzido à nitrito (NO_2^-) pela ação da enzima redutase do nitrato (RN) no citoplasma. O NO_2^- por sua vez é convertido à amônio (NH_4^+) pela redutase do nitrito (RNi) nos cloroplastos em tecidos verdes ou nos plastídeos em tecidos não verdes. A glutamina sintetase (GS) catalisa a união do NH_4^+ com glutamato formando a glutamina, ocorrendo no citosol (se em raízes) ou cloroplastos (se em folhas), que pode ser transportada ou combinada com o 2-oxoglutarato formando o glutamato através da enzima glutamato sintetase (GOGAT) nos cloroplastos.

O excesso de N aplicado pelas adubações pode levar ao acúmulo de NO_3^- nas folhas de tabaco, o que é prejudicial para a qualidade (SIFOLA; POSTIGLIONE, 2003). Altos níveis de adubações nitrogenadas estão associadas com a formação das nitrosaminas específicas do tabaco (TSNAs) (LEWIS et al., 2012). Segundo estes autores, as TSNAs são produzidas através da nitrosação dos alcaloides principalmente durante a cura das folhas de tabaco, mas também pode ocorrer durante o armazenamento e processamento das folhas. Em tabacos com cura ao ar, os agentes nitrosantes vêm de duas fontes: do NO_2^- formado pela RN ou do NO_2^- produzido pela redução do NO_3^- foliar por mediação de microrganismos (LEWIS et al., 2012). Chandrasekhararao et al. (2014) encontraram aumento da atividade da RN e de NO_3^- nas folhas com o aumento de doses de N e concluíram que a atividade desta enzima pode ser usada como indicador da disponibilidade de N para as plantas. Assim, é provável que a atividade da RN possa ser usada como parâmetro para seleção de genótipos eficientes no uso de N, bem como as demais enzimas relacionadas ao ciclo do N. Estes parâmetros relacionados ao N deve ser considerados na seleção de genótipos com alta eficiência de uso de nitrogênio (RUIZ et al., 2006). Para obter menores valores de TSNAs, as estratégias genéticas tem buscado atingir o alcaloide precursor ou o agente nitrosante envolvido (LU et al., 2016). Variedades capazes de reduzir o NO_3^- mais eficientemente ao invés de estocá-lo tem sido o resultado dos métodos

convencionais de seleção, mesmo assim, não são capazes de cessar os riscos de acúmulos a níveis tóxicos (HIREL et al., 2011).

Os níveis de TSNAs nos vários tipos de tabaco são maiores para o grupo Burley (LEWIS et al., 2012). Segundo os autores, estas diferenças são atribuídas aos diferentes tipos de manejo, mas as diferenças genéticas podem também influenciar significativamente. Os tabacos tipo Burley apresentam deficiências no uso e eficiência de N concomitantemente com elevado acúmulo de NO_3^- foliar quando comparado com outros tipos, indicando um possível comprometimento de algum passo do metabolismo do N (LU et al., 2016). Ainda, há uma estreita relação entre o metabolismo do N e do C (NUNES-NESI; FERNIE; STITT, 2010). Assim, há três fatores que são essenciais para reduzir o NO_3^- nas plantas: poder redutor (NAD(P)H), energia (ATP) e esqueletos de C (oxoglutarato e glutamato), que são fornecidos pelo metabolismo do carbono nas plantas. ATP e NADPH são produzidos na etapa fotoquímica e também são usados na etapa bioquímica da fotossíntese. Já o glutamato e o oxoglutarato são produzidos no ciclo de Krebs. Deste modo, a investigação das enzimas do metabolismo do N e de componentes do metabolismo do C são importantes para se estudar a EUN de diversos genótipos de tabaco.

A hipótese desse trabalho é que os genótipos de tabaco apresentam variações quanto à eficiência de uso e responsividade ao N. Mais especificamente, os genótipos eficientes ao uso de N possuem mecanismos fisiológicos como atividade das enzimas do N e atividade fotossintética superiores aos genótipos menos eficientes. O objetivo do primeiro capítulo foi caracterizar diferentes componentes da EUN de diferentes genótipos de tabaco submetidos a níveis adequados e deficientes em N, e classificar os genótipos de acordo com suas eficiências e responsividades ao N. Já o segundo capítulo teve como objetivo avaliar as respostas fisiológicas de genótipos contrastantes quanto à eficiência e responsividade ao suprimento de N quando cultivados sob nutrição deficiente e adequada de N.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1 – GENOTYPIC VARIATION IN NITROGEN USE-EFFICIENCY TRAITS OF 28 TOBACCO GENOTYPES

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Article

Genotypic Variation in Nitrogen Use-Efficiency Traits of 28 Tobacco Genotypes

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Abstract: Knowing the nitrogen use efficiency (NUE) of crops is crucial to minimize environmental pollution, although NUE is rarely provided for numerous genotypes in the tobacco (*Nicotiana tabacum L.*) crop. Through the growth of contrasting genotypes in nutritive solutions, we aimed to characterize five NUE components of 28 genotypes and to classify them according to their efficiency and responsiveness to nitrogen (N) availability. On average, physiological N use efficiency, N harvest index, and N uptake efficiency decreased by 16%, 4%, and 57%, respectively, under N-deficient conditions, while N utilization efficiency decreased by 43% at adequate N supply. The relative efficiency of N use varied from 35% to 59% among genotypes. All genotypes of the Virginia and Maryland varietal groups were efficient, and those of the Burley, Comum, and Dark groups were inefficient, while the responsiveness varied among genotypes within varietal groups, except for Maryland genotypes. Our findings are helpful in indicating genotypes with distinguished efficiency and responsiveness to N supply, which can be further chosen according to soil N level or affordability to N fertilizers worldwide in tobacco crops. In a general framework, this can lead to a more sustainable use of N and can support tobacco breeding programs for NUE.

Keywords: Nitrogen use efficiency; screening; varietal groups; *Nicotiana tabacum L.*, responsiveness; classification; grouping; N availability

1. Introduction

Nitrogen use-efficiency (NUE) improvement for crops is a very important issue to decrease soil, air, and water pollution and, at same time, increase the profitability of landholders through the rational use of mineral nitrogen (N) fertilizers. According to Kant et al. [1], an increase of 1% in NUE could save about 1.1 billion dollars per year. There are different ways to calculate the NUE of a given crop, being dependent on the harvest product of the crop (e.g., leaves, grains, etc.) and in what the researcher is interested to know [2]. The basic concept in our mind is that a crop with high NUE produces more with less.

NUE varies among plant species and, within a certain plant species, it also differs among the varieties that exist; in other words, NUE varies both inter- and intra-species. This variation of NUE is thought to be important as it allows the establishment of different varieties of the same species at different environmental conditions, i.e., different soil N levels. Hence, this implies that plants have different internal mechanisms related to their NUE, or their embedded genetics for simplicity. The first step—and likely the easiest way—to increase the NUE of crops would be to both know and use in the

best way that genetic variability available within a given plant species [3]. However, doing so is a difficult task, as the number of varieties can be large.

In the literature, surveys of NUE traits in different varieties of a given plant species are provided; for example, in sugarcane [4,5], potato [6,7], and rice [8]. However, in most cases, the number of varieties investigated is quite limited. This is understandable due to the time consumption in the conduction of many plants and the laboratory analysis required later. However, a better approach would be to try to embrace the highest number of genotypes as possible, allowing us to know the NUE specifically related to each genotype. This is only possible if the analysis involved in such studies is of low cost and is not time consuming. Thus, this could comprise a greater number of genotypes spanning a wide range of mechanisms related to NUE traits [9].

For this purpose, pot experiments represent a valuable tool for assessment, as the conduction of many genotypes under field conditions is unfeasible, beyond the existing soil variability. The study of genotypes under controlled conditions is faster, allows more genotypes to be investigated, is independent of pathogens and weather [6], and has minimal environmental variability. In addition, when the growing media is a nutritive solution, the evaluation of roots—responsible for nutrient uptake—becomes more reliable, rather than in experiments using pots filled with substrates [10]. Then, after screening for characterization of the NUE by plants, the choice of suitable genotype according to its N demand could be performed according to the area to be cultivated. In this way, farmers would have higher genotype diversity according to soil management conditions, being able to obtain acceptable yields with lower N inputs. Besides this, the knowledge of NUE of different varieties may also provide helpful data for tobacco breeding.

N fertilization in tobacco crops varies widely depending on the genotype. It is known, for example, that the varietal group Burley demands more N than others groups [11], but has similar yields. There are works that show that the difference of N fertilization is even 4-fold higher for Burley compared to other genotypes [12]. For tobacco genotypes, few works have used numerous genotypes in the study of NUE traits [13,14]. Thus, studies of the mechanisms behind NUE traits are limited to a few tobacco genotypes [15,16]. Again, this is comprehensive, since the growing process of many genotypes is time-intensive in terms of labor, and plant analysis is frequently time consuming with associated high costs (e.g., physiological analysis, ^{15}N isotypes). However, a reliable selection of genotypes that differ in their NUE through cheaper and rapid assessment of plants would be interesting prior to the use of valuable sophisticated analysis, but it is expensive and unfeasible to do so in numerous genotypes.

For tobacco crop, N management is of particular interest as this nutrient plays an important role in the formation of nitrogenous compounds, namely, tobacco-specific nitrosamines (TSNAs), consequently linked to the quality of tobacco products. These compounds are carcinogens, found in negligible contents in fresh leaves but increases in the curing process [17]. TSNAs are formed by the reaction of nitrite (which is produced from nitrate (NO_3) uptaken from the soil) with alkaloids [18]. However, high NO_3 contents in the leaves are undesirable. In a general framework, the future tobacco market may require healthier products that comply with health regulatory agencies worldwide. One possible pathway is through the development of more efficient plants in the use of N through the breeding process.

In this sense, a species screening study represents a valuable tool for future studies in a breeding program. Accessing contrasting genotypes regarding NUE will make it possible to know the genes controlling traits related to N use in tobacco, such as chlorophyll and NO_3 content or N metabolism enzymes. For example, two genes were recently identified controlling NUE in tobacco [19]. Thus, the knowledge about the genes related to NUE may contribute to breeding programs aiming to obtain more efficient genotypes that are productive and that contain lower undesirable N compounds.

In light of the scarcity on works assessing a wide range of tobacco genotypes, the aims of this study were (1) to characterize the NUE traits of different *Nicotiana tabacum L.* genotypes when subjected to both a deficient and an adequate N supply, and (2) to classify the genotypes according to their efficiency and responsiveness to N.

2. Materials and Methods

2.1. Tobacco Genotypes and Growth Conditions

A total of 28 tobacco (*Nicotiana tabacum L.*) genotypes were selected from the germplasm bank of the Product Center Americas of Souza Cruz company at Cachoeirinha, Rio Grande do Sul state, Brazil. The cultivars chosen covered five different varietal groups: Virginia, Burley, Comum, Dark, and Maryland (Table S1). The experiment was carried out under greenhouse conditions in Lavras, Minas Gerais state, Brazil, from 20 October to 12 December 2018. Seeds were sowed in seeding trays filled with organic substrate Tabaco−1® (MecPlant, Telêmaco Borba/PR), and then conducted in a floating system with deionized water. Then, 9 days after sowing (DAS), the floating system started to be fertilized with 10 mL L^{-1} of a nutritive solution with the following concentration: 20% of N and K_2O, 10% P_2O_5, 0.15% Mg, 0.05% Fe, 0.025% Mn and Zn, 0.0125% B and Cu, and 0.005% Mo. At 25 DAS, tobacco seedlings were transferred to 30 L plastic trays filled with nutritive solution #1 of Hoagland and Arnon [20] at 20% and 50% of ionic strength (I.S.) for macro- and micronutrients, respectively. At 29 DAS, the nutritive solution was replaced by 40% and 50% of I.S. for macro- and micronutrients, respectively. The nutritive solution was permanently aerated throughout the period of plants adaptation to the hydroponic condition.

After the period of plant adaptation to the nutritive solution, plants of homogeneous size and vigor were selected to be transplanted to pots (3 L, one seedling per pot) with treatments at 31 DAS. The treatment nutritive solutions consisted of two contrasting N concentrations: deficient (2 mM) and sufficient (10 mM), which were established after previous tests with genotypes belonging to the Burley and Virginia groups in concentrations of N ranging from 1 to 12 mM. The concentration of the remaining nutrients was set at 60% and 100% of I.S. for macro- and micronutrients, respectively, based on the concentration of Hoagland and Arnon [20] solution #1 (Table S2). The solutions were renewed at 7, 14, and 18 days after transplanting (DAT), constantly aerated, and the pH adjusted to ~6.0 once per week. The experimental design was completely randomized, in a factorial scheme 28 (genotypes) × 2 (N concentrations, 2 and 10 mM), with four repetitions.

2.2. Plant Analysis and N Use-Efficiency Indexes

Plants were collected at 22 DAT (53 DAS) and roots were rinsed in deionized water. Then, plants were left in the greenhouse for pre-drying over 6 days. After this, plants were separated into roots, stem, and leaves, and dried in an oven with forced air circulation at 65 °C up to constant weight. The dry mass (DM) of each organ was recorded prior to grinding in a Wiley mill, and ground samples were analyzed for N concentration [21]. A standard reference material (NIST® SRM® 1573a tomato leaves) was used in the digestion process to verify the accuracy of the method. N accumulation for each organ was calculated by the product of N concentration and DM.

Five indexes related to N use efficiency were calculated. N use efficiency (NUE; g DM g^{-1} of N) represents the amount of plant DM produced per unit of N accumulated in the plant. Physiological N use efficiency (PNUE; g^2 DM mg^{-1} of N) is the DM produced per N concentration. N harvest index (NHI; %) represents the amount of N in the leaves in relation to N in the whole plant [22]. N uptake efficiency (NUpE; mg N g^{-1} roots) refers to the amount of N in the plant per root mass. Finally, relative N use efficiency (RNUE; %) represents the ratio of DM produced by the plants grown in N-deficient and N-sufficient supplies. The equations of the NUE indexes used in this study are represented below:

$$\text{NUE (g g}^{-1}\text{)} = \text{Plant DM (g)/N accumulation (g)} \quad (1)$$

$$\text{PNUE (g}^2\text{ DM mg}^{-1}\text{ of N)} = \text{Plant DM (g)/N concentration (mg g}^{-1}\text{)} \quad (2)$$

$$\text{NHI (\%)} = (\text{N accumulation in leaves/N accumulation in the plant}) \times 100 \quad (3)$$

$$\text{NUpE (mg N g}^{-1}\text{ roots)} = \text{N accumulation in plant (mg)/root DM (g)} \quad (4)$$

$$\text{RNUE (\%)} = [\text{Plant DM}_{\text{deficiency}}\ (g)/\text{Plant DM}_{\text{adequate}}\ (g)] \times 100 \quad (5)$$

To separate genotypes according to their efficiency and responsiveness to N, we calculated the N use efficiency (NUEf; g DM g^{-1} N) based on the plant DM and N accumulation of plants grown under deficient and adequate N supplies as follows:

$$\text{NUEf (g g}^{-1}) = [\text{Plant DM}_{\text{adeq.}} - \text{Plant DM}_{\text{def.}}]/[\text{N accumulation}_{\text{adeq.}} - \text{N accumulation}_{\text{def.}}] \quad (6)$$

Data underwent analysis of variance (ANOVA) via the Sisvar software version 5.7 [23] and means were compared by Scott–Knott test at $p < 0.05$.

3. Results

3.1. Visual Symptoms of N Deficiency, Dry Mass (DM) Production, N Contents, and N Accumulation in Tobacco Genotypes

Plants grown in N-deficient solution revealed typical symptoms of this nutrient deficiency, that is, older yellowish leaves, while younger leaves showed a slightly lighter green color. A general view of the whole plants conducted in an N-sufficient solution revealed darker green plants, instead of a paler green color visualized in plants grown in a deficient solution, regardless of the genotype assessed (Figure S1). Higher plants were observed under adequate N supply, which was reflected in higher dry mass (DM) of roots, stems, and leaves under this growth condition (Table 1). The order of DM production followed stems < roots < leaves for N-deficient treatment, regardless of the genotype, and roots < stems < leaves on average when plants were grown under adequate N supply. Accordingly, higher root/shoot ratios were observed in plants conducted in an N-deficient solution. The average reduction of DM production when plants were grown under N deficiency was 36%, 73%, and 48% for roots, stems, and leaves, respectively.

Table 1. Means of dry mass (DM; g $plant^{-1}$) of the roots, stems, and leaves and the root/shoot ratio of 28 genotypes of tobacco grown at 2 and 10 mM of nitrogen (N).

Genotypes	DM [1] (g $plant^{-1}$)						Root/Shoot Ratio [2]	
	Roots		Stems		Leaves			
	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM
BAG 06	5.23 ^{B}	8.05 ^{E}	4.20 ^{A}	14.20 ^{B}	12.58 ^{A}	24.31 ^{A}	0.31 ^{E}	0.21 ^{D}
BAT 2101	6.52 ^{A}	10.28 ^{C}	1.70 ^{B}	9.05 ^{E}	10.85 ^{B}	21.39 ^{B}	0.52 ^{A}	0.34 ^{A}
BAT 2301	5.99 ^{A}	8.51 ^{D}	3.77 ^{A}	13.00 ^{C}	11.39 ^{B}	21.75 ^{B}	0.39 ^{C}	0.25 ^{C}
BAT 3004	5.34 ^{B}	8.76 ^{D}	2.56 ^{B}	9.60 ^{E}	9.98 ^{B}	20.55 ^{B}	0.42 ^{C}	0.29 ^{B}
BAT 3201	6.55 ^{A}	9.58 ^{C}	2.88 ^{B}	10.90 ^{D}	10.53 ^{B}	23.08 ^{B}	0.49 ^{B}	0.28 ^{B}
CSC 221	6.08 ^{A}	9.93 ^{C}	3.12 ^{B}	11.43 ^{D}	10.67 ^{B}	20.32 ^{B}	0.44 ^{C}	0.31 ^{A}
CSC 2305	6.07 ^{A}	9.29 ^{C}	2.82 ^{B}	13.60 ^{B}	10.23 ^{B}	22.83 ^{B}	0.47 ^{B}	0.25 ^{C}
CSC 2307	6.38 ^{A}	9.83 ^{C}	2.40 ^{B}	11.54 ^{D}	11.16 ^{B}	22.02 ^{B}	0.47 ^{B}	0.29 ^{B}
CSC 259	6.90 ^{A}	10.61 ^{B}	2.93 ^{B}	12.10 ^{C}	11.13 ^{B}	21.24 ^{B}	0.49 ^{B}	0.32 ^{A}
CSC 2602	6.95 ^{A}	9.79 ^{C}	2.61 ^{B}	10.49 ^{D}	10.62 ^{B}	22.55 ^{B}	0.53 ^{A}	0.30 ^{B}
CSC 302	6.33 ^{A}	11.42 ^{A}	2.87 ^{B}	12.48 ^{C}	10.51 ^{B}	22.56 ^{B}	0.48 ^{B}	0.32 ^{A}
CSC 3702	6.82 ^{A}	11.88 ^{A}	2.17 ^{B}	11.13 ^{D}	11.69 ^{B}	23.09 ^{B}	0.49 ^{B}	0.35 ^{A}
CSC 3703	6.14 ^{A}	10.92 ^{B}	2.25 ^{B}	9.45 ^{E}	11.22 ^{B}	25.53 ^{A}	0.46 ^{B}	0.31 ^{A}
CSC 416	5.17 ^{B}	7.71 ^{E}	3.76 ^{A}	12.18 ^{C}	13.15 ^{A}	25.12 ^{A}	0.30 ^{E}	0.20 ^{D}
CSC 4303	5.98 ^{A}	8.94 ^{D}	4.86 ^{A}	12.38 ^{C}	14.10 ^{A}	26.12 ^{A}	0.31 ^{E}	0.23 ^{C}
CSC 4304	5.64 ^{B}	8.76 ^{D}	3.92 ^{A}	13.73 ^{B}	14.50 ^{A}	26.99 ^{A}	0.31 ^{E}	0.22 ^{D}
CSC 439	5.01 ^{B}	8.62 ^{D}	3.96 ^{A}	13.38 ^{B}	13.15 ^{A}	24.52 ^{A}	0.29 ^{E}	0.23 ^{C}
CSC 444	5.91 ^{A}	8.71 ^{D}	3.82 ^{A}	12.09 ^{C}	15.13 ^{A}	24.97 ^{A}	0.31 ^{E}	0.23 ^{C}
CSC 447	5.48 ^{B}	7.99 ^{E}	3.89 ^{A}	13.23 ^{C}	14.49 ^{A}	25.27 ^{A}	0.30 ^{E}	0.21 ^{D}
CSC 4501	5.43 ^{B}	9.43 ^{C}	4.02 ^{A}	14.76 ^{A}	14.52 ^{A}	24.89 ^{A}	0.29 ^{E}	0.24 ^{C}

CSC 4703	6.12 A	9.30 C	2.92 B	11.89 C	14.90 A	21.89 B	0.34 D	0.27 B
CSC 4704	5.51 B	8.21 D	3.05 B	10.45 D	14.56 A	24.84 A	0.31 E	0.24 C
CSC 4707	5.36 B	7.10 E	4.71 A	15.64 A	13.47 A	27.66 A	0.29 E	0.16 E
CSC 497	4.84 B	8.43 D	4.23 A	13.96 B	13.19 A	25.52 A	0.28 E	0.22 D
CSC 500	5.37 B	7.56 E	3.17 B	10.50 D	14.13 A	20.40 B	0.31 E	0.24 C
Dark O.S.	4.09 B	9.43 C	2.83 B	12.13 C	8.33 B	21.51 B	0.36 D	0.28 B
HB 4488P	5.85 A	8.33 D	3.29 B	12.71 C	10.72 B	24.33 A	0.42 C	0.22 C
New cultivar	5.77 A	9.01 D	2.62 B	9.91 E	14.32 A	26.11 A	0.34 D	0.25 C
Average	5.82	9.16	3.26	12.07	12.33	23.62	0.38	0.26

Means followed by the same letter do not differ significantly ($p > 0.05$) between genotypes by the Scott–Knott test. [1] Higher means were detected for 10 mM for all genotypes assessed. [2] Higher means were detected for 2 mM for all genotypes assessed.

There was a significant variation in DM production of the different organs studied among genotypes. The lowest root DM was 4.09 g plant^{-1} (Dark O.S.) and the highest was 6.95 (CSC 2602) for the N-deficient treatment; otherwise, 7.10 g plant^{-1} was produced by CSC 4707 and 11.88 by CSC 3702 under adequate N supply. For stem DM at N deficiency, it ranged from 1.70 (BAT 2101) to 4.86 g plant^{-1} (CSC 4303), and 9.05 to 15.64 g plant^{-1} for BAT 2101 and CSC 4707, respectively, under adequate N supply. Regarding leaf DM, Dark O.S. and CSC 444 produced the lowest and highest DM, 8.33 and 15.13 g plant^{-1}, respectively, under N deficiency; on the other hand, 20.32 g plant^{-1} was the lowest DM (CSC 221) and 27.66 the highest (CSC 4707). The increase of DM provided by an adequate N supply varied from 32% (CSC 4704) to 131% (Dark O.S.) for roots, 155% (CSC 4303) to 432% (BAT 2101) for stems, and 44% (CSC 500) to 158% (Dark O.S.) for leaves (Table 1). This highlights the importance of N for yield in tobacco production.

Regardless of the organ, higher N contents were detected for plants grown under adequate N supply for all genotypes (Table 2). Among genotypes, the N content varied from 16.66 (BAT 2101) to 20.74 mg g^{-1} (CSC 302) for roots, 13.59 (CSC 497) to 20.94 mg g^{-1} (BAT 2101) for stems, and 13.06 (CSC 4703) to 24.45 mg g^{-1} (Dark O.S.) for leaves when plants were grown in N-deficient solution. Under adequate N supply, the N content ranged from 26.80 (BAT 3201) to 34.54 mg g^{-1} (CSC 4707) in roots, 22.61 (CSC 444) to 28.64 mg g^{-1} (CSC 4704) for stems, and 27.82 (CSC 4707) to 35.81 mg g^{-1} (BAT 3004) for leaves.

Table 2. Means of N content (mg g^{-1}) in the roots, stems, and leaves of 28 genotypes of tobacco grown at 2 and 10 mM of N.

Genotypes	N content (mg g^{-1}) plant^{-1}					
	Roots		Stems		Leaves	
	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM
BAG 06	17.37 A	30.31 B	15.28 C	23.77 B	16.28 B	30.68 B
BAT 2101	16.66 A	29.65 C	20.94 A	28.00 A	16.75 B	33.03 A
BAT 2301	18.17 A	27.14 D	14.87 C	24.50 B	16.33 B	32.85 A
BAT 3004	18.10 A	28.67 C	19.03 B	27.04 A	18.83 B	35.81 A
BAT 3201	19.23 A	26.80 D	16.93 C	23.65 B	17.67 B	34.36 A
CSC 221	18.85 A	28.10 C	14.99 C	25.51 A	18.74 B	34.19 A
CSC 2305	19.03 A	29.70 C	15.65 C	24.14 B	19.57 B	34.47 A
CSC 2307	19.23 A	32.42 B	16.77 C	26.11 A	17.82 B	33.04 A
CSC 259	18.60 A	29.22 C	18.40 B	26.92 A	16.62 B	33.06 A
CSC 2602	18.24 A	29.01 C	17.35 B	27.06 A	17.41 B	33.85 A
CSC 302	20.74 A	28.97 C	18.74 B	23.07 B	17.72 B	32.16 A
CSC 3702	20.23 A	26.99 D	20.38 A	25.71 A	14.94 C	30.75 B
CSC 3703	19.84 A	28.77 C	18.37 B	26.39 A	17.05 B	29.36 B
CSC 416	20.36 A	32.15 B	16.03 C	24.12 B	14.74 C	29.00 B

CSC 4303	19.79 A	31.64 B	14.63 C	24.12 B	13.50 C	29.92 B
CSC 4304	19.38 A	31.54 B	16.08 C	23.02 B	13.18 C	27.96 B
CSC 439	19.23 A	31.01 B	17.12 B	24.06 B	15.18 C	31.21 B
CSC 444	18.54 A	30.42 B	14.81 C	22.61 B	13.41 C	31.32 B
CSC 447	19.43 A	31.77 B	14.91 C	23.87 B	13.88 C	31.18 B
CSC 4501	19.36 A	28.62 C	15.40 C	23.54 B	13.69 C	28.49 B
CSC 4703	20.03 A	31.71 B	18.29 B	26.63 A	13.06 C	31.98 A
CSC 4704	18.24 A	31.03 B	16.16 C	28.64 A	13.79 C	30.99 B
CSC 4707	19.54 A	34.54 A	15.53 C	22.75 B	13.42 C	27.82 B
CSC 497	18.84 A	28.96 C	13.59 C	22.70 B	16.39 B	30.51 B
CSC 500	18.19 A	29.27 C	14.99 C	25.85 A	14.48 C	34.95 A
Dark O.S.	19.03 A	27.40 D	17.56 B	23.98 B	24.45 A	32.60 A
HB 4488P	18.25 A	29.15 C	16.03 C	24.09 B	17.86 B	32.86 A
New cultivar	19.81 A	26.89 D	15.67 C	25.01 B	14.32 C	31.75 A
Average	19.01	29.71	16.59	24.89	16.11	31.79

Means followed by the same letters do not differ significantly ($p > 0.05$) between genotypes by the Scott–Knott test. Higher means were detected in all organs for 10 mM for all genotypes assessed.

The highest N accumulation occurred for all genotypes regardless of the plant organ under adequate N supply (Table 3). N accumulation ranged from 76 (Dark O.S.) to 138 mg N (CSC 3702) in roots, 35.55 (BAT 2101) to 73.22 mg N (CSC 4707) in stems, and 174.71 (CSC 3702) to 212.85 mg N (CSC 497) for leaves when plants were grown under N deficiency. On the other hand, 221.76 (CSC 500) and 330.20 mg N (CSC 302) were the lowest and highest N accumulations in roots whereas, in the stem, this ranged from 246.82 (CSC 3703) to 355.90 mg N (CSC 4707), and from 689.64 (CSC 221) to 827.05 mg N (New cultivar) in the leaves under adequate N supply. Among genotypes, this represents a variation of 81%, 106%, and 22% for roots, stems, and leaves, respectively, under N deficiency, and 49%, 44%, and 20% for roots, stems, and leaves, respectively, when plants were grown in N-sufficient solution. On average, an adequate N supply caused an increase of N accumulation of 145%, 461%, and 284% for roots, stems, and leaves, respectively. The order of N accumulation was stems < roots < leaves for N deficiency, and roots < stems < leaves for adequate N supply on average.

Table 3. Means of N accumulation (mg) in the roots, stems, and leaves for 28 tobacco genotypes grown at 2 and 10 mM of N.

Genotypes	N accumulation (mg) plant^{-1}					
	Roots		Stems		Leaves	
	2 mM	10 Mm	2 mM	10 mM	2 mM	10 Mm
BAG 06	91.02 B	244.36 D	64.22 A	337.34 A	202.71 A	745.81 B
BAT 2101	108.54 B	305.10 A	35.55 A	253.09 D	181.81 A	704.53 C
BAT 2301	108.40 B	230.80 D	55.67 A	317.75 B	185.57 A	710.01 C
BAT 3004	96.48 B	251.36 D	48.58 A	259.20 D	187.65 A	734.77 C
BAT 3201	125.90 A	257.22 C	48.84 A	256.15 D	186.08 A	787.80 A
CSC 221	114.50 A	280.79 B	46.79 A	290.56 C	198.90 A	689.64 C
CSC 2305	115.15 A	275.03 C	43.75 A	327.36 B	198.65 A	785.22 A
CSC 2307	122.23 A	318.42 A	40.23 A	300.60 B	198.41 A	724.76 C
CSC 259	128.67 A	309.69 A	53.85 A	324.82 B	184.77 A	700.05 C
CSC 2602	126.76 A	283.75 B	45.22 A	282.42 C	184.44 A	760.98 B
CSC 302	131.27 A	330.20 A	53.85 A	288.30 C	182.83 A	725.17 C
CSC 3702	138.00 A	320.49 A	44.09 A	285.25 C	174.71 A	708.08 C
CSC 3703	121.72 A	313.82 A	40.81 A	246.82 D	190.20 A	746.71 B
CSC 416	105.18 B	247.94 D	60.11 A	291.35 C	193.12 A	726.13 C
CSC 4303	117.45 A	281.69 B	71.23 A	296.06 C	189.63 A	769.56 B

CSC 4304	109.27 B	275.71 C	63.20 A	316.20 B	190.96 A	750.72 B
CSC 439	96.38 B	267.06 C	68.12 A	321.74 B	199.15 A	758.15 B
CSC 444	109.29 B	264.21 C	56.52 A	272.95 C	202.64 A	779.41 A
CSC 447	106.33 B	253.09 D	58.07 A	315.59 B	201.10 A	776.71 A
CSC 4501	104.96 B	271.13 C	61.90 A	347.38 A	198.28 A	706.71 C
CSC 4703	122.34 A	294.78 B	53.25 A	315.92 B	194.22 A	699.40 C
CSC 4704	100.47 B	254.88 C	49.39 A	286.49 C	200.89 A	756.97 B
CSC 4707	104.71 B	244.91 D	73.22 A	355.90 A	180.71 A	767.62 B
CSC 497	90.63 B	244.73 D	57.61 A	315.74 B	212.85 A	762.15 B
CSC 500	97.55 B	221.76 D	47.60 A	271.01 C	204.69 A	711.63 C
Dark O.S.	76.00 B	257.85 C	48.70 A	290.90 C	199.37 A	698.61 C
HB 4488P	106.69 B	242.99 D	52.90 A	306.16 B	191.37 A	799.77 A
New cultivar	114.25 A	242.07 D	39.99 A	247.36 D	203.89 A	827.05 A
Average	110.36	270.92	52.98	297.16	193.56	743.36

Means followed by the same capital letters do not differ significantly ($p > 0.05$) between genotypes by the Scott–Knott test. Regardless of the organ assessed, higher means were detected for 10 mM for all genotypes assessed.

3.2. N Use-Efficiency Traits

The NUE, i.e., the amount of plant DM produced per unit of N, was higher in plants under N deficiency, regardless of the genotype (Table 4). NUE ranged from 46.94 (Dark O.S.) to 67.50 g DM g^{-1} N (CSC 444) under N deficiency, and from 31.24 (BAT 3004) to 37.02 g DM g^{-1} N (CSC 4501) under adequate N supply. The physiological N use efficiency (PNUE), i.e., plant DM per N concentration, varied between 0.74 (Dark O.S.) to 1.68 g^2 DM mg^{-1} N (CSC 444) for plants subjected to N deficiency, but ranged from 1.21 (BAT 3004) to 1.86 g^2 DM mg^{-1} N (CSC 4707) under adequate N supply. When the amount of N in the leaves per unit of N in the whole plant was calculated, the NHI ranged from 48.98% (CSC 3702) to 61.66% (Dark O.S.) for plants grown under N deficiency, and from 52.50% (CSC 259) to 62.78% (New cultivar) under adequate N supply. The NUpE, i.e., the amount of N in the whole plant by root mass, was between 50.02 (BAT 2101) to 82.53 mg N g^{-1} roots (Dark O.S.) under N deficiency, and from 110.65 (CSC 3702) to 194.02 mg N g^{-1} roots (CSC 4707) under adequate N supply. NUpE was higher for plants grown under adequate N supply regardless of genotype, which was reflected in the lower root/shoot ratio for plants conducted at the aforementioned N concentration (Table 1). In respect to the RENU, in terms of the ratio of plant DM under deficient and at adequate N supplies, 35.16 (Dark O.S.) and 59.17% (CSC 500) were the lowest and the highest values, respectively.

Table 4. N utilization efficiency (NUE; g DM g^{-1} N), physiological N use efficiency (PNUE; g^2 DM mg^{-1} N), N harvest index (NHI; %), N uptake efficiency (NUpE; mg N g^{-1} roots), and relative efficiency of N use (RENU; %) for 28 tobacco cultivars grown at 2 and 10 mM of N

Genotypes	NUE [1] (g DM g^{-1} N)		PNUE (g^2 DM mg^{-1} N)		NHI (%)		NUpE [2] (mg N g^{-1} roots)		RENU
	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM	(%)
BAG 06	61.51 ^{B}	35.08 ^{A}	1.36 ^{Ab}	1.63 ^{Aa}	56.64 ^{Ba}	56.16 ^{Ba}	69.04 ^{A}	165.42 ^{B}	47.23 ^{C}
BAT 2101	58.51 ^{C}	32.24 ^{A}	1.12 ^{Ba}	1.31 ^{Ba}	55.78 ^{Ba}	55.78 ^{Ba}	50.02 ^{B}	122.80 ^{E}	46.92 ^{C}
BAT 2301	60.47 ^{B}	34.33 ^{A}	1.28 ^{Ba}	1.49 ^{Ba}	53.07 ^{Ca}	56.39 ^{Ba}	58.67 ^{B}	148.44 ^{C}	48.97 ^{C}
BAT 3004	53.75 ^{C}	31.24 ^{A}	0.96 ^{Ba}	1.21 ^{Ba}	56.39 ^{Ba}	59.02 ^{Aa}	62.56 ^{B}	142.37 ^{D}	45.97 ^{C}
BAT 3201	55.33 ^{C}	33.44 ^{A}	1.11 ^{Bb}	1.46 ^{Ba}	51.58 ^{Cb}	60.61 ^{Aa}	55.10 ^{B}	136.23 ^{D}	46.06 ^{C}
CSC 221	55.17 ^{C}	33.00 ^{A}	1.10 ^{Bb}	1.38 ^{Ba}	55.22 ^{Ba}	54.87 ^{Ba}	59.36 ^{B}	130.16 ^{D}	47.90 ^{C}
CSC 2305	53.40 ^{C}	32.92 ^{A}	1.03 ^{Bb}	1.51 ^{Ba}	55.50 ^{Ba}	56.60 ^{Ba}	59.15 ^{B}	149.83 ^{C}	41.80 ^{D}
CSC 2307	55.28 ^{C}	32.25 ^{A}	1.11 ^{Bb}	1.40 ^{Ba}	55.00 ^{Ba}	53.97 ^{Ba}	56.94 ^{B}	136.86 ^{D}	46.02 ^{C}
CSC 259	57.08 ^{C}	32.89 ^{A}	1.20 ^{Ba}	1.45 ^{Ba}	50.32 ^{Ca}	52.50 ^{Ba}	53.31 ^{B}	126.83 ^{E}	47.77 ^{C}
CSC 2602	56.59 ^{C}	32.23 ^{A}	1.14 ^{Ba}	1.38 ^{Ba}	51.76 ^{Cb}	57.32 ^{Aa}	51.31 ^{B}	135.77 ^{D}	47.25 ^{C}
CSC 302	53.63 ^{C}	34.60 ^{A}	1.06 ^{Bb}	1.61 ^{Aa}	49.74 ^{Cb}	53.98 ^{Ba}	58.15 ^{B}	117.95 ^{E}	42.41 ^{D}
CSC 3702	58.00 ^{C}	35.07 ^{A}	1.20 ^{Bb}	1.62 ^{Aa}	48.98 ^{Cb}	53.88 ^{Ba}	52.38 ^{B}	110.65 ^{E}	44.97 ^{C}
CSC 3703	55.54 ^{C}	35.10 ^{A}	1.09 ^{Bb}	1.61 ^{Aa}	53.98 ^{Ba}	57.08 ^{Aa}	57.50 ^{B}	120.10 ^{E}	42.78 ^{D}
CSC 416	61.53 ^{B}	35.56 ^{A}	1.36 ^{Aa}	1.60 ^{Aa}	53.94 ^{Ba}	57.40 ^{Aa}	69.36 ^{A}	165.44 ^{B}	49.08 ^{C}
CSC 4303	65.90 ^{A}	35.09 ^{A}	1.65 ^{Aa}	1.68 ^{Aa}	50.16 ^{Cb}	57.16 ^{Aa}	63.66 ^{A}	153.09 ^{C}	53.02 ^{B}
CSC 4304	66.23 ^{A}	36.84 ^{A}	1.59 ^{Aa}	1.83 ^{Aa}	52.55 ^{Ca}	55.93 ^{Ba}	64.68 ^{A}	153.62 ^{C}	48.73 ^{C}
CSC 439	60.87 ^{B}	34.51 ^{A}	1.35 ^{Aa}	1.61 ^{Aa}	54.85 ^{Ba}	56.27 ^{Ba}	72.59 ^{A}	156.69 ^{B}	47.64 ^{C}
CSC 444	67.50 ^{A}	34.74 ^{A}	1.68 ^{Aa}	1.59 ^{Aa}	55.01 ^{Bb}	59.19 ^{Aa}	62.59 ^{B}	151.93 ^{C}	54.43 ^{B}
CSC 447	65.30 ^{A}	34.52 ^{A}	1.56 ^{Aa}	1.62 ^{Aa}	55.02 ^{Ba}	57.70 ^{Aa}	66.78 ^{A}	168.58 ^{B}	51.63 ^{B}
CSC 4501	65.62 ^{A}	37.02 ^{A}	1.57 ^{Aa}	1.82 ^{Aa}	54.28 ^{Ba}	53.38 ^{Ba}	67.41 ^{A}	141.32 ^{D}	48.85 ^{C}
CSC 4703	64.67 ^{A}	32.87 ^{A}	1.55 ^{Aa}	1.42 ^{Ba}	52.51 ^{Ca}	53.38 ^{Ba}	60.49 ^{B}	141.50 ^{D}	55.54 ^{A}
CSC 4704	65.97 ^{A}	33.42 ^{A}	1.52 ^{Aa}	1.47 ^{Ba}	57.27 ^{Ba}	58.30 ^{Aa}	63.67 ^{A}	158.31 ^{B}	53.94 ^{B}
CSC 4707	65.58 ^{A}	36.85 ^{A}	1.54 ^{Ab}	1.86 ^{Aa}	50.35 ^{Cb}	56.12 ^{Ba}	66.93 ^{A}	194.02 ^{A}	46.68 ^{C}
CSC 497	61.52 ^{B}	36.14 ^{A}	1.38 ^{Ab}	1.74 ^{Aa}	59.00 ^{Aa}	57.61 ^{Aa}	75.18 ^{A}	158.70 ^{B}	46.51 ^{C}
CSC 500	64.84 ^{A}	31.90 ^{A}	1.47 ^{Aa}	1.23 ^{Ba}	58.49 ^{Aa}	59.11 ^{Aa}	65.27 ^{A}	160.28 ^{B}	59.17 ^{A}
Dark O.S.	46.94 ^{D}	34.55 ^{A}	0.74 ^{Bb}	1.49 ^{Ba}	61.66 ^{Aa}	56.02 ^{Bb}	82.53 ^{A}	133.76 ^{D}	35.16 ^{E}
HB 4488P	56.60 ^{C}	33.66 ^{A}	1.12 ^{Bb}	1.53 ^{Ba}	54.54 ^{Bb}	59.27 ^{Aa}	60.01 ^{B}	162.16 ^{B}	43.77 ^{D}
New cultivar	63.41 ^{B}	34.19 ^{A}	1.45 ^{Aa}	1.54 ^{Ba}	56.96 ^{Bb}	62.78 ^{Aa}	62.07 ^{B}	146.31 ^{C}	50.37 ^{B}

Average	59.88	34.15	1.30	1.54	54.31	56.71	62.38	146.04	47.88

Means followed by the same capital letters do not differ significantly ($p > 0.05$) between genotypes, and means followed by the same lowercase letters do not differ significantly ($p > 0.05$) between N concentrations. [1] Higher means for 2 mM for all genotypes assessed. [2] Higher means for 10 mM for all genotypes assessed.

3.3. Grouping Tobacco Genotypes According to their Efficiency and Responsiveness to N Supply

Tobacco genotypes were classified into four different groups: efficient and responsive (ER), efficient and non-responsive (ENR), inefficient and responsive (NER), and inefficient and non-responsive (NENR) to N supply. For this, the NUEf and the plant DM under N deficiency for each genotype were used to plot the y and x axis, respectively. The average NUEf and plant DM for all genotypes were calculated, and are represented by the internal lines of the chart in Figure 1. After plotting all genotypes in the chart, each genotype was allocated to one of four quadrants (groups). The genotypes on the right of the chart are efficient, but those on the left are inefficient in N use. Furthermore, genotypes above the horizontal line are responsive, and below are non-responsive to N supply. Interestingly, the 28 genotypes assessed were equally distributed into the four groups. The genotypes belonging to the varietal groups Virginia and Maryland were efficient, while the genotypes of the Burley, Comum, and Dark groups were inefficient in N use. CSC 500 and New cultivar (both Maryland) were non-responsive, while Virginia, Comum, and Burley groups had genotypes with variations in responsiveness to N supply depending on the genotype studied.

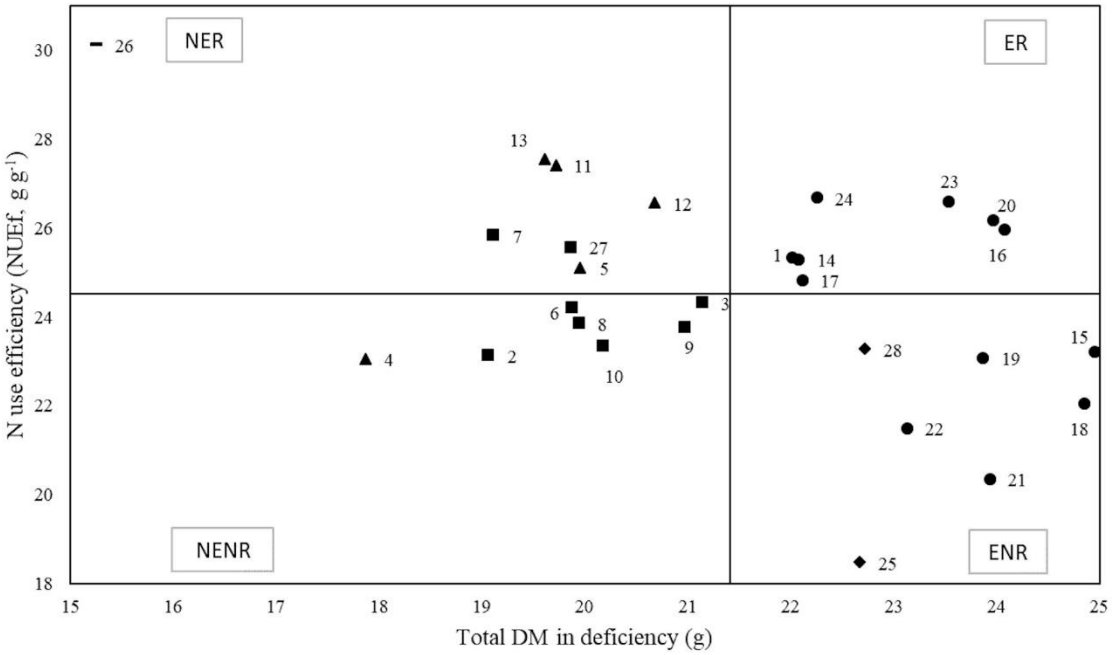

Figure 1. Classification of the 28 tobacco genotypes in response to the total dry mass (DM) produced under N deficiency (g) and the responsiveness to N supply (NUEf; g g^{-1}). Internal lines represent the mean values for the axis of all genotypes. The Virginia, Burley, Comum, Maryland, and Dark varietal groups are represented by circles, squares, triangles, rhombs, and lines, respectively. ER, efficient and responsive; ENR, efficient and non-responsive; NER, inefficient and responsive; NENR, inefficient and non-responsive. For genotypes and their respective numbering, refer to Table S1.

4. Discussion

4.1. Variation of N Utilization Efficiency (NUE) Traits among Genotypes and N Supplies

By the use of two nutritive solutions with contrasting N concentrations, deficient and sufficient in N, we investigated the response of a wide range of tobacco genotypes in terms of N use. The N concentrations chosen in the present study provided plants with either a deficient or an adequate supply of N. This was visually observed by the yellowish color of older leaves compared to the light green color of younger leaves in plants grown in nutritive solution with 2 mM of N, but darker green color of the whole plants grown in 10 mM of N. Furthermore, a decrease in DM, N content, and N accumulation for

roots, stems, and leaves was caused when plants were subjected to N deficiency, regardless of the genotype. This was reflected in a higher root/shoot DM ratio under N deficiency, which typically occurs in plants subjected to N deficiency [24]. A higher ratio root/shoot DM ratio in higher N rates was reported by Brueck and Senbayram [10] in two tobacco varieties; however, the authors attributed this fact to losses of fine roots during the washing of the peat–perlite substrate used for cultivation. Indeed, Poorter et al. [25] relate the challenge of assessing a reliable mass of roots in plants conducted in pot experiments with substrate. Fan et al. [15] found a decrease of root/shoot ratio with increasing N concentration for one cultivar, but not in another one when supplied solely with $N–NO_3$. However, our data are in accordance with the "functional equilibrium" existing between roots and shoots described by Brouwer [26]. When the limiting factor is a nutrient (e.g., N), more biomass is allocated to the roots in comparison to the shoots [25]. Thus, plants favor the organ (roots) responsible for the uptake of the limiting factor (N) that plants are experimenting in a given situation [27]. It is likely that genotypes with a higher root/shoot ratio under N depletion are more sensitive to N deficiency.

The higher production of DM of shoots compared to roots under adequate N supply is related to the capacity of plants to uptake N from the growth media through the roots, as revealed by the NUpE index, calculated as the amount of N in the plant by the mass of the roots. On average, the NUpE of genotypes grown under adequate N supply was 146.04 mg N g^{-1} roots, while plants under N deficiency showed only 62.38 mg N g^{-1} roots. The NHI is calculated as the N accumulation in leaves by the N in the whole plant. In other words, it represents the amount of N that is allocated to leaves by plants. Thus, it may represent an important index for the tobacco market, as leaves are the commercial product of the crop. The NHI was, on average, quite close among plants grown under N-deficient and N-sufficient supplies: 54.31% and 56.71%, respectively. This means that, regardless of the N nutrition status of tobacco genotypes, the N accumulation in leaves compared to the whole plant remains constant in the range of 50%. Thus, provides valuable information regarding N exported from the crop area in a given growing season [28], which may be helpful for N fertilization management. The higher NHIs suggest that these genotypes are more efficient in transferring N to leaves from root N uptake [15].

The DM produced per unit of N, i.e., the NUE index, was higher for plants grown under N deficiency, regardless of the genotype. A decrease of 3–6-fold between the same N concentrations tested in this experiment was found by Fan et al. [15] for two varieties of tobacco. The authors suggest that the highest concentration supplied plants excessively in N at the growth stages tested. However, this can be explained simply by the well-known law of diminishing returns, which postulates that marginal yield decreases as the level of the limiting factor is raised. As the N rates are increased, NUE is decreased, as demonstrated by Sisson et al. [13], in 12 cultivars of tobacco. In this study, 10 mM was the N concentration of the nutritive solution that adequately fertilized tobacco genotypes, and thus, plants grown in it returned 34 g DM g^{-1} N on average, unlike the 60 g DM g^{-1} N produced by plants under N deficiency. Genotypes with higher NUE produce more biomass per unit of N, and they utilize N from the media more efficiently.

The RENU is related to the total DM produced under N-deficient and N-sufficient supplies. It was shown that under N deficiency, the plant DM decreased from 35% (Dark O.S.) to 59% (CSC 500). As leaves are responsible for the most DM in the plant (Table 1), the importance of N fertilization for tobacco genotypes can be confirmed. However, the decrease in plant DM varied considerably among genotypes. Therefore, the genotypic variation should be considered in N fertilizations by landholders.

4.2. Classification of Genotypes: Efficiency and Responsiveness to N Supply of Contrasting Tobacco Genotypes

In this study, we classified the tobacco genotypes according to their efficiency and responsiveness to N supply. This was achieved by the use of plant DM obtained by the growth in an N-deficient nutritive solution, and the NUEf was calculated based on the DM and N accumulation obtained of the plants grown in the contrasting N concentration solutions. The combination of both variables resulted in the separation of genotypes into four groups: ER, ENR, NER, and NENR, being ER the group of genotypes most desirable [28]. We chose a hydroponic condition for this study, as the growth of the tobacco genotypes in soil (i.e., pot or field trial) could result in an unreliable classification due to the

unknown amount of mineral N supplied to plants through organic matter mineralization. In addition, the use of nutritive solutions allows the supply N in concentrations previously established.

The classification performed in this study indicated the Virginia and Maryland varietal groups as efficient in N use, which means they produced plant DM above the average of all of the genotypes assessed. The Burley, Comum, and Dark varietal groups were N-inefficient, that is, they produced below average DM. The responsiveness within varietal groups varied considerably, except for the Maryland and Dark groups. This suggests that even individuals belonging to a same genetic group may have different characteristics in the responsiveness to N supply. For example, CSC 497 and CSC 4703, both belonging to the Virginia group, differ considerably in responsiveness, the first being responsive and the latter non-responsive to N. The data of this study may be interpreted from two interesting and practical points of view. First, the responsiveness to N fertilization in the cultivation of a given genotype may be different from another one, even if both belong to a same varietal group (e.g., CSC 3703 and BAT 3004, CSC 4707 and CSC 4703, and the others in Fig. 1). Thus, N fertilizer management should preferentially be adjusted according to each genotype, rather than considering the same N demand for different genotypes of a same group. Second, the characteristics of efficiency and responsiveness of each genotype allow the choice of suitable genotypes according to soil N level or affordability of N fertilizers by landholders. However, this is dependent on the edaphoclimatic condition requirements and other desirable plant features such as disease resistance, yield, planting season, or cropping cycle.

The present study provides valuable data to allow further deep investigation of the genotypes assessed. This is important for future breeding targeting the improvement of the N metabolism, and consequently, a better N use efficiency for tobacco genotypes. Few studies have considered the use of numerous tobacco genotypes studied concomitantly to characterize N-use efficiency traits (e.g., [14,15]). This is likely due to the time-consuming process involved in plant growth and analysis. However, the screening of contrasting genotypes is a valuable tool to characterize genotypes regarding their N use and selection. For example, modern cultivars showed generally higher NUE, a 50% higher yield compared to older ones in the work of Sisson et al. [13], which compared 12 cultivars. According to the authors, as much is known about genetic diversity coupled with NUE, it is possible to make future improvements in the cultivars. Among six tobacco cultivars, Ruiz et al. [14] selected the one based on the highest NO_3 reductase activity and the lowest foliar NO_3 concentration. The authors successfully obtained plants with higher NUE traits by grafting cultivars with less NUE traits to the best cultivar. However, in our view, a better approach to increase NUE in tobacco crops is firstly to characterize as many genotypes as possible through simple methods. This could then comprise a greater number of genotypes spanning a wide range of mechanisms related to NUE traits [9]. In the present study, through simple data of DM and N content of tobacco genotype organs, we characterized and classified 28 genotypes, which were first thought to have contrasting responses to different N supplies. Thus, our data represent interesting information to further deepen investigation regarding the internal mechanisms related to NUE in tobacco crops. In this way, the contrasting genotypes can be subjected to physiological and molecular studies, providing clues about the physiological traits better related to NUE in those tobacco lines, contributing to tobacco breeding programs. The knowledge of more genes related to NUE may contribute to enlarge the breeding approaches aiming to obtain lower TSNAs in tobacco [19].

The use of efficient genotypes is a strategy to improve NUE in a given crop [28]. In tobacco crops, there are serious concerns regarding the management of N fertilization. First, N rates vary widely among tobacco types, from 56 to 308 kg N ha^{-1} [29,30]. Second, high N rates may increase $N–NO_3$ content in the leaves of tobacco [31], which can be further transformed to $N–NO_2$. Then, $N–NO_2$ reacts with precursor alkaloids, forming TSNAs, widely known to be carcinogenic compounds that affect the health of consumers. Third, nicotine, which is the main alkaloid in tobacco, is an addictive substance that causes harmful effects on human health. Nicotine content in leaves increases with N supply [32,33]; therefore, genotypes with a lower demand for N will probably have a lower concentration of nicotine. Thus, taking into account the reaction of nicotine with $N–NO_2$ to form TSNAs [18], genotypes with higher NUE would be desirable as they would be less harmful to healthy humans because of a lower

TSNA content [30]. Finally, if N–NO_3 undesirably accumulates in leaves, it is likely that a step in N metabolism is impaired [34], which may be caused by excessive use of N fertilizers for any genotype, but such an effect is probably raised in low-efficiency genotypes.

5. Conclusions

The 28 tobacco genotypes studied vary in their efficiency and responsiveness to N supply. The genotypes with the highest efficiency for N supply belong to the Virginia and Maryland varietal groups. The other tobacco genotypes belonging to the Burley, Comum and Dark groups are inefficient in terms of N supply. The tobacco genotypes' responsiveness to N supply varies within each of the varietal groups assessed, except for Maryland.

The discrimination of the contrasting tobacco genotypes regarding their NUE in this study may contribute to tobacco-breeding programs. Furthermore, it helps to choose the most suitable genotype depending on soil N availability or affordability of N fertilizers for landholders worldwide. This can contribute to the improvement of the N use in tobacco crops, while preserving natural resources.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1: Table S1. Tobacco genotypes and their respective varietal groups used in the experiment; Table S2. Stock solutions and amounts used to prepare the nutritive solution with 2 mM of N (deficiency) and 10 mM of N (adequate); Figure S1. Images showing the visual aspect of tobacco genotypes when subjected to N deficiency (2 mM, pots on the left) or adequate N supply (10 mM, pots on the right).

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Table S1. Tobacco genotypes and their respective varietal groups used in the experiment.

Identification	Genotype	Varietal Group
1	BAG 06	Virginia
2	BAT 2101	Burley
3	BAT 2301	Burley
4	BAT 3004	Comum
5	BAT 3201	Comum
6	CSC 221	Burley
7	CSC 2305	Burley
8	CSC 2307	Burley
9	CSC 259	Burley
10	CSC 2602	Burley
11	CSC 302	Comum
12	CSC 3702	Comum
13	CSC 3703	Comum
14	CSC 416	Virginia
15	CSC 4303	Virginia
16	CSC 4304	Virginia
17	CSC 439	Virginia
18	CSC 444	Virginia
19	CSC 447	Virginia
20	CSC 4501	Virginia
21	CSC 4703	Virginia
22	CSC 4704	Virginia
23	CSC 4707	Virginia
24	CSC 497	Virginia
25	CSC 500	Maryland
26	Dark O.S.	Dark
27	HB 4488P	Burley
28	New Cultivar	Maryland

Table S2. Stock solutions and amounts used to prepare the nutritive solution with 2 mM of N (deficiency) and 10 mM of N (adequate).

Stock Solution	mL/L
2 mM	
0.6 M KH_2PO_4	1
1 M KNO_3	2
1 M KCl	1
$CaCO_3$ suspension (3.7533 g/L) [1]	80
0.6 M $MgSO_4.7H_2O$	2
Solution a [2]	1
Fe-EDDHA solution (41.67 g/L of Quelmax®) [3]	2
10 mM	
0.6 M KH_2PO_4	1
1 M $Ca(NO_3)_2.4H_2O$	3
1 M KNO_3	2
0.5 M $Mg(NO_3)_2.6H_2O$	1
0.7 M $MgSO_4.7H_2O$	1
0.5 M K_2SO_4	1
0.5 M NH_4NO_3	1
Solution a [2]	1
Fe-EDDHA solution (41.67 g/L of Quelmax®) [3]	2

[1] $CaCO_3$ suspension was solubilized with HCl until a clear solution was observed. This supplies Ca^{2+} in the treatment with 2 mM through the following stoichiometry: $CaCO_3 + 2\ H^+ \rightarrow Ca^{2+} + CO_2 + H_2O$. pH was adjusted to ~6 after adding this solution, prior to the addition of the remaining stock solutions. [2] Solution a = Micronutrients (except Fe) of Hoagland and Arnon [20]. [3] Quelmax® = commercial product with 6% Fe. Both nutritive solutions have nutrients (except N) set at 60% and 100% of ionic strength for macro- and micronutrients, respectively.

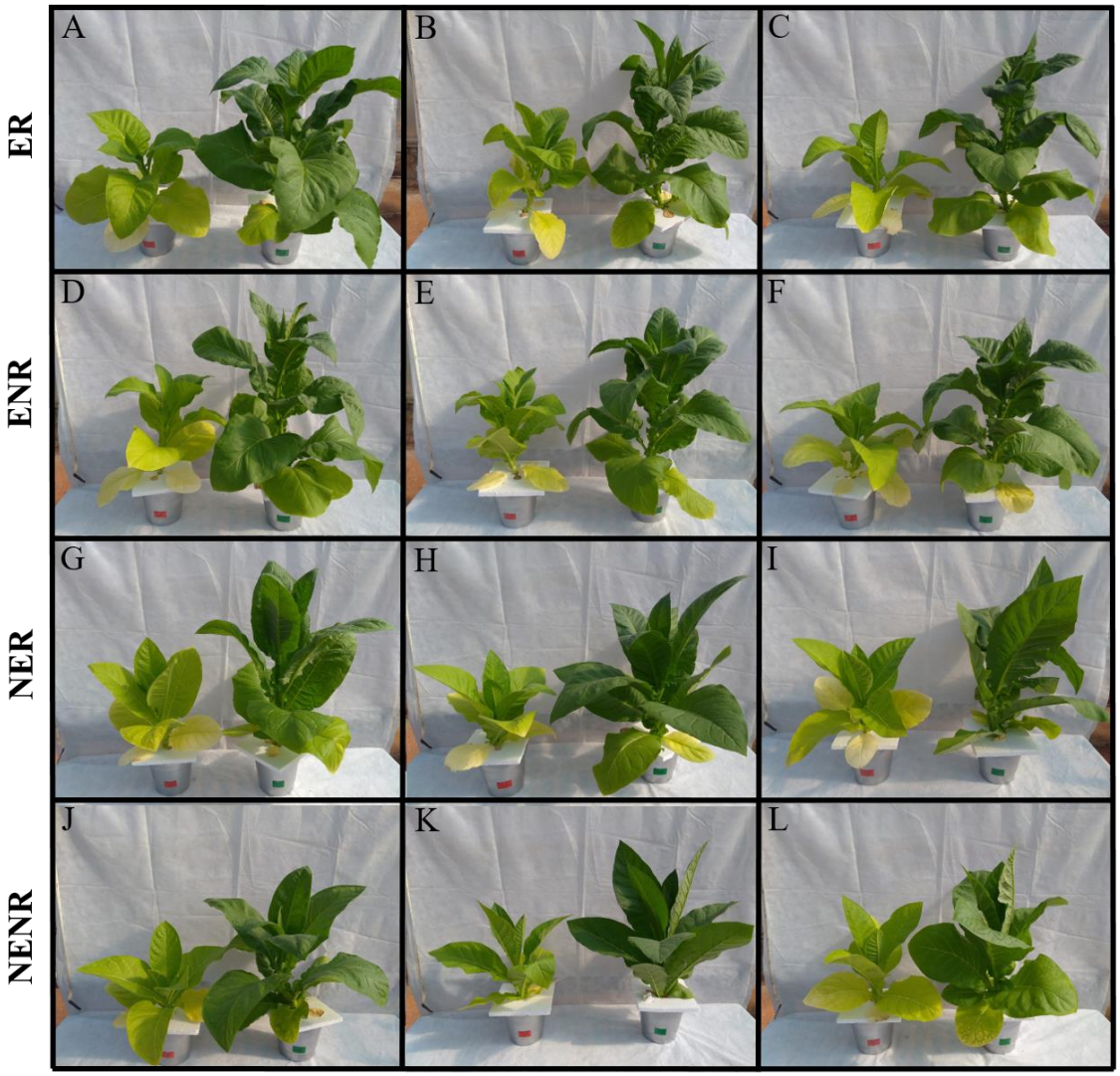


Figure S1. Images showing the visual aspect of tobacco genotypes when subjected to N deficiency (2 mM; pots on the left) or adequate N supply (10 mM; pots on the right). A = CSC 4303, B = CSC 4501, C = CSC 4707, D = CSC 4303, E = CSC 444, F = CSC 4703, G = CSC 2305, H = CSC 3703, I = CSC 302, J = BAT 2101, K = BAT 3004, and L = CSC 2602. ER, efficient and responsive; ENR, efficient and non-responsive; NER, inefficient and responsive; NENR, inefficient and non-responsive.

ARTIGO 2 - N METABOLISM ENZYMES AND GAS EXCHANGES AMONG NUE-CONTRASTING TOBACCO GENOTYPES

Artigo redigido conforme a NBR 6022 (ABNT, 2018) e formatado de acordo com o Manual da UFLA de apresentação de teses e dissertações.

ABSTRACT

Tobacco (*Nicotiana tabacum* L.) is very important non-food crop for many countries. In order to produce acceptable yield, nitrogen (N) fertilizers are essential, however, the excess may cause soil, water and air pollution. Depending on the tobacco genotype to be cropped, N rates differ considerably due to different physiologic and biochemical mechanisms, which are poorly studied. The aim of this study was to evaluate the physiologic responses of contrasted genotypes in efficiency and responsiveness to N supply when grown under deficient and adequate N nutrition. Principal component analysis (PCA) revealed that anthocyanins and flavonoids contents, glutamine synthetase (GS) and intercellular CO_2 concentration (Ci) were related to N deficiency condition, whereas net photosynthetic rate (Pn), activities of nitrate reductase (NR), glutamate synthase (GOGAT), glutamate dehydrogenase (GDH), total dry mass (DM), total N accumulation (NA), instantaneous carboxylation efficiency (k), total chlorophyll, NBI (N balance index), water use-efficiency (WUE), transpiration (E) and stomatal conductance (gs) were associated with adequate N supply. Generally, genotypes efficient and responsive (ER) showed lower anthocyanins but, higher chlorophyll and flavonoids content, higher k, PNUE, NR, GDH and GOGAT activities compared to genotypes inefficient and non-responsive (NENR). Although BAT 2101 and BAT 3004 – both NENRs – showed similar or even higher GS activity, k, flavonoids and chlorophyll content compared to ER genotypes, that did not reflect in similar or higher total DM nor total NA. Under N deficiency, genotypes ER revealed generally higher flavonoids content, PNUE, NR and GDH activities when compared to genotypes NENR under the same condition. The combination of multi-parameters linked to N and C metabolism seems to be decisive for better DM and NA in ER tobacco genotypes. We concluded that the greater total DM and total NA of the ER genotypes is due to the greater pairing of diverse parameters of both C and N metabolism here investigated.

Keywords: Nitrate reductase. Glutamine synthethase. Glutamate synthase. Glutamate dehydrogenase. Gas exchanges. Flavonoids. Anthocyanins. Nitrogen balance index. Chlorophyll. Dualex. *Nicotiana tabacum* L. PCA.

1 INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is a non-food crop very important for the economy of many countries. In 2019, tobacco was produced in 3.6 M ha worldwide, resulting in a production of 6.7 M tons. The main countries producers are China, India and Brazil, which all together accounted for ~63% of the world production in 2019 (FAOSTAT, 2020). To ensure productivity, nitrogen (N) is an important nutrient for this crop (CHAOQIANG JIANG; CHAOLONG ZU; HUOYAN WANG, 2015; ISABELLA; GIAMPAOLO; ALBINO, 2017; ZOU et al., 2017; CHEN et al., 2020; SOARES et al., 2020). However, the application of this nutrient may cause water (KAISER et al., 2015) and air pollution, mainly for genotypes with higher N demands (BOETTCHER et al., 2020), representing concerns with N fertilizers management. Then, the increase of NUE in tobacco should be pursued to ensure a friendly-environmental activity without compromising profitability.

In tobacco, there is another particular concern regarding N fertilization. As N rates increases, the nitrate (NO_3^-) content in leaves is also enhanced, which is associated to the formation of tobacco-specific nitrosamines - TSNAs (SHI et al., 2013; LI et al., 2017a), which are carcinogenic compounds. So, this imposes one more reason to find out ways to reduce the impacts to smokers' healthy. One pathway to improve tobacco quality is certainly through the production of leaves with the minimal NO_3^- content as possible. If higher NO_3^- is incorporated in organic compounds such as proteins, amino acids and others, it is likely that yield also increases. After the uptake by roots and translocation to leaves, NO_3^- begins to be metabolized by sequential enzymes of the N metabolism. Firstly, NO_3^- is reduced to nitrite (NO_2^-) by the nitrate reductase (NR) enzyme in cytoplasm. NO_2^- is highly toxic to plants so it is rapidly reduced to ammonium (NH_4^+) through nitrite reductase (NiR) in chloroplasts in green tissues or in plastids in non-green tissues. Then, glutamine synthetase (GS) reduces NH_4^+ to glutamine, occurring in the cytosol (if in roots) or chloroplasts (if in leaves), which can be transported to others parts of the plant, but also can be converted to glutamate by the glutamate syntethase (GOGAT) in chloroplasts, so glutamate is finally used to form other amino acids.

Another important nutrient for plant yield is carbon (C). The photosynthetic N use efficiency (PNUE) is calculated by the ratio of Pn and foliar N, indicating the amount of CO_2 incorporated for a given amount of N foliar. Since the enzymes of the N metabolism represent the way that plants possess to assimilate N inorganic forms to organic compounds, and is tightly linked to the C metabolism, both mechanisms play a crucial role in NUE in tobacco and consequently on yield. It is expected that genotypes with more NUE have higher biomass, show

higher N metabolism enzymes with diminished NO_3^- contents in leaves, but that depends on the N supply level (FAN et al., 2018).

In the literature, comparisons among different tobacco genotypes studying aspects of the N or C metabolism is very scarce and limited to few materials. LI et al. (2017b) found lower pigment, chlorophyll, carotenoids, Pn, NR and GS in two Burley compared with other two flue-cured tobaccos in nutritive solution under 4 mM N. They found the suppression of genes related to N and C metabolism in burley type. In a field and pot experiment, two flue-cured tobacco presented higher NR activity per N applied and lower NO_3^- content than two burley ones (LI et al., 2017a). NR increased with increasing levels of NO_3^- supply in two tobacco genotypes, while GS activity in leaves increased for one genotype and the another one decreased in the highest N level (FAN et al., 2018). All these valuable studies suggest the contribution of many mechanisms that culminate with lower response of one genotype compared to another one, despite of the limitation of genotypes assessed.

Furthermore, there is a need to investigate the internal mechanisms responsible for differences observed in the field, in terms of higher yield with less N rates in tobacco (e.g., flue-cured types) in comparison with less efficient ones (e.g. Burley) tobacco genotypes. For this, it is important to keep similar and controlled cultivation conditions among the plots for N investigations. Nevertheless, experiments in soil (field or pot) impose complexity to the N dynamic, giving to uncertainties of the amount and form of N supplied to plants.

In our previous study (ANDRADE et al., 2020) we screened 28 different genotypes regarding their efficiency and responsiveness to N supply, which represents relevant data to landholders and breeders when choosing the material to be cultivated or improved. However, aiming to go further, the next step is to find out the physiological mechanisms behind the yielding capacity of tobacco genotypes under contrasting N availabilities. In the light of the complex C and N metabolisms interplay (NUNES-NESI; FERNIE; STITT, 2010), the aim of the present study was to evaluate the physiologic responses of contrasted genotypes with known efficiency and responsiveness to N supply when grown under deficient and adequate N nutrition.

2 MATERIAL AND METHODS

2.1 Tobacco genotypes

The tobacco (*Nicotiana tabacum L.*) genotypes investigated in this study were chosen based on our previous work which we classified 28 tobacco genotypes according to their efficiency and responsiveness to N supply (ANDRADE et al., 2020). Briefly, seven genotypes were classified in each one of the contrasting groups of efficient and responsive (ER) and non-efficient and non-responsive (NENR). Thus, in the present study we selected three genotypes within each group which showed the lowest and the highest N use efficiency (NUE) and one genotype with the highest NUE of the NENR group, and one with the lowest NUE of the ER group. Therefore, genotypes used in the present study were CSC 4501, CSC 4707, CSC 497, CSC 439 (ERs), BAT 3004, BAT 2101, CSC 2602 and BAT 2301 (NENRs). All ERs genotypes belongs to the varietal group 'Virginia' and NENRs are 'Burley', with exception of BAT 3004 belonging to the group 'Comum'.

2.2 Growth conditions and experimental design

The experiment was carried out under greenhouse conditions in Lavras, Minas Gerais state, Brazil. Seeds were sowed in seeding trays filled with organic substrate MecPlant Tabaco−1® (MecPlant, Telêmaco Borba/PR), and then conducted in a floating system with deionized water. Then, 14 days after sowing (DAS), the floating system started to be fertilized with 10 mL L^{-1} of a nutritive solution with the following concentration: 20% of N and K_2O, 10% P_2O_5, 0.15% Mg, 0.05% Fe, 0.025% Mn and Zn, 0.0125% B and Cu, and 0.005% Mo. At 29 DAS, tobacco seedlings were transferred to 30 L plastic trays filled with nutritive solution #1 of Hoagland and Arnon (HOAGLAND; ARNON, 1950) at 20% and 100% of ionic strength (I.S.) for macro- and micronutrients, respectively. At 35 DAS, the nutritive solution was replaced by 40% and 100% of I.S. for macro- and micronutrients, respectively. The nutritive solution was permanently aerated throughout the period of plants adaptation to the hydroponic condition, and the water lost by transpiration was daily replaced.

After the period of plant adaptation to the nutritive solution, plants of homogeneous size and vigor were selected to be transplanted to pots (3 L, one seedling per pot) with treatments at 40 DAS. The treatments consisted of two nutritive solutions with contrasting N concentrations: deficient (2 mM) and adequate (10 mM), similarly as done previously (ANDRADE et al.,

2020), being that 5% of N-NH4+ is presented in the adequate level. Therein, the concentration of the remaining nutrients was set at 60% and 100% of I.S. for macro- and micronutrients, respectively, based on the concentration of Hoagland and Arnon (HOAGLAND; ARNON, 1950) solution #1 (APPENDIX A). Both nutritive solutions did not show any nutrient deficiency in plants besides the N deficiency at 2 mM. The solutions were renewed at 7, 14, 21, 25, 28 and 32 days after transplanting (DAT), constantly aerated, and the pH adjusted to ~6.0 once per week. To control *Alternaria spp.*, applications of fungicide Nativo® (Bayer) composted of tebuconazole (triazole) + trifloxystrobin (strobilurin) with mineral oil (Assist®, Basf) were performed at 19 and 26 DAT. The experimental design was a block-randomized, in a factorial scheme 8 (genotypes) × 2 (N concentrations, 2 and 10 mM), with six blocks.

2.3 Dry mass (DM), N accumulation (NA) and root measurements

Plants were collected at 37 DAT (77 DAS) at phenological stages (CORESTA, 2019) 1108/1109 for N deficient plants and 1109 to 1113 for N adequate level. Roots were detached from shoots and immersed in alcohol 20% and kept under 4 °C until further analysis. Then, shoots were left in the greenhouse for pre-drying over 10 days. After this, leaves and stems were separated and dried in an oven with forced air circulation at 65 °C up to constant weight. The dry mass (DM) of each organ was recorded prior to grinding in a Wiley mill, and ground samples were analyzed for N concentration (TEDESCO et al., 1995). A standard reference material (NIST® SRM® 1573 a tomato leaves) used in the digestion process revealed an average recovery of 108%, used to correct the N concentration. N accumulation (NA) for each organ was calculated by the product of N concentration and DM of the organ.

For determination of volume, surface area and diameter of roots, a small portion of roots (R_{saf}) were carefully sampled and spread over a rectangular acrylic bowl with a water film in it, and images were taken through a scanner connected to a computer. Images processing were performed in Safira software (JORGE; RODRIGUES, 2008) and an object of known length was used to calibrate it. R_{saf} were dried, and the DM was recorded to calculate the volume (v), surface area (sa) and diameter (d) of the entire root, which followed the equation v/sa/d of entire Roots = [DM of entire roots x i] / DM of R_{saf}, where i is the value obtained for the v, sa or d measured in the R_{saf}.

2.4 Non-destructive analysis of physiological parameters

The last fully expanded leaf from the apex of each plant was marked by a string tied in its petiole to ensure the performance of different assessments in the same leaf. These analyses are described below.

2.4.1 Leaf gas exchange measurements

Net photosynthetic rate (Pn), stomatal conductance (gs), intercellular CO_2 concentration (Ci) and transpiration rates (E) were measured using a portable infrared gas analyzer (LCp+ ADC bioscientific limited, UK). Photosynthetic active radiation (PAR) and CO_2 concentration in the reference chamber were set to 1000 µmol m^{-2} s^{-1} and 400 µmol of CO_2 mol^{-1} air, respectively. The flow in the leaf chamber was 200 µmol s^{-1} and the T_{leaf} was about 27 ^{o}C. Each reading was recorded after stabilization of Pn, performed at 33 and 34 DAT between 8:00 and 10:30 h. Data represent means of three readings taken in different points in the middle third of the last fully expanded leaf on each plant. The following indexes were calculated: water use efficiency - WUE (Pn/E), instantaneous carboxylation efficiency - k (Pn/Ci) and photosynthetic N use-efficiency - PNUE (Pn/ leaves NA).

2.4.2 Chlorophyll, flavonoids, anthocyanins and nitrogen balance index (NBI) assessments by Dualex

To assess non-destructively the contents of chlorophyll, flavonoids, anthocyanins and NBI (ratio between chlorophyll and flavonoids), we used the Dualex 4 Scientific (FORCE-A, Orsay, France) device. Data are means of four readings that were undertaken in different points in the middle third of the last fully expanded leaf on each plant. Readings were performed at 35 DAT.

2.5 Biochemical analysis

After data collection described above, the marked leaf was detached from stem, its middle third was sampled with a scissors, wrapped in aluminum foil and immediately immersed in liquid N. Then, these samples were conducted to the laboratory and stored in an ultra-freezer at -80 ^{o}C until assays described below were initiated.

2.5.1 Biochemical determination of chlorophyll and carotenoids

Photosynthetic pigments were biochemically determined according to (LICHTENTHALER; BUSCHMANN, 2001 with adaptation). About 0.1 g of the leaf tissue was put in an aluminum foil-covered glass bottle and incubated in 10 mL of acetone 80% in the refrigerator until the tissue had totally bleached out. The final volume was completed to 10 mL when necessary before the supernatant was subjected to spectrophotometric readings at 663,2 nm, 646,3 nm and 470 nm. The readings obtained from the mentioned wavelengths were used to calculate the chlorophylls 'a', 'b' and carotenoids contents, respectively. Dilutions were performed when necessary. Total chlorophyll is the sum of 'a' and 'b'.

The correlation between the chlorophyll quantification by Dualex and by the biochemical method was plotted to develop predictive relation among them (APPENDIX B). For this, it was proposed a reference curve with increasing N concentrations (0.5; 2; 6; 10 and 14 mM of N) for the genotypes with opposite NUE (i.e. BAT 3004 and CSC 497).

2.5.2 Activity of the enzymes related to the N metabolism

About 1g of fresh leaf (excluding the main vein) was ground in liquid N with 10% of PVPP and stored in falcon tubes at -80 °C until further processing. Then, the powder obtained was solubilized in 5 mL of extraction buffer containing the potassium phosphate buffer (100 mM, pH 7.5), ethylenediamino tetra-acetic acid (EDTA) 5 mM, dithiothreitol (DTT) 2 mM and phenylmethylsulfonyl fluoride (PMSF) 1 mM. The extract was homogenized and centrifuged at 16000 g, at 4 ^{o}C, for 20 minutes. Then, the supernatant was collected and used to determine the activity of nitrate reductase (NR), glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH).

For the determination of the NR activity (BERGES; HARRISON, 1995 with adaptation), aliquots of the enzymatic extracted were added to the incubation buffer containing potassium phosphate buffer (100 mM, pH 7.5), and potassium nitrate 10 mM. Then, the medium was incubated at 30 °C for three minutes. After the incubation period, the reaction was initiated by the addition of 0.2 mM b-NADH, followed immediately by the reading on a spectrophotometer. RN activity was determined by monitoring the oxidation of b-NADH, at 340 nm, for 10 minutes, at one-minute intervals. For the enzyme activity calculations, only the last five minutes of reading were used. The molar extinction coefficient used was 6.22 mM^{-1} cm^{-1}.

GS activity (RATAJCZAK; RATAJCZAK; MAZUROWA, 1981) was determined by the addition of aliquots of the enzymatic extract to the reaction medium containing Tris-HCl (100 mM, Ph 7.5), mercaptoethanol 10 mM, MgSO4.7H2O 20 mM, NH_2OHCl 15 mM, monosodium glutamate 50 mM and ATP 10 mM. Then, the medium was incubated at 30 ^oC for 30 minutes. The reaction was stopped by adding 1 mL of a solution composed of FeCl3 370 mM, HCl 670 mM and TCA 200 mM, followed by centrifuging at 16,000g for 5 minutes. The supernatants were removed and used for reading at 540 nm. The quantification of the chelate Fe-L-glutamyl-γ-hydroxamate (GHA) produced by the reaction was performed based on the standard curve with known concentrations of ɤ-glutamyl hydroxamate.

GOGAT activity (PIMENTA et al., 1989, with adaptation) was determined by the addition of aliquots of the enzymatic extracted in an incubation buffer containing Tris-HCl (70 mM, pH 7.8), L-glutamine 9 mM and 2-oxoglutarate 9 mM. The medium was incubated at 35 °C for five minutes, followed by the addition of b-NADH 0.2 mM and monitoring of the oxidation of b-NADH, at 340 nm for 10 minutes, at one-minute intervals. For the enzyme activity calculations, only the last five minutes of reading were used. The molar extinction coefficient used was 6.22 mM^{-1} cm^{-1}.

GDH activity determination (GROAT; VANCE, 1981) was performed through the addition of aliquots of the enzymatic extracted to the incubation medium containing Tris-HCl (100 mM, pH 7.8), 2-oxoglutarate 10 mM, $CaCl_2$ 4 mM and $(NH_4)_2SO_4$ 100 mM. This medium was incubated at 30 $^{\circ}C$ for 3 minutes. After the incubation, the reaction was initiated by the addition of 0.2 mM b-NADH followed immediately by readings in the spectrophotometer. GDH activity was determined by monitoring the oxidation of b-NADH, at 340 nm, for 10 minutes, at one-minute intervals. For the enzyme activity calculations, only the last five minutes of reading were used. The molar extinction coefficient used was 6.22 mM^{-1} cm^{-1}.

2.6 Statistical analysis

Firstly, the dataset obtained in this study was subjected to a Pearson's correlation to find the correlated parameters (APPENDIX C). The excess of parameters highly correlated (i.e., $r>0.7$, $p<0.05$) was retired to avoid redundancy in the principal component analysis (PCAs, Origin 2015 – OriginLab, JOLLIFE; CADIMA, 2016). Ellipses in charts represent the samples within the chosen confidence level (95% confidence level). The coefficients of each parameter were performed through correlation matrix. Then, an ANOVA was performed for all parameters

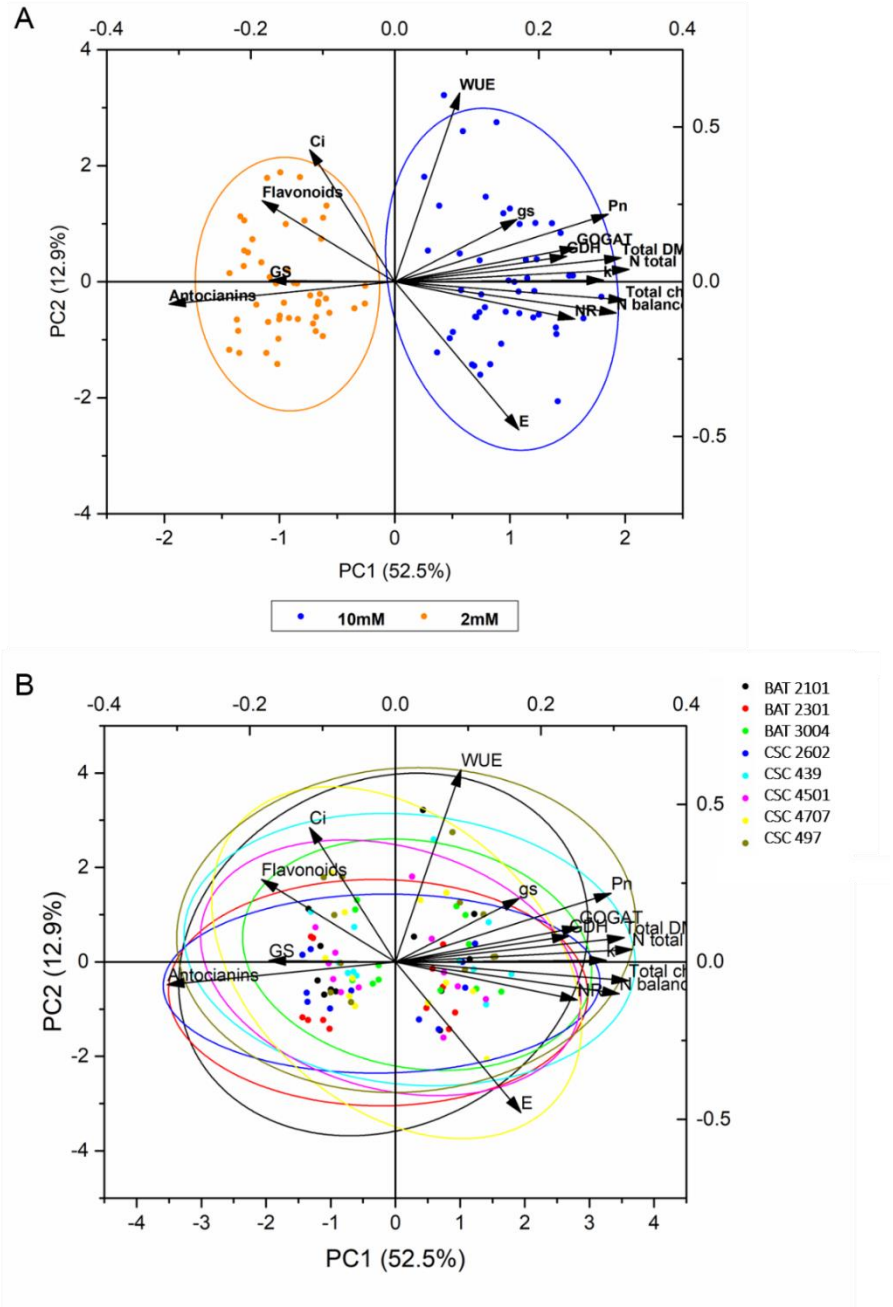
and when differences were detected, means were compared by the Scott-Knott test at $p < 0.05$ in Sisvar software (FERREIRA, 2011).

3 RESULTS

3.1 Principal component analysis (PCA)

When only the response of the studied parameters was observed in the two nitrogen conditions, it was defined clearly two groups of parameters highly related depending of the N level on what plants were grown (FIGURE 1A). Flavonoids, Ci, GS activity and anthocyanins contents are parameters related with N deficiency condition, whereas Pn, NR, GOGAT, GDH, total DM, total N, k, total chlorophyll and NBI had a strong relation with N adequate condition. WUE, E and gs also have a relation with adequate N condition, but weaker. When genotypes are discriminated in the same PCA, it notes that CSC 2602 and BAT 2301 behave more similarly, while the other genotypes are more comprehensive (FIGURE 1B). 65.4% of the variance was explained in this PCA, being that PC1 explained 52.5% of the variance, being this responsible for separating the contrasting N conditions. PC2 explained 12.9% of the variance. Although the PCA comprising all genotypes in both N conditions had explained satisfactorily the data variation, it was not possible to discriminate the similarities among the genotypes in a conclusive way. In this way, we performed others PCAs in the attempt to visualize any possible similarity among genotypes belonging to the same group according to our previous classification (ANDRADE et al., 2020).

Figure 1 – Principal Component Analysis of biochemical and physiological parameters discriminating the N availability effect (A) and tobacco genotypes (B) under N deficiency and adequate N supply.

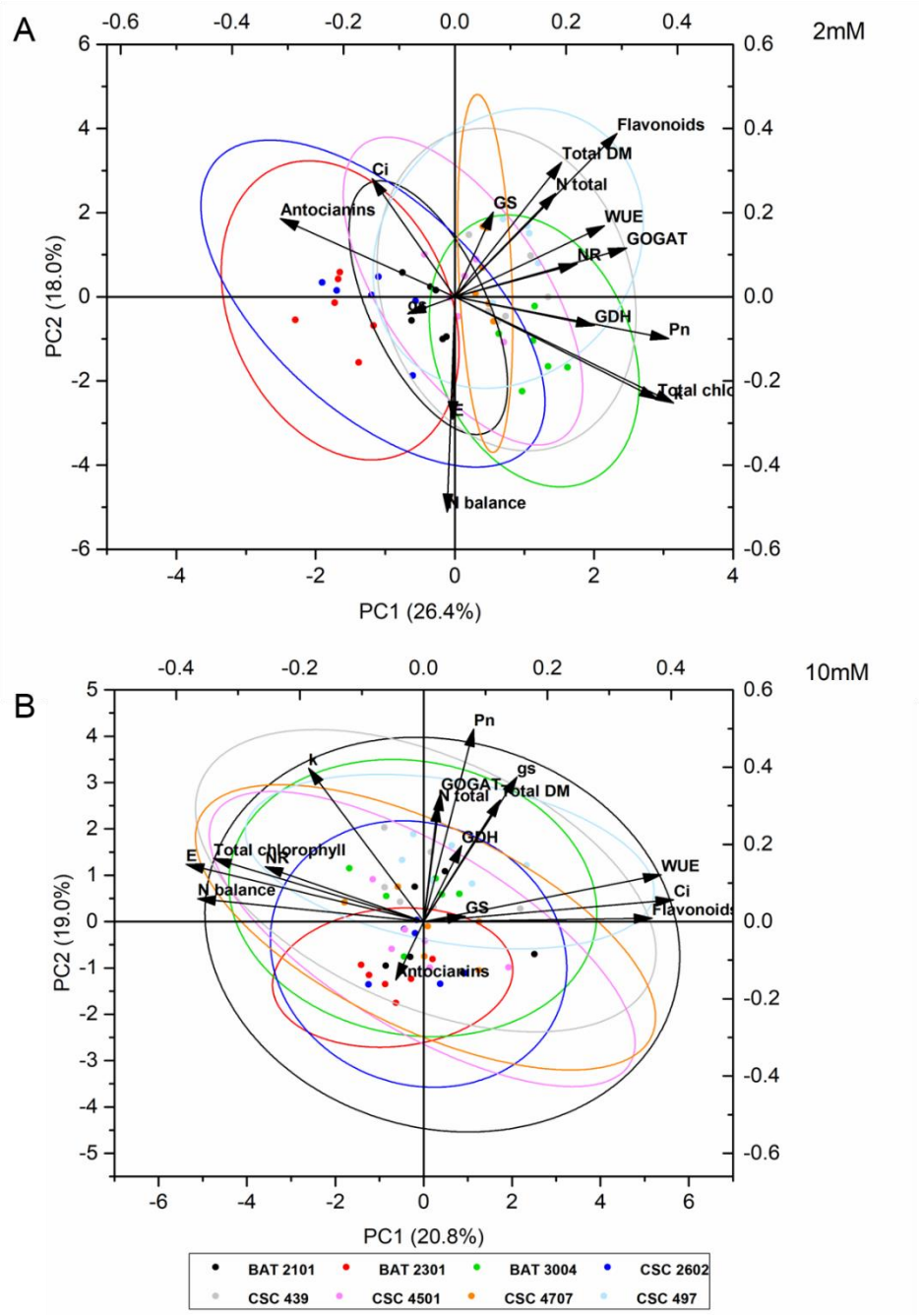


Fonte: Do autor (2021).

When the response of the genotypes in each one of the N levels was assessed separately, the variance was 44.4% explained for N deficiency condition (FIGURE 2A) and 39.8% for adequate N condition (FIGURE 2B). Under N deficiency, the genotypes belonging to NENR group were allocated more on the left of the PC1, except BAT 3004. Although CSC 4501 is

ER, there was a trend of this genotype to allocate more on the left of PC1. When the genotypes are studied in the adequate N condition, it is not clear a discrimination according to the group that a given genotype belongs. It is noted that BAT 2301 and CSC 2602 have a similar response, as well as CSC 4707 and CSC 4501, and CSC 439 and CSC 497. Interestingly, BAT 2101 is highly favored when submitted to adequate N nutrition.

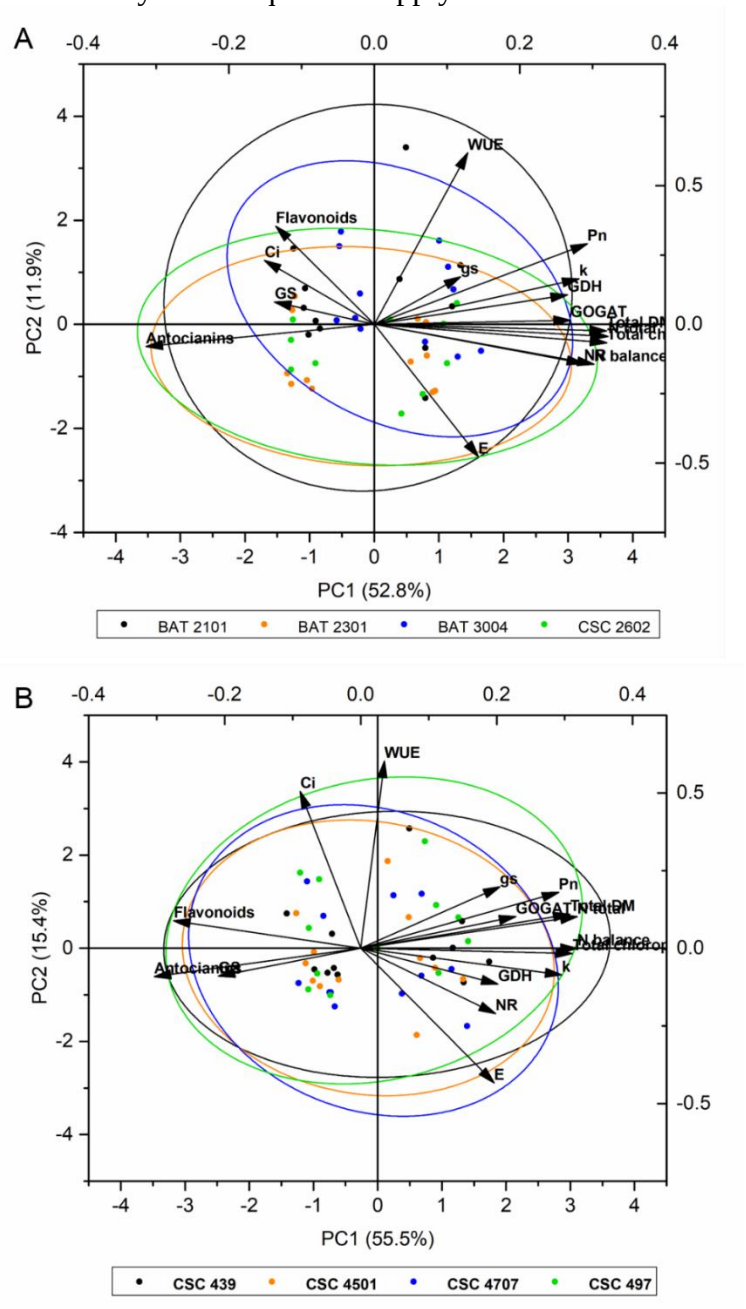
Figure 2 – Tobacco genotypes distribution in Principal Component Analysis as affected by deficiency (A) or adequate N nutrition (B).



Fonte: Do autor (2021).

When both groups NENR and ER are assessed separately, there was a greater explanation of the data variance, 64.7% (FIGURE 3A) and 70.9% (FIGURE 3B) respectively, being that PC1 explained the majority part of the variance in both situations. CSC 2602 and BAT 2301 are similar in the NENR group, whereas the pairs CSC 4501 and CSC 4707, and CSC 439 and CSC 497 are similar in the ER group.

Figure 3 - Distribution of NENR (A) and ER (B) tobacco genotypes as affected by the biochemical and physiological parameters in the Principal Component Analysis under N deficiency and adequate N supply.

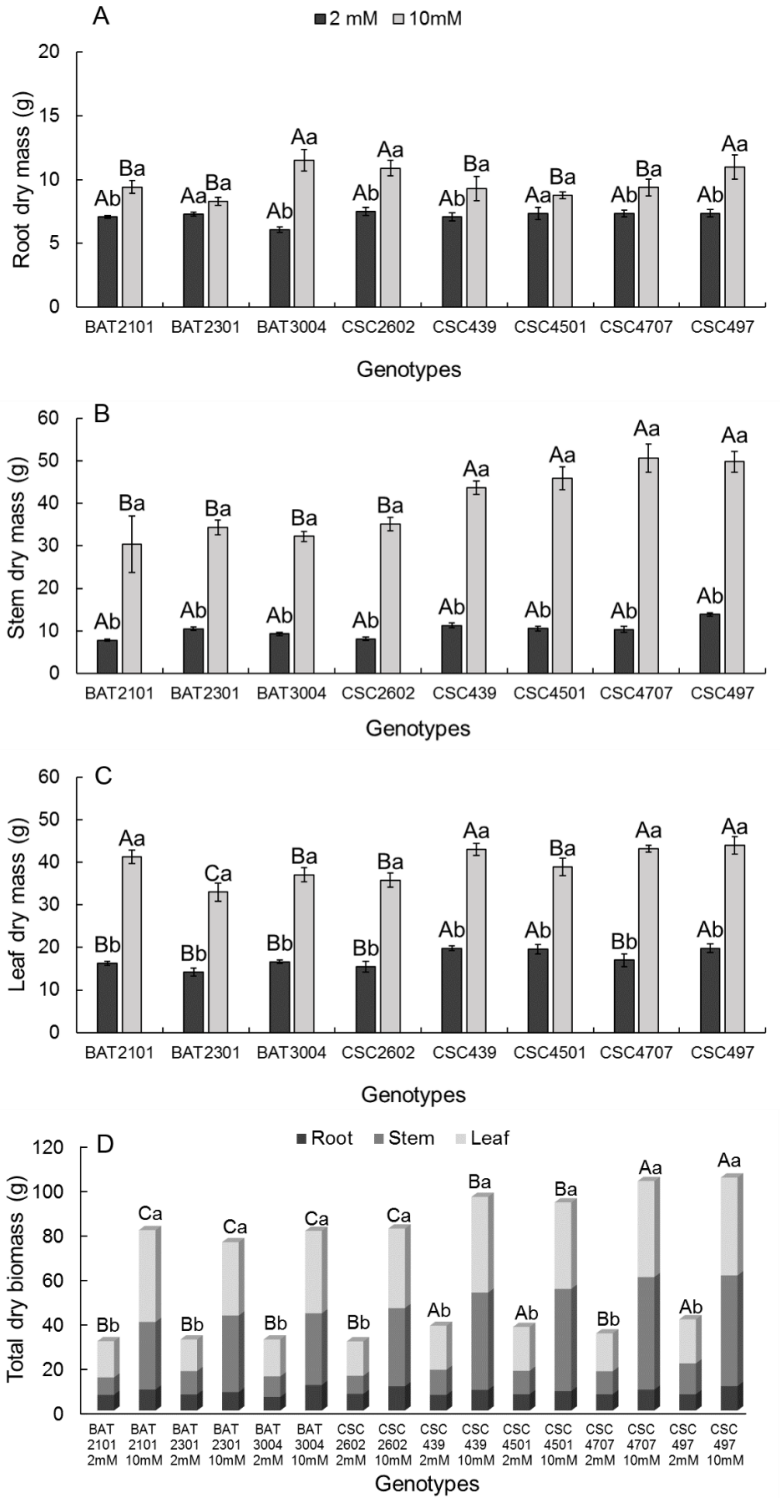


Fonte: Do autor (2021).

3.2 Dry mass (DM) and N accumulation (NA)

The interaction genotypes x N concentration was significant for DM of root, stem, leaves and for the whole plant. Under adequate N nutrition, BAT 3004, CSC 2602 and CSC 497 had higher root DM, by the other hand, under N deficiency all genotypes showed similar root DM (FIGURE 4A). All genotypes showed higher root DM when grown at adequate N nutrition, except for BAT 2301 and CSC 4501 that had similar root DM regardless N nutrition level. At adequate N nutrition, all genotypes belonging to the ER group, i.e. CSC 439, CSC 4501, CSC 4707 and CSC 497 had higher stem DM, on contrary, there was no differences in stem DM among genotypes under N deficiency (FIGURE 4B). The production of stem was greater for all genotypes when N was supplied at adequate level. For leaves production, it followed the order BAT 2101, CSC 439, CSC 4707, CSC 497 > BAT 3004, CSC 2602, CSC 4501 > BAT 2301 under adequate N nutrition (FIGURE 4C). CSC 439, CSC 4501 and CSC 497 had higher production of leaves under N deficiency. All genotypes had more leaves DM when grown at adequate N nutrition. The total plant DM production under adequate N supply followed the order CSC 4707, CSC 497 > CSC 439, CSC 4501 > BAT 2101, BAT 2301, BAT 3004 and CSC 2602 (FIGURE 4D). Under N deficiency, CSC 439, CSC 4501 and CSC 497 had greater plant DM. The production of plant DM was greater when genotypes were grown under adequate N nutrition.

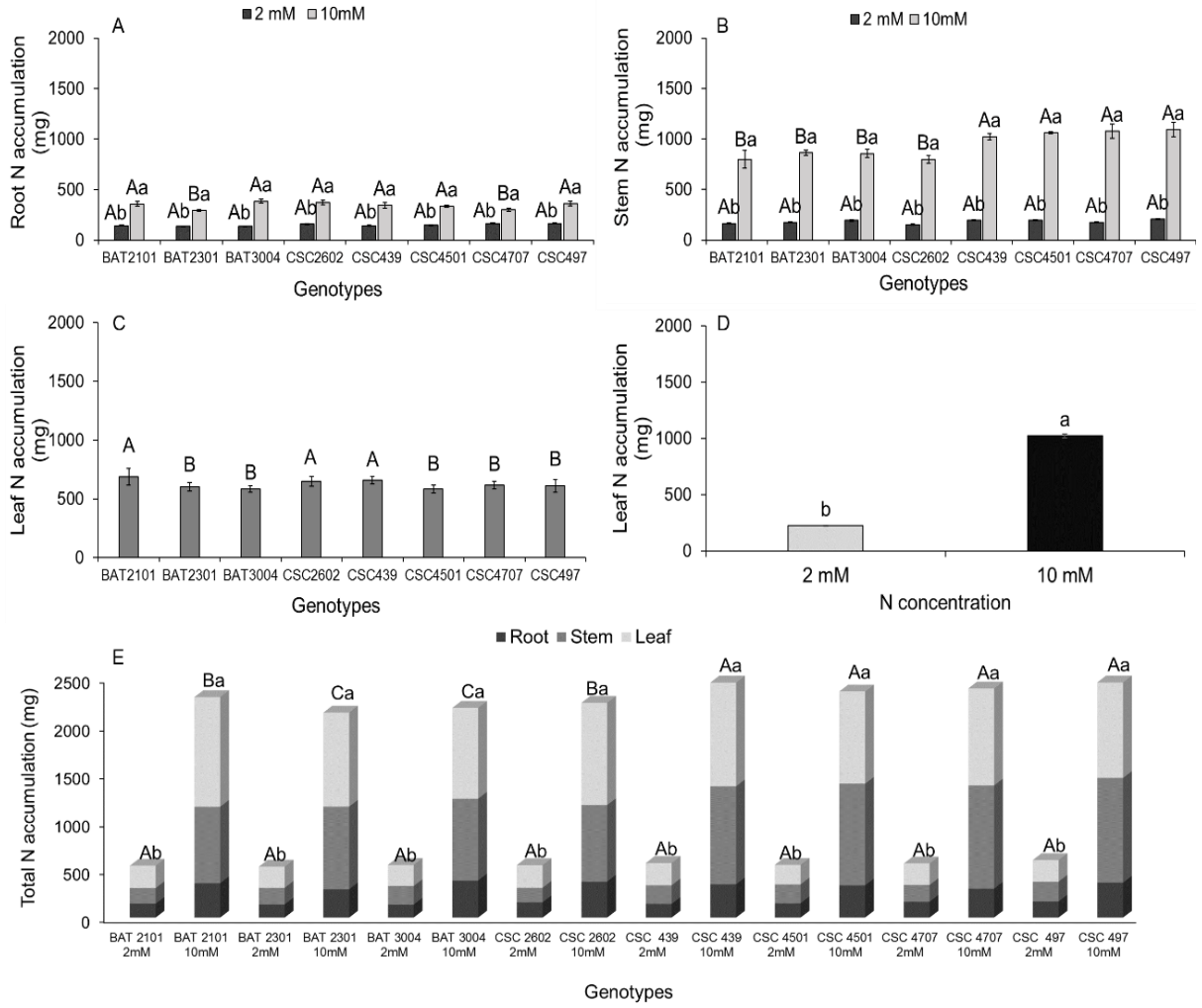
Figure 4 – Dry mass of roots, stem, leaves and total of different tobacco genotypes as affected by N availability.



*Genotypes followed by the same capital letters do not differ (p> 0.05) from each other within each N concentration, while same lowercase letters do not differ (p> 0.05) between N concentration by the Scott-Knott test. Bars represent the standard error of the mean (n=6).
Fonte: Do autor (2021).

For N accumulation (NA) in roots, stem and in the whole plant, there was a significant interaction of genotypes and N levels. For leaves NA, there was significant differences for genotypes and N levels. NA in root were lower in BAT 2301 and CSC 4707 under adequate N nutrition, by the other hand, there was no differences among genotypes when grown under N deficiency (FIGURE 5A). The root NA was greater for all genotypes when N was adequately supplied. Regarding NA in stem, genotypes of the ER group, i.e. CSC 439, CSC 4501, CSC 4707 and CSC 497 were greater at adequate N supply, but under N deficiency, the genotypes had similar stem NA among them (FIGURE 5B). Under adequate N nutrition, all genotypes had higher stem NA compared to the growth under N deficiency. Leaves NA was greater for BAT 2101, CSC 2602 and CSC 439 (FIGURE 5C). The growth at adequate N supply caused higher leaves NA in comparison to the growth at N deficient nutritive solution (FIGURE 5D). Plant NA followed the order CSC 439, CSC 4501, CSC 4707, CSC 497 > BAT 2101, CSC 2602 > BAT 2301, BAT 3004 under adequate N supply (FIGURE 5E). All genotypes had similar plant NA at N deficiency, being that all genotypes had higher plant NA when grown at adequate N nutrition.

Figure 5 – Nitrogen accumulation in roots (A), stem (B), leaves (C, D) and in the whole plants (E) of tobacco genotypes as affected by N availability

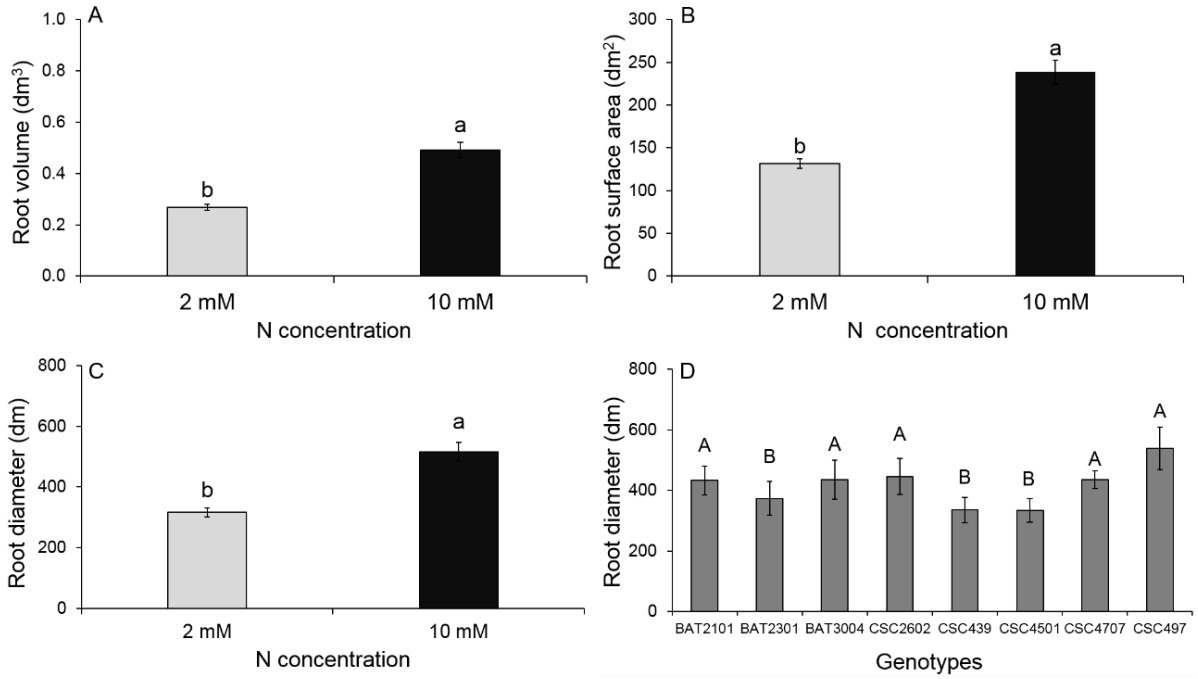


*Genotypes followed by the same capital letters do not differ (p> 0.05) from each other within each N concentration, while same lowercase letters do not differ (p> 0.05) between N concentration by the Scott-Knott test. Bars represent the standard error of the mean (n=6).
Fonte: Do autor (2021).

3.3 Root measurements: volume, surface area and diameter

There was a significant difference among the N levels studied for root volume and surface area (FIGURE 6A, B). For root diameter, there was significant differences for genotypes and N levels (FIGURE 6C, D). Higher volume, surface area and root diameter occurred when genotypes were grown at adequate N level. Lower root diameter was produced by BAT 2301, CSC 439 and CSC 4501.

Figure 6 – Root volume (A), surface area (B) and diameter (C, D) as affected by the N availability in tobacco genotypes.

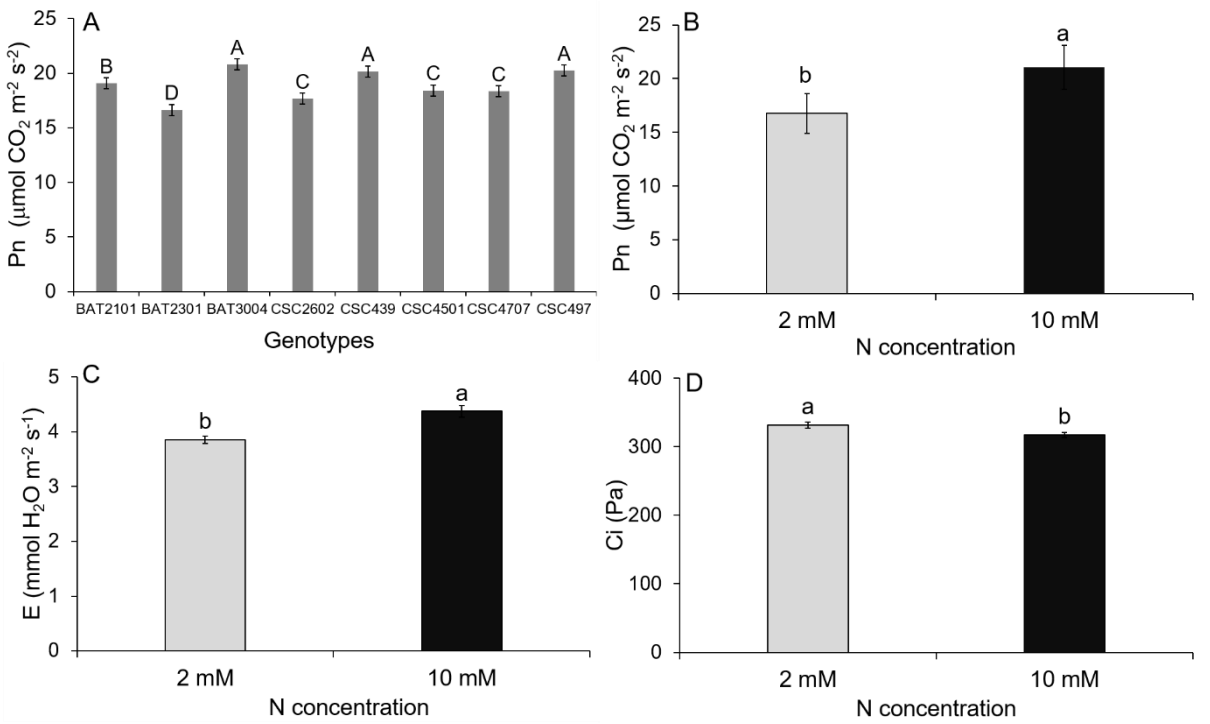


*Genotypes followed by the same capital letters do not differ (p> 0.05) from each other, while same lowercase letters do not differ (p> 0.05) between N concentration by the Scott-Knott test. Bars represent the standard error of the mean (n=6).
Fonte: Do autor (2021).

3.4 Leaf gas exchange measurements

The net photosynthetic rate (Pn) was significantly affected by genotypes and N levels (FIGURE 7A, B), while transpiration rate (E) and leaf intercellular CO_2 concentration (Ci) were affected only by N levels (FIGURE 7C, D). The Pn followed the order BAT 3004, CSC 439, CSC 497 > BAT 2101 > CSC 2602, CSC 4501, CSC 4707 > BAT 2301. Higher Pn and E were found under adequate N supply, while higher Ci was found under N deficiency.

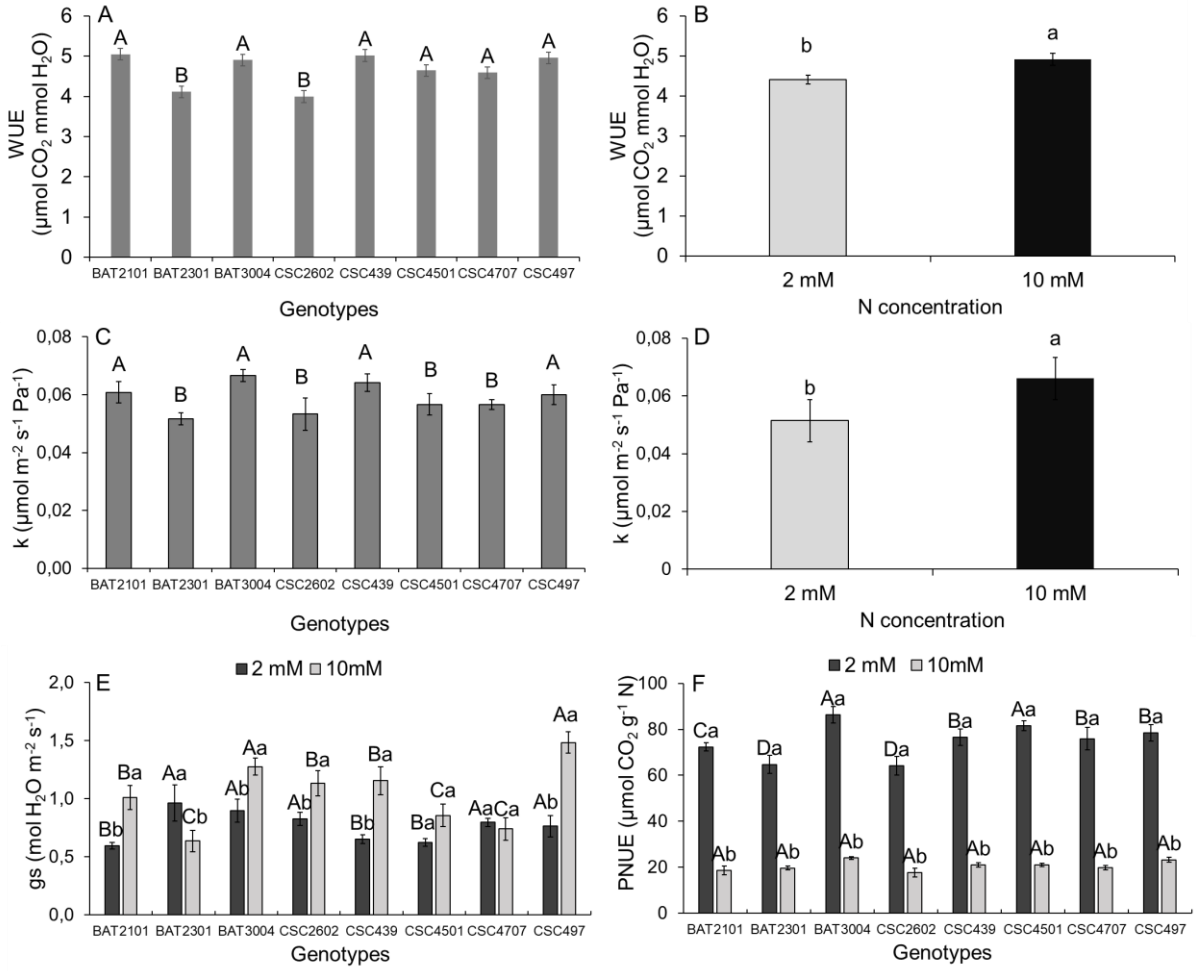
Figure 7 – Net photosynthetic rate (Pn, in A and B), transpiration rate (E, in C) and intercellular carbon concentration (Ci, in D) of tobacco genotypes as affected by the N availability.



*Genotypes followed by the same capital letters do not differ (p> 0.05) from each other, while same lowercase letters do not differ (p> 0.05) between N concentration by the Scott-Knott test. Bars represent the standard error of the mean (n=6).
Fonte: Do autor (2021).

The WUE and k were significantly influenced by genotypes and N levels, while gs and PNUE were influenced by the interaction genotypes and N levels. CSC 2602 and BAT 2301 had the lower WUE values (FIGURE 8A), while the growth of genotypes at adequate N level revealed higher WUE (FIGURE 8B). In respect to k, BAT 3004, CSC 439, BAT 2101 and CSC 497 had higher values (FIGURE 8C). The cultivation under adequate N supply caused higher k values (FIGURE 8D), while gs followed the order BAT 3004, CSC 497 > BAT 2101, CSC 2602, CSC 439 > BAT 2301, CSC 4501, CSC 4707, but at N deficiency, BAT 2101, CSC 439 and CSC 4501 had the lower values (FIGURE 8E). CSC 4501 and CSC 4707 showed similar gs regardless N nutrition level. Under adequate N supply, PNUE did not differ among genotypes, while under N deficiency the order followed BAT 3004, CSC 4501 > CSC 439, CSC 4707, CSC 497 > BAT 2101 > BAT 2301, CSC 2602 (FIGURE 8F). All genotypes had higher PNUE under N deficiency.

Figure 8 – Water use-efficiency (WUE, A and B), carboxylation efficiency (k, C and D), stomatal conductance (gs, E) and photosynthetic N use-efficiency (PNUE, F) of tobacco genotypes as affected by N availability.



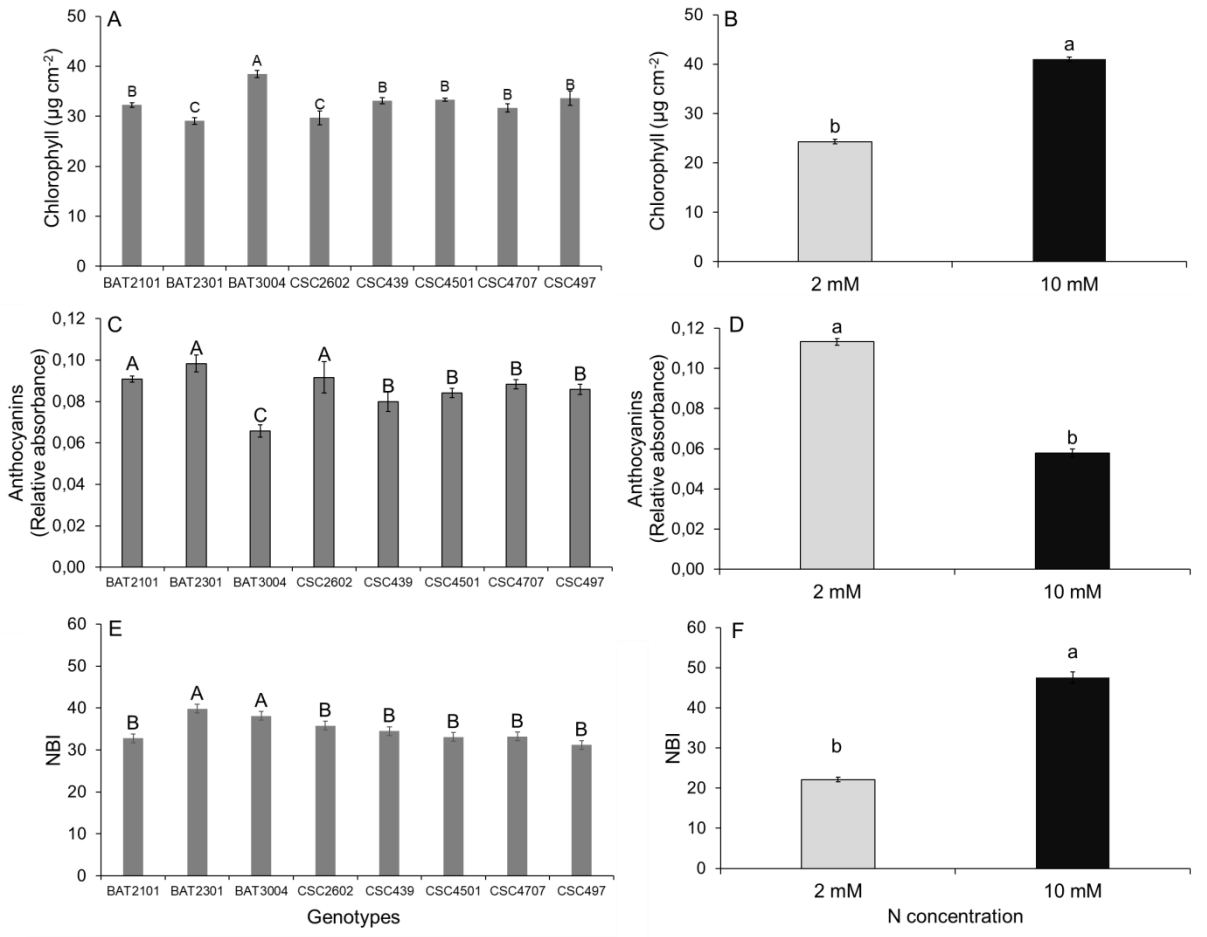
*Genotypes followed by the same capital letters do not differ (p> 0.05) from each other within each N concentration, while same lowercase letters do not differ (p> 0.05) between N concentration by the Scott-Knott test. Bars represent the standard error of the mean (n=6).
Fonte: Do autor (2021).

3.5 Chlorophyll, flavonoids, anthocyanins and nitrogen balance index (NBI) measured by Dualex

Chlorophyll content, anthocyanins and nitrogen balance index were influenced by genotypes and N levels (FIGURE 9), while flavonoids were affected by the interaction of genotypes and N levels (FIGURE 10). The chlorophyll content followed the order BAT 3004 > BAT 2101, CSC 439, CSC 4501, CSC 4707, CSC 497 > BAT 2301, CSC 2602 (FIGURE 9A), being that growth under adequate N supply caused higher chlorophyll content (FIGURE 9B) and lower anthocyanins (FIGURE 9D). The order for anthocyanins was BAT 2101, BAT

2301, CSC 2602 > CSC 439, CSC 4501, CSC 4707, CSC 497 > BAT 3004 (FIGURE 9C). The NBI was higher for BAT 2301 and BAT 3004 (FIGURE 9E), while the cultivation under adequate N nutrition reflected in higher NBI value (FIGURE 9F).

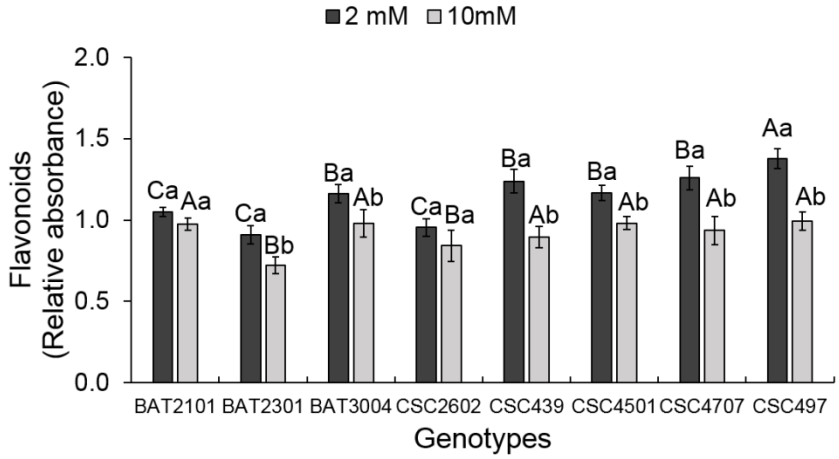
Figure 9 – Chlorophyll (A, B) and anthocyanin contents (C, D), and NBI (E, F) of tobacco genotypes as affected by N availability.



*Genotypes followed by the same capital letters do not differ (p> 0.05) from each other, while same lowercase letters do not differ (p> 0.05) between N concentration by the Scott-Knott test. Bars represent the standard error of the mean (n=6).
Fonte: Do autor (2021).

Under adequate N supply, BAT 2301 and CSC 2602 showed lower flavonoids content, and at N deficiency, the order was CSC 497 > BAT 3004, CSC 439, CSC 4501, CSC 4707 > BAT 2101, BAT 2301, CSC 2602 (FIGURE 10). All genotypes showed higher flavonoids content when grown under N deficiency, except BAT 2101 and CSC 2602 that had similar values regardless N supply.

Figure 10 – Flavonoids content in tobacco genotypes as affected by N availability.

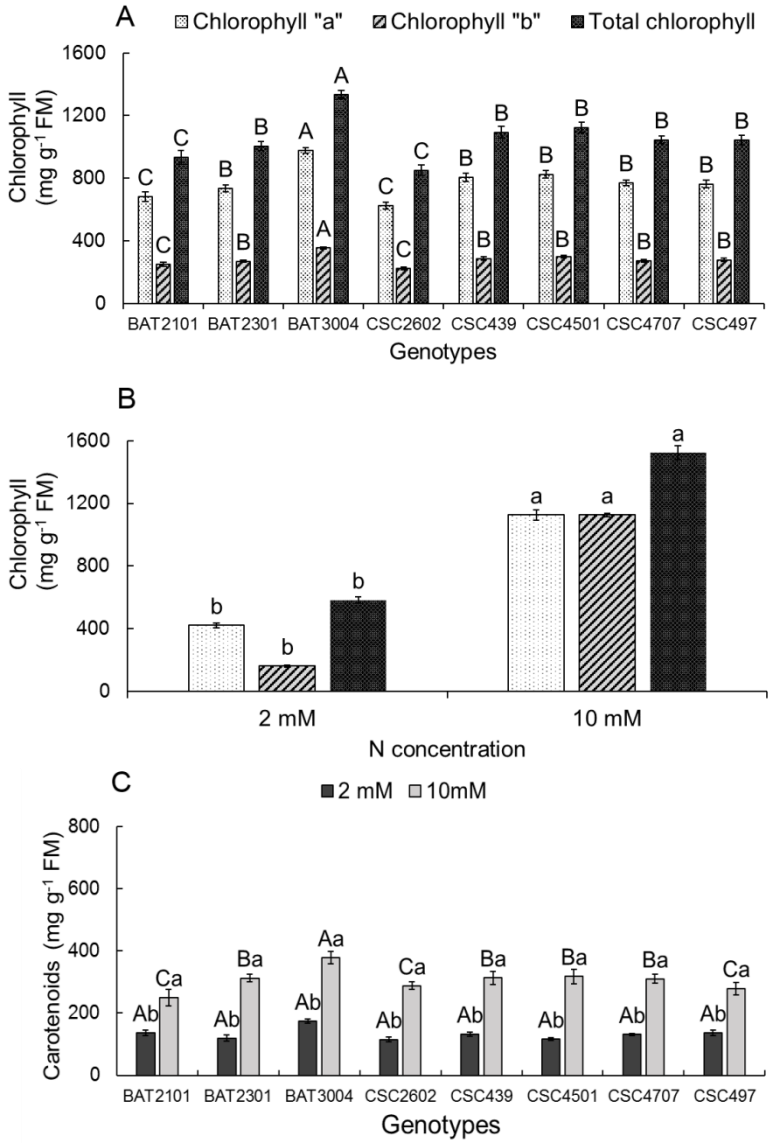


*Genotypes followed by the same capital letters do not differ (p> 0.05) from each other within each N concentration, while same lowercase letters do not differ (p> 0.05) between N concentration by the Scott-Knott test. Bars represent the standard error of the mean (n=6).
Fonte: Do autor (2021).

3.6 Biochemical determination of chlorophyll and carotenoids

Chlorophyll a, b and total were affected by genotypes and N levels, while carotenoids content was influenced by the interaction of both factors studied. The order was BAT 3004 > CSC 4501, CSC 439, CSC 4707, CSC 497, BAT 2301 > BAT 2101, CSC 2602 for chlorophyll a, b and total (FIGURE 11A), being the growth at adequate N level caused higher contents (FIGURE 11B). There were no differences among genotypes under N deficiency in respect to carotenoids content, but when plants were grown at adequate N level, the order was BAT 3004 > BAT 2301, CSC 439, CSC 4501, CSC 4707 > BAT 2101, CSC 2602, CSC 497 (FIGURE 11C). Under this condition, all genotypes showed higher carotenoids content compared to N deficient nutritive solution.

Figure 11 – Chlorophyll a, b, total (A, B) and carotenoids (C) contents in tobacco genotypes as affected by N availability.



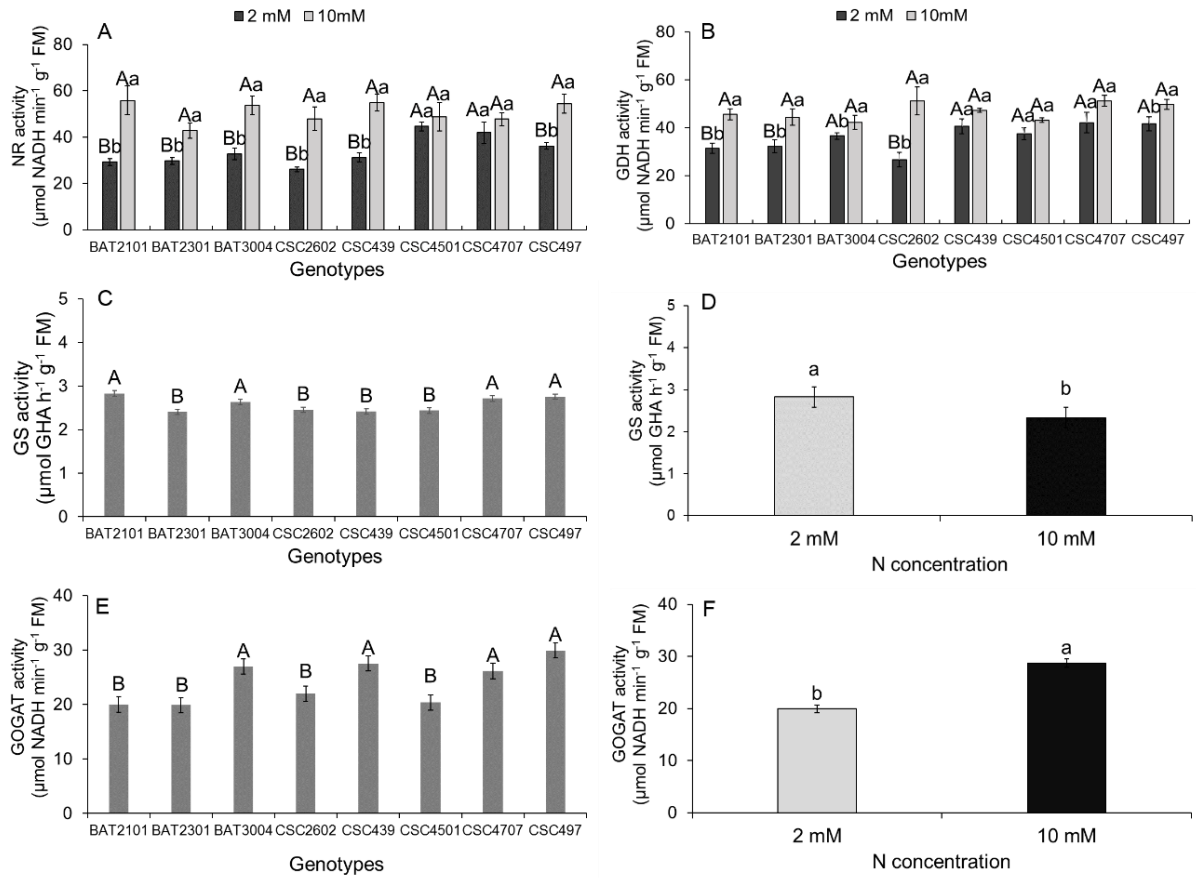
*Genotypes followed by the same capital letters do not differ (p> 0.05) from each other within each N concentration, while same lowercase letters do not differ (p> 0.05) between N concentration by the Scott-Knott test. Bars represent the standard error of the mean (n=6).
Fonte: Do autor (2021).

3.7 Activity of the enzymes related to the N metabolism

NR and GDH activities were significantly influenced by the interaction of genotypes and N levels, while GS and GOGAT were influenced by genotypes and N levels. Under adequate N nutrition, there was no differences among genotypes, but when they were subjected to N deficiency, CSC 4501 and CSC 4707 had higher NR activity (FIGURE 12A). Generally,

NR activity decreased with N deficiency, except for CSC 4501 and CSC 4707 that were statistically similar regardless N nutrition. In respect to GS activity, BAT 2101, BAT 3004, CSC 4707 and CSC 497 were superior among the others genotypes (FIGURE 12C), being that the growth under adequate N supply showed lower GS activity (FIGURE 12D). GOGAT activity was greater in BAT 3004, CSC 439, CSC 4707 and CSC 497 (FIGURE 12E), being the higher activity occurrence when genotypes were adequately supplied in N (FIGURE 12F). Finally, there was no differences among genotypes for GDH activity when subjected to adequate N nutrition (FIGURE 12B). On contrary, for N deficiency BAT 2101, BAT 2301 and CSC 2602 had lower GDH activity. Generally, GDH decreased with N deficiency, except for CSC 439, CSC 4501 and CSC 4707 that were statistically similar regardless N level.

Figure 12 – Activities of nitrate reductase (NR, A), glutamate dehydrogenase (GDH, B), glutamine synthetase (GS, C and D) and glutamate synthase (GOGAT, E and F) in tobacco genotypes as affected by N availability.



*Genotypes followed by the same capital letters do not differ (p> 0.05) from each other within each N concentration, while same lowercase letters do not differ (p> 0.05) between N concentration by the Scott-Knott test. Bars represent the standard error of the mean (n=6).
Fonte: Do autor (2021).

4 DISCUSSION

The greater DM production and NA of a group of genotypes over another one is due to physiological and biochemical mechanisms that allow the expression of such characteristics. In this study, the ER genotypes produced plants with higher DM and N accumulated in them in comparison to NENR group. In general, we observed that ER genotypes had the following responses: lower anthocyanins content, higher chlorophyll and flavonoids content, higher k, WUE, PNUE, NR, GDH and GOGAT activities in relation to NENR ones. Moreover, under N deficiency ER genotypes showed higher flavonoids content, PNUE and activities of NR and GDH that allowed them a better performance than NENR genotypes.

In our previous study (ANDRADE et al., 2020), we assessed 28 tobacco genotypes of different varietal groups that were classified according to their efficiency and responsiveness to the use of nitrogen. For this, two robust indicators easy to obtain were used: DM and NA in plants. Thus, plants that produced above-average DM under N deficiency were classified as efficient (E), and those with below-average DM as inefficient (NE). Responsiveness was determined using the NUE index, which was calculated based on the DM and NA of the plants under both conditions of N availability (deficiency and adequate). So, plants that had above-average NUE were classified as responsive (R) and plants with NUE below the average as non-responsive (NR). Thus, four groups were formed from the combination of efficiency and responsiveness: efficient responsive (ER), efficient and non-responsive (ENR), inefficient and responsive (NER), inefficient and non-responsive (NENR). Therefore, this classification has brought important implications in practical terms, as it can help landholders in making decisions about the most suitable genotype based on the N availability in the soil or according to the affordability to invest in nitrogen fertilizers.

Although the methodology described above is quite useful, it only indicates the genotype based on the ability to produce DM and accumulate N. More important than that is to identify the internal mechanisms involved in the genotype differentiation, which may help in future breeding programs to increase the NUE in tobacco. This may allow breeders to be more assertive for improving tobacco NUE. The levels of NO_3^- in leaves have been higher in the varietal group Burley compared with flue-cured type (LI et al., 2017b), which is undesirable since the increase in nitrate is correlated with the formation of TSNAs, carcinogenic compounds (LEWIS et al., 2012). In this way, we chose contrasting genotypes in NUE, i.e. ER and NENR, to investigate the ecophysiological and biochemical traits under contrasting N availability, in the same conditions that were performed in our previous experiment. As expected, under

adequate N conditions, the ER genotypes had higher DM production and NA (FIGURES 4D, 5E) than the NENR genotypes. However, under N deficiency, ERs had higher DM (except CSC 4707) and similar NA compared to NENR genotypes (FIGURES 4D, 5E). That demonstrates the effectiveness of the methodology used in the separation of genotypes in our previous study, even when the crops are carried out at different seasons of the year.

In the present study, we evaluated several physiological and biochemical parameters and, to better understand them, we used the PCA statistical tool, where we can see more clearly how the parameters and genotypes behaved. Ci, flavonoids, GS activity and anthocyanins clearly driven the variation of the data under N deficiency, meaning through the ANOVA statistically higher Ci, GS activity, anthocyanins and flavonoids levels under N deficiency, with no statistical difference in only two out of eight genotypes for this last parameter. Since this information encompasses both ER and NENR genotypes, that suggests that the responses obtained from these parameters are of widespread occurrence regardless of the tobacco genotype evaluated.

The similarity between CSC 2602 and BAT 2301 identified in Fig. 3A and 1B under both N conditions share similar anthocyanin and chlorophyll contents, WUE, k and GS activity, which caused the similarity of these genotypes. In contrast, these genotypes differ in terms of Pn, which shows that the superiority in photosynthesis of CSC 2602 is not able to differentiate it from BAT 2301. The similarity among them observed when adequately supplied in N (FIGURE 2B) refers to the production of flavonoids also in this condition (FIGURE 10), now demonstrating the similarity in the response of this parameter. However, both differ in gs, meaning that CSC 2602 is not able to differ from BAT 2301 even having higher gs under adequate N nutrition.

The similarity between CSC 2602, BAT 2301 and BAT 2101 under N deficiency (FIGURE 2A) was caused by similar flavonoids and GDH activity at this condition. ERs genotypes, with exception of CSC 4501, were related to parameters associated with adequate nutrition, suggesting that there are some efforts of the ER genotypes through the parameters of adequate nutrition shown in Figure 1A to contribute to the production of greater DM of this group even in the N deficiency, corroborating with data of higher DM shown in Figure 4D. However, BAT 2101 differs by presenting higher PNUE and lower gs than both CSC 2602 and BAT 2301 under N deficiency. Interestingly, BAT 3004 did not follow the same trend as the others NENR genotypes (FIGURE 2A), showing a response more similar to that of ER genotypes.

The similarity between CSC 4707 and CSC 4501, and CSC 439 and CSC 497 under both N conditions (FIGURE 3B) was provoked by the shared parameter within these groups of k, Pn and NR activity. It is noted that BAT 2101 is quite favored when placed in an N adequate supply (FIGURE 2B), although that did not reflect into greater DM production (FIGURE 4D). However, there is a similarity regarding the GDH activity, chlorophyll and anthocyanins contents in both N concentrations for all the four genotypes. In N deficiency, there was a similarity of the pairs above for NR activity.

There were no differences among genotypes for Ci, which suggests that this parameter is affected to a greater extent. A decreasing in the Ci / Ca (ratio between intercellular and CO_2 in the environment) with an increase in the supply of N in tobacco was previously found (BRUECK; SENBAYRAM, 2009), which is in line with our results. The higher Ci under N deficiency is also observed in other crops (LI et al., 2012; LIU et al., 2020), being related to the limitation of CO_2 capture under this condition. E was also lower for deficient plants as well as gs for the majority of them, suggesting a diffusive limitation of photosynthesis. In addition, plants under N deficiency also showed lower k, which explains the accumulation of Ci as a consequence of the lower efficiency of the photosynthetic apparatus in this condition, indicating a biochemical limitation of photosynthesis. The reduction in photosynthetic activity was previously attributed to the lower amount of the Rubisco enzyme and, consequently, its activity in nitrogen deficient plants (GAO et al., 2018).

The parameters Pn, E, k and chlorophyll were also reduced under N deficiency, while gs was reduced in most genotypes, which is in agreement with other studies (BRUECK; SENBAYRAM, 2009; HUANG et al., 2016; LIN et al., 2017), showing that the photosynthetic apparatus is injured by N deficiency. In general, ER genotypes performed better than the NENR genotypes regarding Pn, k, PNUE (this one at N deficiency) and chlorophyll content, which can partially respond to the better contribution of these genotypes in producing more DM and accumulating more N. Lower Pn values occurred for two burley genotypes compared to flue-cured ones under N deficiency (LI et al., 2017b), which corroborates with the results of this study.

Here, it is worth highlighting the interrelationship between carbon and nitrogen metabolism, culminating in the end with the production of DM and NA in plants, which ultimately determines whether the genotype is ER or NENR. The first step for the entry of C into the plant begins with opening the stoma, where C-CO_2 is gained and water vapor is lost, evaluated by gs and E respectively. E was unaffected by genotypes, only by the availability of N, which suggests that it is an insensitive indicator to differ the contrasted genotypes assessed.

In other words, this is not a factor that is determining the difference between the genotypes, as well as Ci. With respect to gs, this was in general higher under adequate N nutrition, and under N deficiency the NENR genotypes are statistically similar, or even present higher values in relation to RE. However, there was no trend that led the differentiation of the groups using gs data, again suggesting that it was not decisive for the separation of the groups.

After C-CO_2 has entered the leaf, the next stage is carboxylation, the first stage of the Calvin cycle. In it, the rubisco enzyme incorporates CO_2 and water to ribulose-1,5-bisphosphate generating two molecules of 3-phosphoglycerate. The carboxylation efficiency (k) varied according to the genotypes, being greater under adequate nutrition of N. Here the distinction between the groups begins to be explained, where the ER genotypes obtained higher k in relation to the NENR, with exceptions of BAT 2101 and BAT 3004. That was reflected in photosynthesis (Pn), where the highest values were obtained under adequate N nutrition and the ER were higher in relation to the NENR, with the exceptions of BAT 2101 and BAT 3004. Both genotypes differed from the others in the group NENR. First, that was demonstrated when BAT 3004 was the only genotype of the NENR group that was located close to the ER genotypes in the analysis of PCA (FIGURE 2A). Second, BAT 2101 under N deficiency was related to a few parameters evaluated (FIGURE 2A), but was greatly favored when properly nourished (FIGURE 2B). Third, these genotypes were statistically superior to the other NENRs in the levels of chlorophyll, Pn, GS activity, k, PNUE and flavonoid levels (this under adequate nutrition) and numerically for NR activity under adequate N nutrition and flavonoid levels under N deficiency. Although both genotypes perform better in terms of the parameters discussed compared to the other NENRs, that did not reflect statistically greater production of DM of the plants, while the highest NA occurred for BAT 2101 and CSC 2602 (not BAT 3004) in this group. That suggests the importance of different traits of the C and N metabolism to be paired so that plants accumulate more DM and have more N accumulated.

For the first time, the values of leaf pigments obtained indestructibly by Dualex device are presented for the tobacco crop affected by the availability of N. The highest values of NBI and chlorophyll content and lower values of flavonoids content at adequate N condition are in agreement with other crops in field (COELHO et al., 2012 for potato; QUEMADA; GABRIEL; ZARCO-TEJADA, 2014 for maize; GABRIEL et al., 2017 for maize; BEN ABDALLAH; PHILIPPE; GOFFART, 2018 for potato; DONG et al., 2020 for maize) or greenhouse (DA SILVA et al., 2020 for peper) conditions. Higher anthocyanin values were found in the absence of nitrogen fertilization in the field for corn (ZHANG; TREMBLAY; ZHU, 2012). The higher levels of anthocyanins and flavonoids under N deficiency may be related to the prevention of

sugar accumulation in conditions not favorable to the plant, ensuring the maintenance of the photosynthetic apparatus for a longer time (LO PICCOLO et al., 2018). That is, under N deficiency, the plant changes its reserve allocation pattern in order to maintain homeostasis (MENG et al., 2018; LUO et al., 2019). This is in line with the results of the present study, which also show that ER genotypes in general showed statistically lower levels of anthocyanins, corroborating the results of better DM and NA.

The nitrate (NO_3^-) uptake by the roots is first reduced to nitrite by the nitrate reductase (NR), producing nitrite (NO_2^-). Under N deficiency, the genotypes CSC 4501 and CSC 4707 showed higher NR compared to the other genotypes, being a differential of these genotypes, but at adequate N supply all genotypes obtained similar activities. Nitrite (NO_2^-) is then reduced to ammonium (NH_4^+) by nitrite reductase, which is later combined with glutamate by glutamine synthetase (GS) to form glutamine. The N deficiency caused greater activity of the GS, where CSC 4707, CSC 497 (ERs), BAT 2101 and BAT 3004 (NENRs) had the greatest GS activities among the genotypes. Greater GS activities in conditions under N deficiency have been reported in the literature (LOTHIER et al., 2011 for *Arabidopsis*; RUBIO-WILHELMI et al., 2012 for tobacco; WEI et al., 2018 for tobacco). In this case, under N deficiency there is an increase in a typically root isoform (GS1) of the enzyme in leaves, which may even be related to the re-assimilation of ammonium released by the breakdown of amino acids in senescent leaves and not to the N assimilation process (LOTHIER et al., 2011; MOISON et al., 2018). Also, due to the importance of GS for N re-assimilation resulting from photorespiration (KOCHEVA et al., 2020), it may be that the increase in enzyme activity is related to this function, but likely lower compared to plant adequately nourished with N.

The glutamine produced can react with 2 oxoglutarate to produce glutamate through glutamate synthase (GOGAT). The higher activities of this enzyme occurred under adequate N condition and for the ER genotypes, except CSC 4501 (the lower of ERs) and BAT 3004 (the higher of NENRs). Glutamate can also be generated alternatively by glutamate dehydrogenase (GDH), from ammonium and 2 oxoglutarate. Under N deficiency, the ER genotypes obtained greater GDH activities than the other genotypes, except for BAT 3004, the one of the exceptions of this study.

During the N reduction process, reducing power, energy and carbon skeletons (KOCHEVA et al., 2020) are provided by the C metabolism from the photochemical step and/or from the respiratory pathway. On the other hand, the assimilated N is required for the synthesis of chlorophyll (whose precursor is aminolevulinic acid produced from glutamate) and proteins (rubisco and many others). Finally, the high demand for reducing power and energy by the

enzymes of nitrogen metabolism can also be beneficial for the consumption of the excess reducing power produced in the photochemical step, in order to delay or prevent photoinhibition. Thus, the highest levels of chlorophyll, k, Pn, RN, GOGAT and GDH justify the higher DM production and NA in ERs plants.

The allocation of DM between shoot and root is dependent on the condition of N availability. Under N deficiency there is an increase in the root: shoot ratio (LUO et al., 2019). In the present study, plants under N deficiency had a greater investment in root DM compared to plants under adequate N nutrition. This response is common in plants under N deficiency and can be explained by changes in the pattern of carbon and nitrogen allocation in at this condition. Thus, lower translocation of N occurs to the shoots, whereas carbon (starch or sucrose) is also translocated to the roots, allowing greater root growth (GAO et al., 2018). The greater root growth is directly related to the need for greater absorption of N and other macro and micronutrients (LUO et al., 2019). The volume and surface area of the roots were similar between the genotypes, which means that these factors do not contribute to differ genotypes. However, the higher root diameter observed in BAT 2101, BAT 3004 and CSC 2602 among NENRs may be associated with higher values for these genotypes of Pn. Among ERs, the higher root diameter in CSC 4707 and CSC 497 corresponds to higher values of GS activity. That probably occurs due to effects in root system as a consequence of the imbalance among the C and N metabolism.

In this study, we found that stem is an organ that has a considerable amount of DM and NA, but that is more relevant in the ER tobacco genotypes. That occurred probably due to the stage that plants were assessed, i.e. after 77 days of growth, which may be different in the end of the crop cycle. The stem was considered the main organ of carbon stock in tobacco plants (BRUECK; SENBAYRAM, 2009). On average, under N adequate condition for the NENRs genotypes, the production of leaf DM is greater (46% of the total DM) than that of stem (41% of the total DM). For the ERs, the opposite occurs with 43% of the DM of the whole plant being allocated in leaves, while the stem is formed by more DM, 48%. The NA showed the same trend of allocation of DM, with NENR genotypes presenting on average 47 and 37% of the NA in leaves and stems, respectively, but ERs showed more N accumulated in stems than in leaves, on average 44 and 42% respectively.

Under N deficiency the allocation of DM in stems is lower, on average 28% and 30% for NENR and ER respectively, and 50% in leaves for both groups. The NA occurs similarly, with 42 and 39% in leaves for the NENR and ER respectively, and 31 and 34% in stem for the NENR and ER genotypes respectively.

While under adequate N nutrition there is an average increase in leaf dry mass of the NENR to ERs genotypes of 15% and a decrease of 4.3% for roots, the increase in stem is much more expressive, 43.7%. Under deficiency this increase in stem is also relevant, 29%, while leaves and roots have a smaller increase, 21.9% and 4.3% respectively from NENR to ER. The NA under adequate nutrition of N from NENRs to ERs decreases on average by 4.6% for roots and increases by 28% for stem; under deficiency the increases are 13 and 8% for stems and roots respectively. The NA in leaves in both conditions of N decreases by 2% from NENR to ER. Roots always represent the lowest DM production for all genotypes in both conditions of N.

Again, stem has large DM and NA, and thus influenced when the genotypes were previously classified. ERs are genotypes with better performance in general, not only for their higher photosynthetic performance, but also for the greater activity of enzymes related to the assimilation and incorporation of N in plants. Due to the close link between C and N metabolism in plants, the most efficient genotypes are superior in assimilating carbon and nitrogen in a more paired way. According to Kocheva et al. (2020) the most efficient genotypes in the N-use present a better performance under deprivation of this nutrient. Under this condition for our tobacco plants, the ER genotypes showed generally greater flavonoids content, greater PNUE, greater NR and GDH activities compared to NENR. That suggests that, in general, these genotypes, in addition of performing better in the assimilation of carbon, are able to ensure greater accumulation of DM as well as NA because they are able to alter the source-sink relationships for the production of secondary compounds, in our case the flavonoids avoiding, for example, inhibition of photosynthesis by the excess of carbohydrates (MENG et al., 2018; LUO et al., 2019). The greater activity of RN in general under N deficiency suggests that they also ensure a greater amount of N or guarantee greater transport of N to shoots despite the limiting conditions. In general, the higher GDH activity under this condition can contribute to the close link between this enzyme and the respiratory cycle, beyond the occurrence to ER genotypes in having higher activity of the enzymes of the GS/GOGAT cycle.

5 CONCLUSIONS

There are differences in the nitrogen use efficiency and responsiveness among the diverse tobacco genotypes here investigated. Although both NENR genotypes, BAT 2101 and BAT 3004, having some enzymatic and photosynthetic parameters higher or similar to ER, that do not mean higher DM and NA, which shows that C and N metabolism parameters need to be more paired to express higher yield and NA phenotypes. For that, plants need to present better N assimilation, closely associated with greater C assimilation as well. Surely, it is desired plants that assimilate and allocate biomass in the harvestable product.

The greater performance in NR and GDH activities, flavonoids content and PNUE under N deficiency is a differential of the ER genotypes. The ER genotypes perform satisfactorily under N adequate level, but the mechanisms they resort when subjected to N deficiency allow them having better performance than NENR genotypes. So breeders should focus in those genotypes with higher development under N limitation. It is likely that more than a single parameter is needed to obtain an increasingly efficient and nitrogen-responsive tobacco genotype.

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APPENDIX A – NUTRIENT SOLUTIONS FOR THE DIFFERENT N TREATMENTS USED IN THE EXPERIMENT

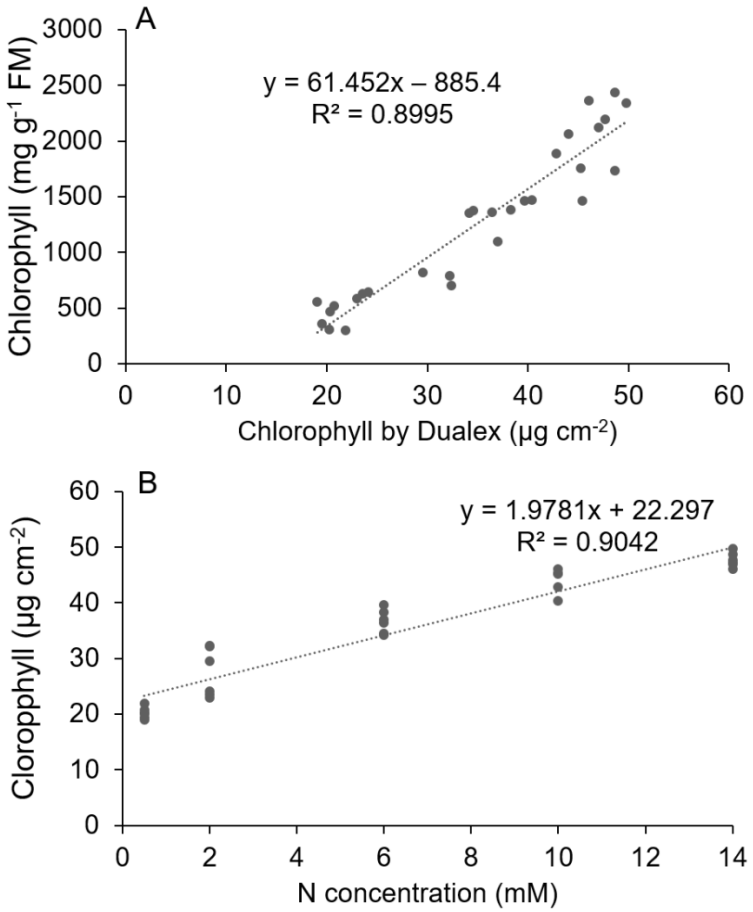
Table – Stock solutions and amounts used to prepare the nutritive solutions used in the experiment. Solutions of 2 mM and 10 mM were used for the conduction of the experiment. Solutions of 0.5 mM, 6 mM and 14 mM were used in the calibration experiment for the chlorophyll quantification (BAT 3004 and CSC 497).

Stock Solution	mL/L
----------0.5 mM ----------	
0.6 M KH_2PO_4	1.0
1 M KNO_3	0.5
1 M KCl	2.5
$CaCO_3$ (3.7533 g L^{-1})	80.0
0.6 M $MgSO_4.7H_2O$	2.0
Solution a	1.0
Fe-EDDHA Solution (41.67 g L^{-1} of Quelmax®)	2.0
----------2 mM ----------	
0.6 M KH_2PO_4	1.0
1 M KNO_3	2.0
1 M KCl	1.0
$CaCO_3$ (3.7533 g L^{-1})	80.0
0.6 M $MgSO_4.7H_2O$	2.0
Solution a	1.0
Fe-EDDHA Solution (41.67 g L^{-1} of Quelmax®)	2.0
----------6 mM ----------	
0.6 M KH_2PO_4	1.0
1 M Ca $(NO_3)_2.4H_2O$	3.0
1 M KCl	3.0
0.6 M $MgSO_4.7H_2O$	2.0
Solution a	1.0
Fe-EDDHA Solution (41.67 g L^{-1} of Quelmax®)	2.0
----------10 mM----------	
0.6 M KH_2PO_4	1.0
1 M $Ca(NO_3)_2.4H_2O$	3.0
1 M KNO_3	2.0
0.5 M $Mg(NO_3)_2.6H_2O$	1.0
0.7 M $MgSO_4.7H_2O$	1.0
0.5 M K_2SO_4	1.0
0.5 M NH_4NO_3	1.0
Solution a	1.0
Fe-EDDHA Solution (41.67 g L^{-1} of Quelmax®)	2.0
----------14 mM----------	
0.6 M KH_2PO_4	1.0
1 M $Ca(NO_3)_2.4H_2O$	3.0
1 M KNO_3	3.0
0.6 M $MgSO_4.7H_2O$	2.0
1 M NH4NO3	2.5
Solution a	1.0
Fe-EDDHA Solution (41.67 g L^{-1} of Quelmax®)	2.0

[1] $CaCO_3$ suspension was solubilized with HCl until a clear solution was observed. This supplies Ca^{2+} in the treatment with 0.5 and 2 mM through the following stoichiometry: $CaCO_3 + 2\ H^+ \rightarrow Ca^{2+} + CO_2 + H_2O$. pH was adjusted to ~6 after adding this solution, prior to the addition of the remaining stock solutions. [2] Solution a = Micronutrients (except Fe) of Hoagland and Arnon (HOAGLAND; ARNON, 1950). [3]Quelmax® = commercial product with 6% Fe. The nutritive solutions have nutrients (except N) set at 60% and 100% of ionic strength for macro- and micronutrients, respectively.

APPENDIX B – CHLOROPHYLL DETERMINATION WITH DUALEX X BIOCHEMICAL METHODOLOGY

Figure – Relation between chlorophyll determination by Dualex and the chlorophyll quantified biochemically (A) and relation between the N concentration increases with chlorophyll determined by the Dualex in tobacco leaves.



APPENDIX C – PEARSON'S CORRELATION

Figure – Heat map of the Pearson-correlations of the parameters assessed.