

ERIC VICTOR DE OLIVEIRA FERREIRA

**INDICADORES FISIOLÓGICOS DO STATUS DE NITROGÊNIO EM
PLANTAS DE EUCALIPTO**

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Solos e Nutrição de Plantas, para obtenção do título de *Doctor Scientiae*.

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BIOGRAFIA

ERIC VICTOR DE OLIVEIRA FERREIRA, filho de Wilson Rodrigues Ferreira e Vicentina de Paula, nasceu em São Paulo-SP, em 15 de junho de 1980.

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Em março do mesmo ano iniciou o curso de mestrado no Programa de Pós-graduação em Ciência do Solo da Universidade Federal do Rio Grande do Sul (UFRGS), sendo orientado pelo prof. Ibanor Anghinoni, obtendo seu título em fevereiro de 2009.

Neste mesmo mês do referido ano, ingressou no curso de doutorado do Programa de Pós-graduação em Solos e Nutrição de Plantas da Universidade Federal de Viçosa (UFV), sob a orientação do prof. Roberto Ferreira de Novais, concluindo-o em março de 2013.

Em 2012 participou do programa de doutorado sanduíche pelo CNPq (SWE) na Duke University, North Carolina-EUA, junto ao laboratório do prof. Robert Jackson.

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RESUMO

FERREIRA, Eric Victor de Oliveira, D.Sc., Universidade Federal de Viçosa, março de 2013. **Indicadores fisiológicos do status de nitrogênio em plantas de eucalipto.** Orientador: Roberto Ferreira de Novais. Co-orientadores: Ivo Ribeiro da Silva e Nairam Félix de Barros.

Embora o nitrogênio (N) seja um nutriente requerido em grandes quantidades pelas plantas, os efeitos de sua aplicação na produção de matéria seca do eucalipto são divergentes, existindo também diferenças nutricionais entre espécies desse gênero. As análises foliares são um método auxiliar de avaliação do status nutricional das plantas, principalmente no caso do N em que o diagnóstico da sua disponibilidade para as plantas, baseado em análises do solo, é dificultado pela complexidade de suas reações no solo. O teor foliar de N total tem sido a ferramenta mais amplamente utilizada para verificação de possíveis deficiências deste nutriente nas plantas. Contudo, algumas limitações de seu uso com respeito a esta proposta têm sido relatadas. Dessa maneira, os objetivos deste trabalho foram avaliar indicadores alternativos do status de N, como teores de N-NH_4^+ , N-NO_3^- , N/P foliar, clorofila, atividade foliar das enzimas redutase do nitrato (RN) e glutamina sintetase (GS) e leituras de clorofila pelo SPAD (Soil Plant Analysis Development), em substituição ao seu teor total, em clones de eucalipto cultivados em diferentes condições de disponibilidade de N. Adicionalmente, o comportamento diferencial desses clones com relação ao crescimento, nutrição mineral e trocas gasosas também foi avaliado. Para tanto, foram conduzidos dois experimentos, sendo um em casa de vegetação e outro em campo. No primeiro caso, foi utilizado um fatorial 2×6 em delineamento de blocos casualizados, sendo dois clones de eucalipto (VM-01 e I-144) e seis doses de N (0; 0,74; 2,93; 4,39, 5,85 e 8,00 mmol L^{-1} de NH_4NO_3) em solução nutritiva. O experimento de campo constituiu-se de um fatorial $2 \times 2 \times 2 \times 3$ em delineamento inteiramente casualizado, com duas épocas de amostragem (seca e chuvosa), duas regiões (Pompeu e João Pinheiro-MG), dois clones (VM-01 e I-144) e três posições de coleta das folhas na copa das árvores (base, meio e ápice). Os teores de N-NH_4^+ (raiz) e N-NO_3^- (raiz e folha) representaram bem o status de N dos clones em casa de vegetação, porém eles não apresentaram o mesmo comportamento em plantas cultivadas a campo. A N/P, clorofila e as leituras SPAD tiveram comportamento similar à aplicação de N (modelos quadrático e raiz quadrático) em casa de vegetação, com maiores coeficientes de correlação (r) para o clone VM-01. Nas

diferentes épocas de avaliação no campo, tanto a N/P quanto a clorofila, não refletiram o real status de N dos solos. As leituras SPAD não foram consideradas boas indicadoras do status de N, pois o clone que tinha maiores valores (I-144) foi o que apresentou os menores teores foliares de N total. Este último não foi considerado um bom indicador do status de N, visto que o mesmo não apresentou os mais altos valores de r em casa de vegetação. Ainda assim, desses indicadores avaliados, ele refletiu melhor as variações no status de N dos solos nas diferentes épocas de avaliação sob condições de campo. O clone I-144 absorveu menos N (e sintetizou menos proteína), porém apresentou maior atividade da RN e da GS com maior produção de matéria seca, comparativamente ao VM-01, em casa de vegetação. A atividade dessas enzimas foi negativamente influenciada pelo maior suprimento de N, provavelmente por um efeito inibidor tanto do NO_3^- (substrato da RN) como do NH_4^+ (substrato da GS) em altas concentrações. Tanto em casa de vegetação como em campo, as maiores atividades da RN e GS estiveram associadas à menor disponibilidade de N no meio de cultivo das plantas, como, a princípio, uma maior eficiência enzimática nesta condição. A atividade das referidas enzimas apresentou também correlação negativa com o teor foliar de N total dos clones cultivados em casa de vegetação, com destaque para RN. Os clones apresentaram comportamento diferencial à aplicação de N em solução nutritiva, com o VM-01 sendo superior em altura, altura/diâmetro do colo e teores de macronutrientes e de Cu, Fe, Mo e Zn. Porém, o clone I-144 teve maior produção de matéria seca da raiz e total, raiz/parte aérea e diâmetro do colo, além de maior condutância estomática (g_s) e transpiração (E). Portanto, o clone I-144, independentemente do menor teor foliar de N total, reduziu e assimilou mais N, o que se converteu em melhor crescimento, assim apresentando maior eficiência de utilização deste nutriente.

ABSTRACT

FERREIRA, Eric Victor de Oliveira, D.Sc., Universidade Federal de Viçosa, March, 2013. **Physiological indicators of nitrogen status in eucalyptus plants.** Adviser: Roberto Ferreira de Novais. Co-advisers: Ivo Ribeiro da Silva and Nairam Félix de Barros.

Although nitrogen (N) is a nutrient required in large amounts by plants, the effects of its application on dry matter production for eucalyptus are divergent, also with nutritional differences among species of this genus. Leaf analysis is an auxiliary method for assessing nutritional status of plants, mainly for N, because the diagnosis of its availability for plants, based solely on soil analysis, is difficult due to the complexity of its soil reactions. Total N leaf concentration has been widely used for verification of possible deficiencies of this nutrient in plants. However, some limitations of its use with regard to this proposal have been reported. Hence, the objectives of this work were to assess alternative indicators of N status in substitution to total N for eucalyptus clones grown under different N availability, such as: chlorophyll, N-NH_4^+ and N-NO_3^- concentrations, leaf N/P, nitrate reductase (NR) and glutamine synthetase (GS) activities, and chlorophyll readings by SPAD (Soil Plant Analysis Development). In addition, it was also evaluated the differential behavior of these clones regarding growth, mineral nutrition, and gas exchanges. In doing so, two experiments were carried out, one in the greenhouse and another in the field. The greenhouse experiment was a 2 x 6 factorial in a randomized complete block design consisting of two eucalyptus clones (VM-01 and I-144) and six doses of N (0, 0.74, 2.93, 4.39, 5.85 and 8.00 mmol L⁻¹ of NH_4NO_3) in nutrient solution. The field experiment was a 2 x 2 x 2 x 3 factorial in a randomized complete design, with two seasons (dry and rainy), two regions (Pompeu and João Pinheiro-MG), two clones (VM-01 and I-144), and three positions of leaf sampling in the crown (base, middle, and apex). The concentrations of N-NH_4^+ (root) and N-NO_3^- (root and leaf) well represented N status of clones in greenhouse; however, they did not show the same behavior in plants grown in the field. The N/P, chlorophyll, and SPAD readings had similar responses to N application (quadratic and square root models) in greenhouse, with the highest correlation coefficients (r) for the clone VM-01. Among different seasons, N/P and chlorophyll did not reflect the real soils N status in the field. SPAD readings were not considered good indicators of N status either, due to the clone that showed the highest readings (I-144) was the one with the lowest total N

concentrations. Total N was also not the best indicator of N status, once it did not show the highest r in the greenhouse experiment. Nevertheless, of all indicators evaluated, it better represented the variations of soils N status in the different seasons under field conditions. Clone I-144 absorbed less N (and synthesized less protein), although it showed higher NR and GS activities with higher dry matter production, compared to VM-01, in greenhouse. The activity of these enzymes was negatively influenced by greater N supply, probably due to an inhibiting effect of NO_3^- (substrate of NR) and NH_4^+ (substrate of GS) in high concentrations. Not only in greenhouse, but also in the field, the highest activities of NR and GS were associated with lower N availability in the cultivation medium of plants. The activity of these enzymes also showed negative correlation with leaf total N of the clones grown in greenhouse, mainly for NR. Clones showed differential behavior to N in solution, with VM-01 being superior in height and height/collar diameter and leaf concentrations of macro and of Cu, Fe, Mo and Zn. In the other hand, clone I-144 had greater production of root and total dry matter, root/shoot and collar diameter, as well as greater stomatal conductance (g_s) and transpiration (E). Therefore, clone I-144, regardless of the lower total N leaf concentration, reduced and assimilated more N, which was converted in better growth, making it the more efficient clone in the utilization of this nutrient.

GENERAL INTRODUCTION

The worldwide use of N fertilizer in agriculture has increased to about 80 million t year⁻¹, with an important role in keeping food production in pace with population growth (Olfs et al., 2005). However, the doubling of worldwide food agricultural production over the past four decades has been associated with a 7-fold increase in the use of N fertilizers, with consequent impact on the diversity and functioning of ecosystems (Hirel et al., 2007).

Although N is a nutrient required in large amounts by plants (Cantarella, 2007), the effects of its application on eucalyptus' dry matter production are divergent (Jesus et al., 2012). The response of eucalyptus to N, when it occurs, it is between 10 and 20 % of increase in dry matter, but it seems to decrease or even disappear with cultivation time. According to Gonçalves (2013)¹, in the initial phase, in which growth is faster, the N demand is greater, so biogeochemical cycling would not be enough to supply the demand of this nutrient by eucalyptus and so it would respond to N fertilization. By contrast, in a subsequent phase (older plants) N demand decreases and biogeochemical cycling of N also increases (more leaves fall from the trees with greater amount of litter on the soil surface), and thus the cycling would supply the requirement of the eucalyptus and would happen no response to N.

As a result, forestry companies have used little or no N fertilization on their sites. Though, high export rates, low fertilization rates, and the depletion of this nutrient in the soil, indicate that intensively managed forests respond to N fertilization after several cultivation cycles (Pulito, 2009). The N content exported in the stem (wood plus bark) of seven year old eucalyptus is 150 kg ha⁻¹ of N, for a yield of 50 m³ ha⁻¹ year⁻¹ (data estimated by NutriCalc 2001). Leite et al. (2010) found a deficit (mean of five regions) of 345 kg ha⁻¹ of N during three cycles of cultivation of eucalyptus in the Vale do Rio Doce region, Minas Gerais State, which can result in significant reduction on N levels in the soil and an unsustainable practice in the long term.

Thus, questions as whether or not eucalyptus responds to N and why of this fact, are relevant. The N from the mineralization of soil organic matter (SOM) would be enough to attend the demand of eucalyptus species, as suggested (Pulito, 2009)? This author says that the mean demand of this species (20 to 50 kg ha⁻¹ year⁻¹) is satisfied by

¹ Personal communication.

N from mineralization of SOM (30 to 40 kg ha⁻¹ year⁻¹). Another hypothesis that has also been recently raised would be a possible involvement of microorganisms (diazotrophic bacteria, for example) performing the fixation of atmospheric N₂ to supply the N deficit in the eucalyptus sites.

The complexity of the reactions of N in soil hampers the diagnosis of its availability for plants based solely on soil analysis, contrary to what occurs successfully for other nutrients (Cantarella, 2007). Thus, leaf analysis arises as an alternate method for assessing N status of plants. While Nutree ® does not recommend or recommend only 20 kg ha⁻¹ in topdressing for each 1 g kg⁻¹ less than 17 g kg⁻¹ of total N in leaf analysis performed in eucalyptus up to two year old, some private companies' professionals have recommended from 100 to 150 kg ha⁻¹ of N, very discrepant condition. The Diagnosis and Recommendation Integrated System (DRIS) for N in eucalyptus has shown values equal or greater than zero, thus it does not recommended fertilization with this nutrient. But, on the other hand, imagines also that N deficiency problems can be no detected in standard routine analysis (leaf total N concentration).

Although leaf analyses of the total N concentration are routine, reports suggest that its use with the proposal of nutritional diagnosis has some limitations (Chapin et al., 1982; Perdomo et al., 2007; Rubio-Covarrubias et al., 2009). For example, leaf total N can be insensitive to seasonal demand of this nutrient by plants and show limited response to high N supply for trees. Thus, evaluation of the concentrations of N-NH₄⁺ and N-NO₃⁻, leaf N/P, chlorophyll and enzyme activities, in addition to SPAD (Soil Plant Analysis Development) readings, have been proposed as alternative indicators of N to diagnose nutritional status of plants and, thus, contribute to manage of its fertilization.

In contrast with conifers from temperate regions, little is known about the processes of N storage and remobilization in eucalyptus (Wendler et al., 1995), as well as fragmented knowledge of the biochemical and molecular mechanisms that control uptake, assimilation and recycling of N (Mickelson et al., 2003). Therefore, in view of the insensitivity of leaf total N, we aimed to investigate the concentrations of N-NH₄⁺ and N-NO₃⁻, leaf N/P, chlorophyll and the enzymatic activities of nitrate reductase (NR) and glutamine synthetase (GS), as well as SPAD readings, as possible indicators of the N status in eucalyptus plants.

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CHAPTER 1 – Is total nitrogen leaf concentration a good indicator of the status of this nutrient for eucalyptus clones?

Abstract- Total N leaf concentration has been widely used for verification of possible plant deficiencies of this nutrient in routine analysis. However, reports have shown some limitations of its use with respect to plant nutritional diagnosis. Hence, the aim this work was to evaluate the total N concentration and compare it with other alternative indicators of the N status in eucalyptus clones, under different conditions of N supply. For this purpose, two experiments were conducted, one in the greenhouse and another in the field. The greenhouse experiment was a 2 x 6 factorial in a randomized complete block design consisting of two eucalyptus clones (VM-01 and I-144) and six doses of N (0, 0.74, 2.93, 4.39, 5.85 and 8.00 mmol L⁻¹ of NH₄NO₃) in nutrient solution. The field experiment was a 2 x 2 x 2 x 3 factorial in a randomized complete design, with two seasons (dry and rainy), two regions (Pompeu and João Pinheiro, Minas Gerais State, Brazil), two clones (VM-01 and I-144), and three positions of leaf sampling in the crown (base, middle and apex). We evaluated the concentrations of total N, N-NH₄⁺ and N-NO₃⁻; chlorophyll; leaf N/P and SPAD (Soil Plant Analysis Development) readings (only in greenhouse). The concentrations of N-NH₄⁺ (root) and N-NO₃⁻ (root and leaf) showed potential for predicting eucalyptus N requirements in greenhouse, however, these mobile N forms (mainly N-NO₃⁻) did not represent well the soil N levels in the field. It was observed similar responses for leaf N/P, chlorophyll and SPAD readings to N application in greenhouse (quadratic and square root models), and the N/P and chlorophyll did not reflect the real differences in soil N status in the field. The total N leaf concentration is not good indicator of N status, because it did not have highest correlation coefficient in greenhouse. Nevertheless, of all indicators evaluated, it better represented the variations in soils N status for eucalyptus clones under field conditions.

Keywords: *ammonium, chlorophyll, leaf N/P, nitrate, SPAD.*

INTRODUCTION

The complexity of the reactions of N in soil hampers the diagnosis of its availability for plants based solely on soil analysis, contrary to what occurs successfully for other nutrients (Cantarella, 2007). Thus, leaf analysis arises as an alternate method for assessing N status of plants. According to Smith & Lorenagan (1997), plant analysis

is regarded as reasonably reliable method of assessing N status of crops because it avoids many of the problems associated with soil N tests. The use of plant analysis is based on the idea that the plant itself is the best indicator for N supply from soil during its growth period (Olfs et al., 2005).

Assessments made directly on the plants, which integrate all soil and environmental factors, are auxiliary to the recommendation of N fertilization, as the concentration of nitrate (N-NO_3^-) in leaves or petioles of various cultures (Cantarella, 2007), the total N leaf concentration in perennial crops (Raij et al., 1997), in addition to readings of chlorophyll by SPAD- Soil Plant Analysis Development (Cantarella, 2007). Olfs et al. (2005) point out the total N concentration or the ratio of this with its critical level, the plant-sap or petiole N-NO_3^- and the chlorophyll concentration of leaves as plant indicators most commonly used as base for N fertilizer recommendation. However, in routine analysis of most laboratories the total N concentration has been used (Rubio-Covarrubias et al., 2009), being this tool the most widely used for verification of possible deficiencies of this nutrient (Araújo, 2007).

On the other hand, the use of total N leaf concentration, with the purpose of nutritional diagnosis, presents some limitations. Reports have shown a limited response of leaf total N to high N supply in woody crops (Alva et al., 1998). It has been observed that total N concentrations increase rapidly in response to N fertilization when plants are N deficient, but once N demand for growth is satisfied, it increases only slightly or not at all, becoming difficult to diagnose areas with excessive applications of N fertilizers (Rubio-Covarrubias et al., 2009). It was recognized in onion under greenhouse conditions that there is a period from the beginning of maximum leaf growth to the following 30 days in which total N concentration is stabilized (Alcántar et al., 2002). In *Eucalyptus globulus* and *E. grandis*, leaf total N did not differ between responsive and non-responsive sites (Perdomo et al., 2007). Also, leaf total N can be insensitive to seasonal adjustment of nutrient use (Chapin et al., 1982).

A desirable N indicator should be sensitive to a broad range of N supply and also predict plant growth (Rubio-Covarrubias et al., 2009). According to Rambo et al. (2010), an ideal indicator of N status must have a reproducible relation with its level in the soil-plant system and be able to detect deficiency or excess of this nutrient. In this manner, N compounds such as N-NO_3^- in petioles of kiwi fruit leaves and chlorophyll in leaves of American sycamore have been proposed as more sensitive indicators of N fertilization than total N concentration in perennial species (Rubio-Covarrubias et al.,

2009). These authors comment also that mobile N forms as N-NO_3^- and N-NH_4^+ and chlorophyll have shown advantages over total N in many crops.

Maier & Chvyl (2003) report that when decisions on crop nutrient requirements are based on plant analysis data, relationships among nutrients within the plant should be considered to ensure correct interpretation, for example, the synergism between N and P in potato. Thus, the leaf N/P has been extensively used to guide the fertilization with these nutrients in eucalyptus in Australia and New Zealand (Judd et al., 1996; Knight & Nicholas, 1996; Perdomo et al., 2007). And finally, as a nondestructive and alternative indirect evaluation method of plant N status in real-time, Madeira et al. (2009) suggest SPAD (Soil Plant Analysis Development) to monitor N concentration in *E. globulus* (up to two year old). The N concentration is related to chlorophyll concentration in leaves (Alcántar et al., 2002). However, the relationship between chlorophyll concentrations and SPAD values is not always linear (Fontes, 2011).

Nutrition differences have been found among eucalyptus species (Santana et al., 2002), as observed to N (Pinto et al., 2011), and also among different positions of leaves in the tree crown (Turnbull et al., 2007). This last fact is due to differences in light availability in the tree crown and the relationship between N concentration and irradiance (Le Roux et al., 1999), that is, N is preferentially allocated to foliage in areas of higher irradiance (Givnish, 1988). However, relationships between N distribution and light gradients are inconsistent (Turnbull et al., 2007). Additionally, N is a mobile nutrient in plants (Marschner, 2012). Therefore, it is important to assess these differences among species as well as among crown positions of eucalyptus trees.

Despite many attempts, there is no consensus regarding the standardization of a better N indicator in plants. This lack of consensus demonstrates a clear need for research on plant N status. Therefore, the objectives of this study were to evaluate alternative indicators of N status (N-NH_4^+ , N-NO_3^- , N/P and chlorophyll), in addition to its total concentration and SPAD readings in eucalyptus clones under different conditions of N supply in greenhouse and in field.

MATERIAL AND METHODS

Two experiments were conducted, one in greenhouse and another in the field. The greenhouse experiment was carried out in nutrient solution, which allows a better control on the experimental conditions. The field experiment was carried out in commercial eucalyptus plantations (Vallourec & Mannesmann Tubes Company) to

validate the variables under study, since they reflect the real condition of the eucalyptus management. The sites were chosen due to their contrasting soil physical and chemical properties (Table 1).

Table 1. Soil physical and chemical properties¹ of the experimental sites on beginning of the evaluations (Sept./11)

Layer	Po ²	JP ³	Po	JP	Po	JP	Po	JP	Po	JP
	pH		SOM ⁴		P		K		Clay	
---cm---	-----H ₂ O-----		---dag kg ⁻¹ ---		-----mg dm ⁻³ -----				-----%-----	
0-10	5.17	5.15	3.53	1.89	2.83	6.21	44	16	75	17
10-20	4.90	4.86	2.66	1.40	4.05	13.61	36	14	74	17
20-40	4.64	4.73	2.07	1.11	15.76	8.57	31	10	74	16
40-60	4.73	4.74	1.70	0.78	3.98	2.28	19	6	73	18
<u>60-100</u>	4.80	4.50	1.29	0.52	1.12	1.17	10	2	75	21
Mean	4.85	4.79	2.25	1.14	5.55	6.37	28	10	74	18
	Ca ²⁺		Mg ²⁺		Al ³⁺		H+Al		CEC ⁵	
---cm---					-----cmol _c dm ⁻³ -----					
0-10	1.44	0.45	0.35	0.35	0.59	0.41	6.65	4.56	2.49	1.25
10-20	0.75	0.17	0.21	0.18	0.95	0.57	6.65	4.38	2.00	0.95
20-40	0.30	0.13	0.10	0.13	1.17	0.54	6.32	3.78	1.65	0.83
40-60	0.23	0.05	0.08	0.09	0.91	0.53	5.41	3.21	1.26	0.68
<u>60-100</u>	0.12	0.01	0.06	0.05	0.65	0.48	4.68	2.80	0.85	0.55
Mean	0.57	0.16	0.16	0.16	0.85	0.51	5.94	3.74	1.65	0.85

¹Mean values of samples collected in line and interline. ²Pompeu (Po) and ³João Pinheiro (JP) regions. ⁴Soil organic matter (SOM) = organic C x 1.724 - Walkley-Black. pH in water- 1:2.5 ratio. P and K (Mehlich 1). Clay determination according to Ruiz (2005). Ca²⁺, Mg²⁺ and Al³⁺ (KCl 1 mol L⁻¹). H + Al (calcium acetate 0.5 mol L⁻¹). ⁵Cation exchange capacity (CEC)= Ca²⁺+ Mg²⁺+ K + Al³⁺.

Greenhouse experiment

This trial was carried out at Federal University of Viçosa, Viçosa (20° 45' S, 42° 52' W), MG, Brazil, for three months (from July to September 2011). The experiment was a 2 x 6 factorial in a randomized complete block design with five replications, consisting of two eucalyptus clones (VM-01 and I-144) and six doses of N (0, 0.74, 2.93, 4.39, 5.85 and 8.00 mmol L⁻¹ of NH₄NO₃) in nutrient solution. The clones have different nutritional efficiencies in relation to N (Pinto et al., 2011); VM-01 (*Eucalyptus urophylla* vs *E. camaldulenses*) is considered N demanding in contrast to clone I-144 (*E. urophylla* vs *E. grandis*). It was used the nutrient solution of Clark (1975), modified to contain the N-NH₄⁺/N-NO₃⁻ equal to 1 (Locatelli et al., 1984) and the double of P concentration (Caldeira et al., 1994). The nutrient solution was maintained under

permanent aeration, with pH 5.5 ± 0.05 (Locatelli et al., 1984), and it was changed weekly using deionized water. In the first thirty days of the experiment, the plants were grown in collective plastic trays (11 L) and the nutrient solution concentration was gradually increased week by week (25, 50, 75 and 100 % of the original concentration). Later, two homogeneous plants were selected regarding the height and vigor, and then these plants were transplanted to individual pots (6 L) representing an experimental unit. The plants were approximately 50 days old at the beginning of the experiment and they were originated from tube seedlings.

At the end of the experiment were taken readings of chlorophyll with SPAD (Soil Plant Analysis Development; SPAD-502 model) in the two youngest fully expanded leaves of each plant, taking the average of six consecutive measurements, from 8 to 10 am. To evaluate the chlorophyll concentrations, two discs (0.78 cm^2 each) were collected on the same leaves evaluated with the SPAD from each plant. Finally, after sixty days in nutrient solution with the treatments, the plants were harvested and oven dried ($60^\circ\text{C}/72 \text{ h}$) to determine the dry matter, and they were then ground (Wiley mill) for chemical assessments.

Field experiment

The sites were located in the southeast of Brazil, Minas Gerais State, and the areas and species descriptions as well as some agronomic practices adopted are presented in table 2. This experiment was a $2 \times 2 \times 2 \times 3$ factorial scheme in a randomized complete design with five replications, with two evaluation times (September/2011 and February/2012), two regions (Pompeu and João Pinheiro municipalities), two eucalyptus clones (VM-01 and I-144), and three positions of leaf sampling in the crown (base, middle and apex). The evaluation times in September and February represented the dry and rainy seasons respectively, and the samplings were performed at the end of each season (Table 2; Figure 1). The regions Pompeu and João Pinheiro have clay and sandy soils, respectively (Table 1). Each plot consisted of five eucalyptus rows with six plants each row, with 262.5 m^2 in both regions, according to the spacing of sites (Table 2). In addition to the fertilizations with N-P-K (Table 2), the areas also received 2.5 (Pompeu) and 2.0 (João Pinheiro) t ha^{-1} of lime, 0.8 t ha^{-1} of gypsum and 9 L ha^{-1} of ammonium borate (1.22 kg ha^{-1} of B, aerial application). Application of 4 kg ha^{-1} of B as ulexite (10 % of B) in the soil was also performed in Pompeu.

The trunk circumference measured at 1.3 m (breast height) from the ground of all trees (30) located within the evaluation plot was recorded and then averaged. Later, five representative trees were chosen within this range in each plot to measure the height (h) so as to estimate the trunk solid volume $[(\pi (\frac{dbh^2}{4}) h) / 2]$, dbh= diameter at breast height; Figures 1a and b]. Of these five trees, four were used as reference for soil sampling and another tree (fifth) was used for leaf sampling. The soil samples were collected in the layers of 0-10, 10-20, 20-40, 40-60 and 60-100 cm of depth at four locations/sampling place (line and interline) per plot. These soil samples were air dried, grounded and sieved (2 mm) before of laboratory analysis.

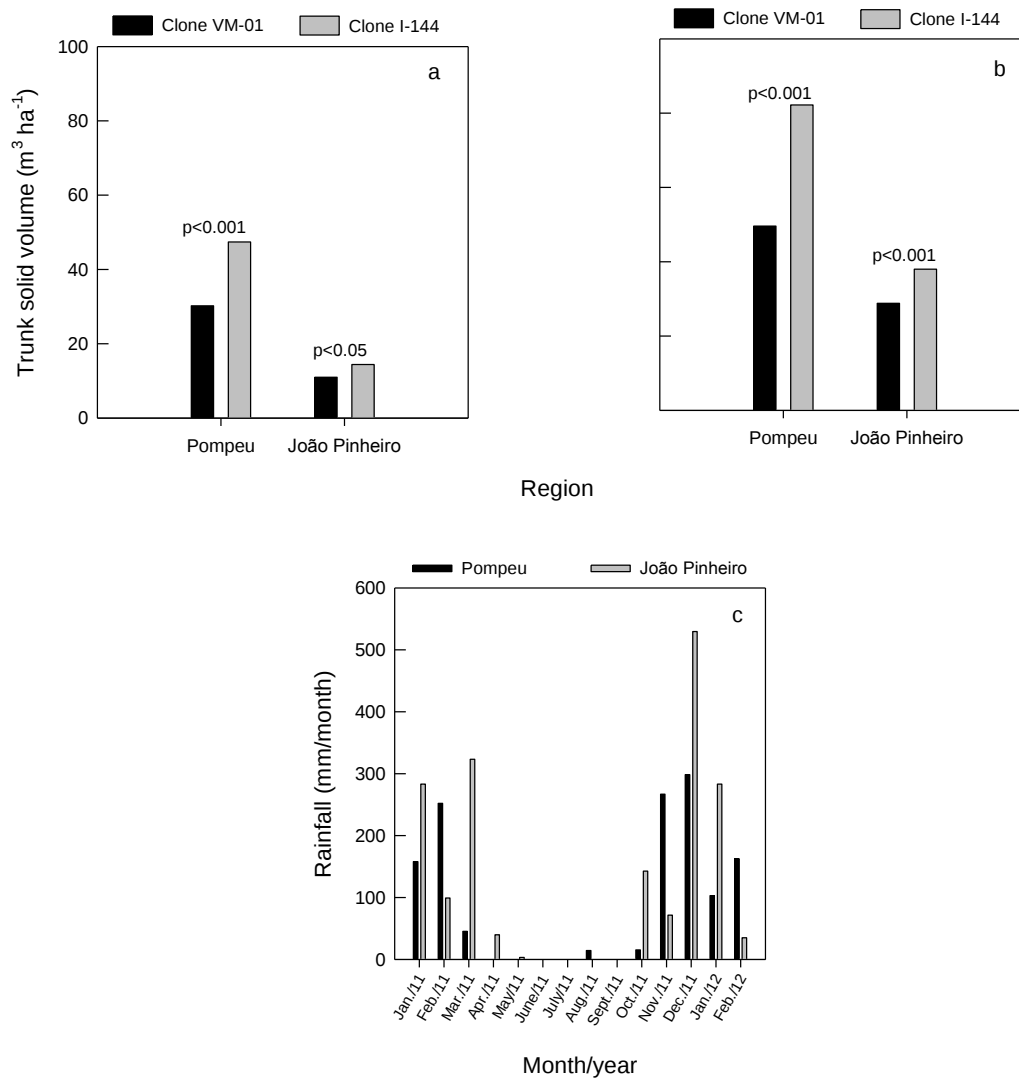


Figure 1. Trunk solid volume of eucalyptus clones grown on field in the regions of Pompeu (clay soil) and João Pinheiro (sandy soil) under dry season-Sept./11 (a) and rainy season-Feb./12 (b) and rainfall occurred in these regions from Jan./2011 to Feb./2012 (c). Significance of p-value by F test.

Table 2. Description of sites located in the State of Minas Gerais-MG, Brazil, belonging to Forestry Company Vallourec & Mannesmann Tubes (V&M Tubes) used for the field experiment

Region	Coordinate/ altitude	Soil	Clone	Specie	Planting Date	Stocking Density	Fertilization			Sampling (season)	
							kg ha ⁻¹ (N-P-K)			Dry	Rainy
		Latossolo Vermelho Amarelo ¹				plants ha ⁻¹	At planting	7 map ³	18 map ³		
Pompeu	18°53' S 45°02' W/ 654 masl ⁴	(Oxisol) ²	VM- 01	<i>E. urophylla</i> vs <i>E.</i> <i>camaldulenses</i>	04-15- 10	1333 (3.0x2.5m)	360 (10- 27- 10)	240 (23- 00- 21)	350 (23- 00- 21)	09/14/11	02/09/12
			I-144	<i>E. urophylla</i> vs <i>E. grandis</i>							

		Neossolo Quartzarênico ¹									
João Pinheiro	17°30' S 46°07' W/ 533 masl ⁴	(Entisol) ²	VM- 01	<i>E. urophylla</i> vs <i>E.</i> <i>camaldulenses</i>	05-03- 10	1333 (3.0x2.5m)	300 (10- 27- 10)	250 (23- 00- 23)	350 (23- 00- 23)	09/16/11	02/07/12
			I-144	<i>E. urophylla</i> vs <i>E. grandis</i>							

¹Brazilian classification (Embrapa, 2006). ²Soil Taxonomy. ³Months after planting. ⁴Meters above sea level.

For plant sampling, only fully expanded leaves were collected (180 leaves/tree), with 60 leaves for each crown position, between middle and tips of the branches and from two and three branches of different sides of the plant, in the morning (from 8 to 12 h). The trees crowns were measured and then divided them into three parts (base, middle and apex) and the leaf sampling was done within the mean height of each part. In these same leaves, we also extracted two discs (0.78 cm²) for chlorophyll determination. During the second sampling (February 2012), we chose another tree to collect the leaves in each plot, since interference could exist with the previous sampling (September 2011). The leaves drying and grinding procedure was the same used for greenhouse experiment.

Laboratory analyses

The leaf samples were analyzed for total N, N-NH₄⁺, N-NO₃⁻, chlorophyll and P concentrations. Greenhouse samples were also evaluated for the concentrations of root total N, N-NH₄⁺ and N-NO₃⁻. Total N was determined by Kjeldahl (Bremner, 1996), whereas the mineral N forms (N-NH₄⁺ and N-NO₃⁻) were analyzed according to Tedesco et al. (1995). Chlorophylls (a and b) were extracted in saturated dimethylsulphoxide (DMSO) with CaCO₃ in water bath (65 °C) by 1 h (greenhouse experiment) and by 2 h (field experiment), times defined in previous tests, and the absorbance measured at 649 and 665 nm. Chlorophylls (a and b) were calculated according to Wellburn's (1994) equations. From chlorophylls (a and b) we got the total chlorophyll. Phosphorus was extracted in HCl (0.01 mol L⁻¹), after mineralization of the samples by calcination in muffle furnace (500 °C) and quantification in ICP-OES (optical emission spectrophotometry) to calculate leaf N/P. Soil samples were analyzed for total N (Kjeldahl method), N-NH₄⁺ (Kempers & Zweers, 1986) and N-NO₃⁻ (Yang et al., 1998). The pH in water (1:2.5 ratio), organic C (Walkley-Black), P and K (Mehlich-1), texture (Ruiz, 2005), Ca²⁺, Mg²⁺ and Al³⁺ (KCl 1 mol L⁻¹) and H + Al (calcium acetate 0.5 mol L⁻¹) were also determined. The cation exchange capacity was calculate as (CEC)= Ca²⁺ + Mg²⁺ + K⁺ + Al³⁺. The organic C concentrations were transformed to soil organic matter (SOM = organic C x 1.724) (Alvarez V. et al., 1999).

Statistical analyses

All data were tested by variance analysis (F test; p<0.05) to verify the effect of the studied factors on measured variables using SAS software (SAS, 2004). We fitted

equations and chose the significant model ($p < 0.05$) with highest R^2 . We estimated concentrations of leaf critical range associated to 90 and 100 % of maximum growth, derived from equations fitted to dry matter production of plants and total N and N/P data. We also fitted Pearson correlation coefficients (r) between N supply (doses) and the indicators of N status and total dry matter of eucalyptus clones. In the case of field experiment, we adopted 10 % ($p < 0.10$) as significant effect for F test. For this last analysis, we evaluated the main effect and only some interactions of greater interest in the study, due to the large number of possible interactions. The means were compared by Tukey ($p < 0.05$) and all figures were done with the SIGMAPLOT software.

RESULTS AND DISCUSSION

The N doses increased the concentrations of total N, $N-NH_4^+$ and $N-NO_3^-$ in leaves and roots of both eucalyptus clones (Figure 2). For total N in root, clone VM-01 had Pearson correlation coefficient (r) lower than clone I-144, but in leaf it was inverse with general r of 0.73 (Table 3). There was no significant difference ($p > 0.05$) between the clones with respect to root total N, however leaves differed significantly, and the clone VM-01 showed the highest values (Figures 2a and b). Pinto et al. (2011) assessed the nutritional efficiency of eucalyptus clones and found differences in the N use efficiency between the clones VM-01 and I-144. The first clone was found to have a lower efficiency, which indicates its greater demand for this nutrient.

Total N leaf concentrations estimated with 2.93 mmol L⁻¹ of NH_4NO_3 (dose of Clark's solution) were 34.93 g kg⁻¹ for VM-01 and 30.73 g kg⁻¹ for I-144. Previous studies have shown values of total N concentration for *Eucalyptus spp.* from 14 to 35 g kg⁻¹ (Shedley et al., 1995; Leite et al., 2011). Dechen & Nachtigall (2007) report the range from 20 to 50 g kg⁻¹ of leaf total N as ideal for the best plant growth, however we consider these range too wide. The total N critical range for clone VM-01 is from 30.60 to 36.54 g kg⁻¹, referring to estimated doses which provided 90 and 100 % of dry matter production (1.63 and 3.80 mmol L⁻¹ of NH_4NO_3 , respectively). For clone I-144, the estimated doses of 1.90 and 4.55 mmol L⁻¹ of NH_4NO_3 were related to the leaf critical range of total N from 28.30 to 32.30 g kg⁻¹ (Figure 2b). In the general mean, the total N leaf concentrations found in the field were smaller (20.33 g kg⁻¹; Figure 3) than those found in the greenhouse (28.05 g kg⁻¹; Figure 2b), because the plants were older than the greenhouse plants.

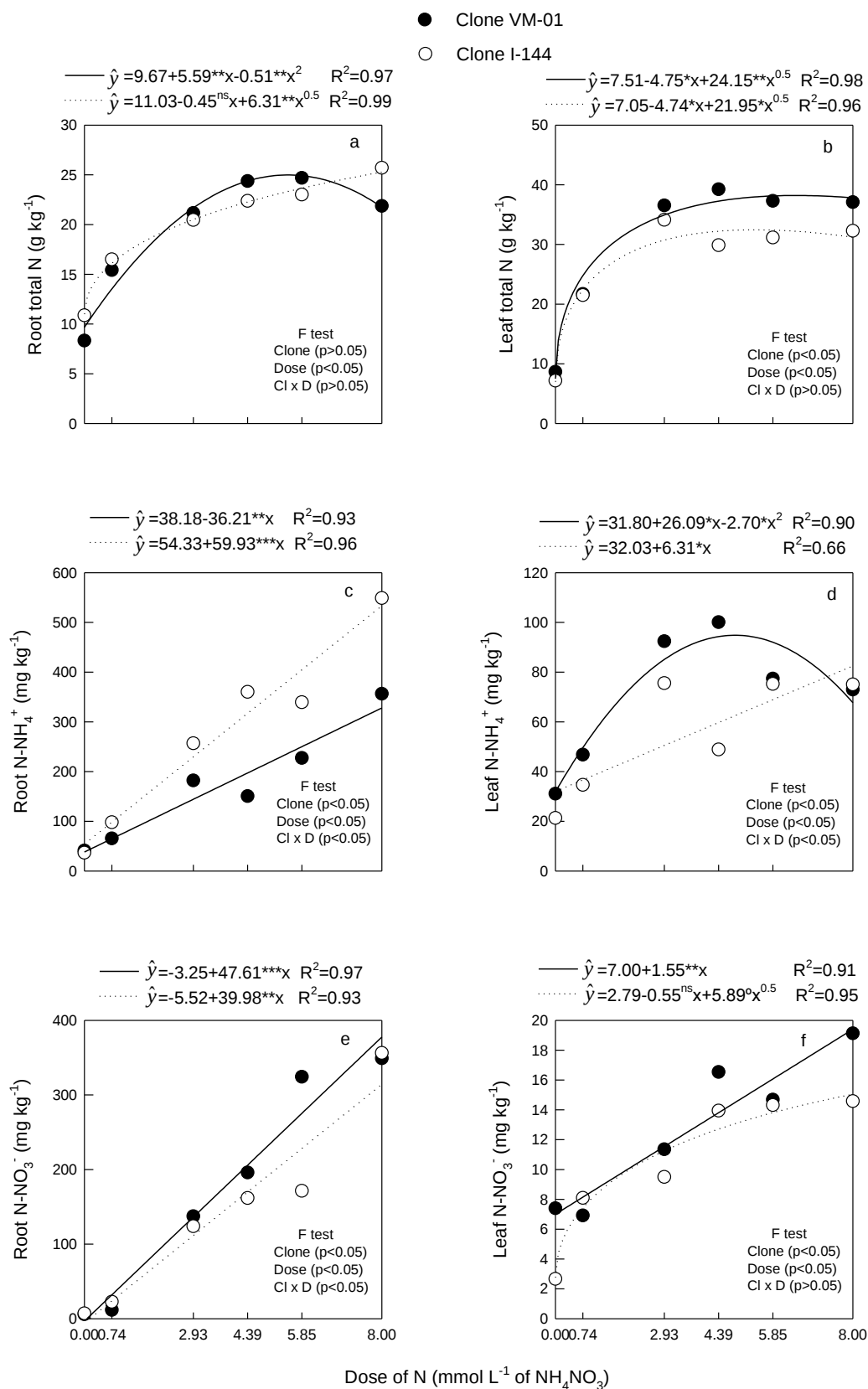


Figure 2. Concentrations of total N in root (a) and in leaf (b), N-NH₄⁺ in root (c) and in leaf (d) and N-NO₃⁻ in root (e) and in leaf (f) of young eucalyptus clones under N doses in nutrient solution. ^{ns}, °, *, ** and ***, represent, respectively, not significant and significant at 10, 5, 1 and 0.1 % by F test (p<0.05). Cl (clone) and D (dose).

Table 3. Pearson correlation coefficients (r) between N supply (doses) and indicators of N status and total dry matter (TDM) of eucalyptus clones grown in nutrient solution

Clone	Total N		N-NH ₄ ⁺		N-NO ₃ ⁻		N/P	Chlorophyll	SPAD	TDM
	root	leaf	root	leaf	root	leaf		leaf		-
VM-01	0.69	0.79	0.81	0.51	0.92	0.85	0.74	0.71	0.85	0.53
I-144	0.78	0.69	0.92	0.64	0.88	0.75	0.52	0.69	0.57	0.47
General*	0.73	0.73	0.82	0.55	0.90	0.78	0.60	0.65	0.58	0.47

* Pearson correlation coefficients (r) considering the data of both clones.

The optimal total N leaf concentration for tree growth decreases with age (Rubio-Covarrubias et al., 2009), due to a combination of factors, such as dry matter production rate exceeding soil N uptake rate (Smith & Lorenagan, 1997). In the field, total N leaf concentrations were different for both soils with greater concentrations in the clay soil in the dry season (Figure 3a), so they represented well the differences on soil total N concentrations between the regions in this season (Table 4). However, on rainy season total N leaf concentrations were greater in the region of sandy soil and thus they did not represent well the soil total N, since the clay soil also had greater total N concentrations in this season.

Soils with higher clay content offer greater protection for organic matter and greater N accumulation in soil (Cantarella, 2007). In the rainy season there is a chance of greater soil organic matter mineralization and as the clay soil has more organic matter (Table 1) and total N (Table 4), it has higher potential of N release for plant uptake, we would hope greater total N leaf concentrations in this situation. Greater total N leaf concentrations were verified on rainy season for both soils (Figure 3b). Therefore, as there was no constancy in the total N leaf concentrations between different soils regions to each season (Figure 3a), their concentrations cannot be considered a good indicator of its status for eucalyptus clones.

The clones also differed in total N with greater values for VM-01 in the middle of the crown and in the crown apex in I-144 (Figure 3c). There were differences in both clones in total N in different positions of crown; these concentrations increased from crown base to apex of trees (Figure 3d), due to N being a mobile element in plants (Marschner, 2012) and also preferentially allocated to foliage in areas of higher irradiance (Givnish, 1988). Thus, as there was no difference in total N between crown middle and apex to clone VM-01 and between base and middle to clone I-144, the

crown middle could be recommended for leaf sampling. For *E. globulus* sites, irrespective of their fertilization times, leaf total N did not show a good relationship with N response in Uruguayan soils (Perdomo et al., 2007). Rubio-Covarrubias et al. (2009) found that total N concentration showed the highest and most consistent association with the supply of this nutrient and growth of nectarine trees. Leaf analysis performed in different sites did not support the widespread use of total N concentration as an indicator of its deficiency in *E. nitens* (Smethurst et al., 2004). These results indicate a lack of consensus with the total N use as indicator of its status and also a need for more research in this issue. Thus, the total N use for leaf diagnosis is not reliable because it does not represent well its availability in the soil.

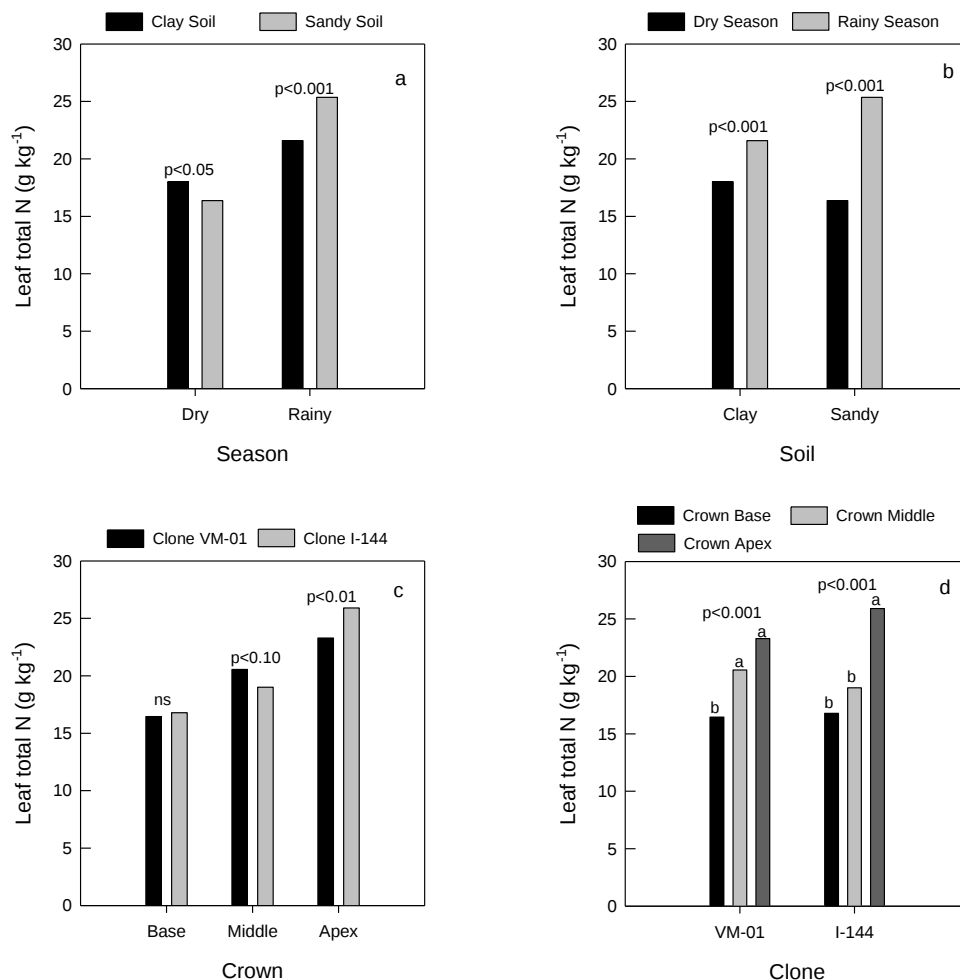


Figure 3. Total N leaf concentrations of eucalyptus clones in different soils for each season (a), in different seasons for each soil (b), in different clones for each position of leaves collect in the crown (c) and in different crown positions for each clone (d). ^{ns} represents not significant at 10 % by F test ($p > 0.10$). Means with the same letter, in each clone, are not significantly different by Tukey ($p < 0.05$).

In general, soil total N and N-NH_4^+ concentrations were higher in the soil upper layers (Table 4) because in these layers there is more organic matter (Table 1) and more than 95 % of soil total N is present in organic forms (Cantarella, 2007). In contrast, there was no standard distribution for the soil N-NO_3^- concentrations in the different layers, probably due the N-NO_3^- ions having high mobility and leaching in soils (Groffman, 2000). The plants absorb most of the soil N as N-NH_4^+ and N-NO_3^- (Marschner, 2012). In all conditions of different regions, seasons, clones and soils depths there was a predominance of N-NH_4^+ concentrations over N-NO_3^- (Table 4). This fact is explained due to this specie being generally cultivated under acid soils with low natural fertility (Gama-Rodrigues et al., 2005) where the nitrification is lower due to bacteria that perform this process (*Nitrosomonas* and *Nitrobacter*) are sensitive to lower pH (Moreira & Siqueira, 2002; Table 1). As consequence, eucalyptus species preferentially absorb more N-NH_4^+ than N-NO_3^- (Barros & Novais, 1996; Figures 2, 4 and 5). Preferential uptake of N-NH_4^+ in relation to N-NO_3^- can constitute an advantage, given the lower metabolic energy use, since it does not require the action of root N-NO_3^- reductase (Grespan et al., 1998). It would be advantageous for eucalyptus that seems to reduce further N-NO_3^- in the leaves than in the roots, as verified by too lower N-NO_3^- concentrations found in the leaves in relation to root (Figures 2e and f).

The N-NH_4^+ and N-NO_3^- concentrations showed good results in the greenhouse experiment; there was a linear increase in their concentrations in the roots of both clones with the N doses (Figures 2c and e), with high r (Table 3). General r (both clones) was 0.82 and 0.90 for N-NH_4^+ and N-NO_3^- , respectively. For leaf N-NO_3^- concentrations there was also good correlation (general $r=0.78$) and a linear response for clone VM-01 and square root for I-144 to N doses (Figure 2f). In contrast, to leaf N-NH_4^+ , there was lower r for both clones; 0.51 (VM-01) and 0.64 (I-144). It was observed quadratic response in VM-01 and low determination coefficient ($R^2=0.66$) in I-144 for leaf N-NH_4^+ (Figure 2d). Generally the mobile N forms have shown a higher rate of leaf accumulation than total N with increasing N supply (Rubio-Covarrubias et al, 2009). Therefore, these authors point out that the stable N forms (N total and chlorophyll) are poor indicators in conditions of high N supply in which N-NH_4^+ and N-NO_3^- concentrations were highly responsive, suggesting its usefulness as N status indicators for nectarine trees in these conditions. However, the good performance observed for N-NH_4^+ and N-NO_3^- concentrations for plants grown in nutrient solution (Figure 2) did not occur in the field (Figures 4 and 5).

Table 4. Concentrations¹ of N (total, ammonium and nitrate) in the soils under eucalyptus clones in different regions (Pompeu and João Pinheiro) and seasons (Dry and Rainy)

	Pompeu				João Pinheiro			
	VM-01		I-144		VM-01		I-144	
	Dry	Rainy	Dry	Rainy	Dry	Rainy	Dry	Rainy
Layer	Total N							
---cm---	-----dag kg ⁻¹ -----							
0-10	0.11	0.11	0.11	0.11	0.06	0.06	0.07	0.06
10-20	0.09	0.09	0.10	0.09	0.04	0.05	0.05	0.05
20-40	0.08	0.08	0.08	0.07	0.04	0.03	0.04	0.04
40-60	0.07	0.06	0.07	0.07	0.03	0.02	0.03	0.03
<u>60-100</u>	0.06	0.05	0.08	0.06	0.02	0.01	0.03	0.03
Mean	-----0.08 a-----				-----0.04 b-----			
	N-NH ₄ ⁺							
	-----mg kg ⁻¹ -----							
0-10	40.26	30.70	37.27	32.25	26.17	23.68	20.76	27.32
10-20	30.65	27.33	30.75	27.90	22.82	24.76	18.56	26.03
20-40	25.43	27.04	21.74	27.73	18.62	22.06	19.61	36.36
40-60	24.04	32.18	23.86	24.83	20.48	19.05	16.63	34.22
<u>60-100</u>	25.87	31.15	22.41	24.08	20.79	18.18	17.47	24.52
Mean	-----28.37 a-----				-----22.91b-----			
	N-NO ₃ ⁻							
	-----mg kg ⁻¹ -----							
0-10	0.55	0.54	0.86	0.51	0.69	0.54	0.72	0.44
10-20	0.49	0.41	0.57	0.57	0.59	0.47	0.61	0.61
20-40	0.48	0.28	0.52	0.44	0.56	0.46	0.51	0.29
40-60	0.60	0.36	0.55	0.38	0.56	0.34	0.64	0.29
<u>60-100</u>	0.60	0.25	0.93	0.57	0.88	0.52	1.10	0.52
Mean	-----0.52 a-----				-----0.57 a-----			

¹Mean values of samples collected in line and interline. Pompeu and João Pinheiro represent regions with clay and sandy soil, respectively. Means with the same letter, in the rows, are not significantly different by Tukey (p<0.05). Total N (Kjeldahl's method), N-NH₄⁺ (Kempers & Zweers, 1986) and N-NO₃⁻ (Yang et al., 1998).

The N-NH_4^+ leaf concentrations were higher in the sandy soil region in both dry and rainy seasons (Figure 4a), therefore it did not reflect their soil concentrations due to clay soil having a greater concentration this ion (Table 4). The N-NH_4^+ leaf concentrations were smaller in the rainy season, for both clay and sandy soils (Figure 4b), in contrast that was also observed with their concentrations on soil (Table 4). There was difference in N-NH_4^+ leaf concentrations between the clones only on the crown apex, with greater values for I-144, differently to what happened in the greenhouse (Figure 2d and 4c). It was observed that higher N-NH_4^+ leaf concentrations only on crown apex in clone I-144 and in VM-01 there was no difference in their concentrations among the crown positions (Figure 4d).

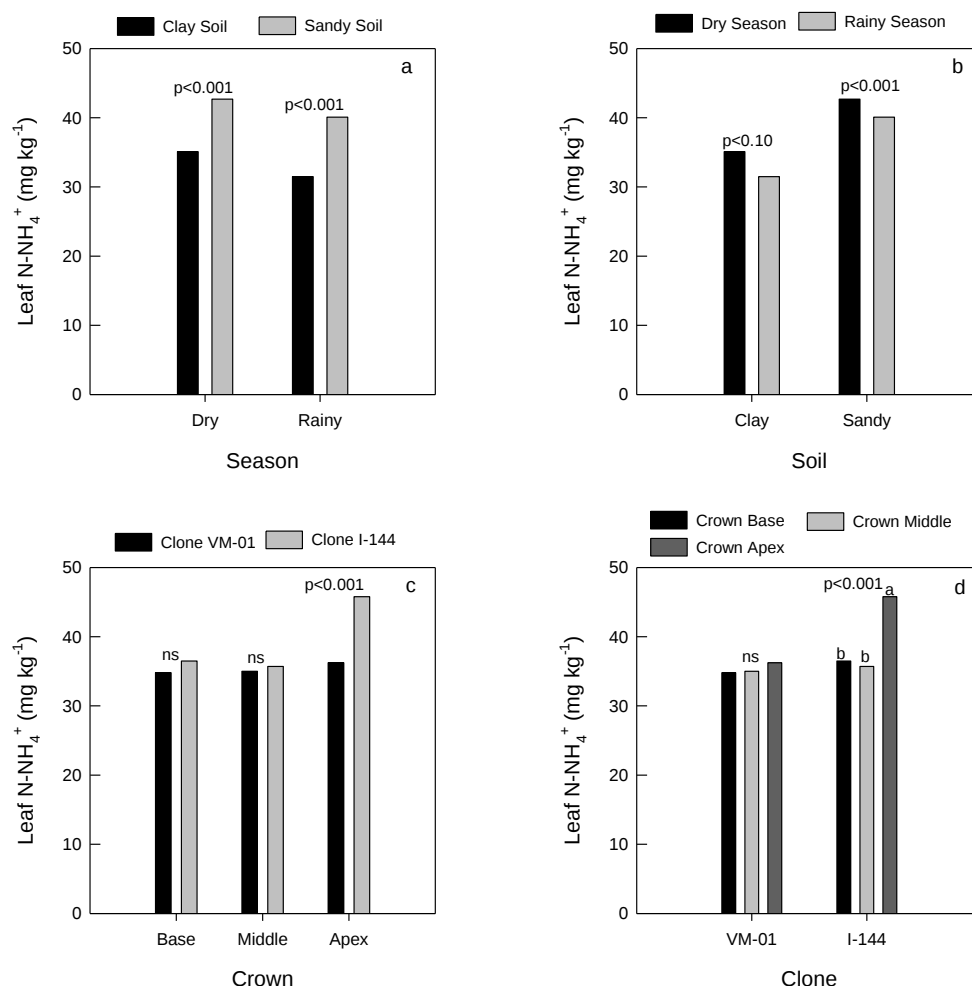


Figure 4. Leaf N-NH_4^+ concentrations of eucalyptus clones in different soils for each season (a), in different seasons for each soil (b), in different clones for each position of leaves collect in the crown (c) and in different crown positions for each clone (d). ^{ns} represents not significant at 10 % by F test ($p > 0.10$). Means with the same letter, in each clone, are not significantly different by Tukey ($p < 0.05$).

For the N-NO_3^- leaf concentrations, except between soils on dry season, there was no difference among soils, seasons, clones and crown positions (Figures 5a, b, c and d), probably as a consequence of not having existed differences in their concentrations in the soil (Table 4). Concentrations of N-NO_3^- were very low for the purposes of diagnosis in canola (Hocking, 2001). Thus, it has been suggested that the combination of both indicators (stable compounds- N total and chlorophyll and N mobile forms- N-NH_4^+ and N-NO_3^-) could be used to diagnose the N status over a wide range of availability this nutrient in soil (Rubio-Covarrubias et al., 2009).

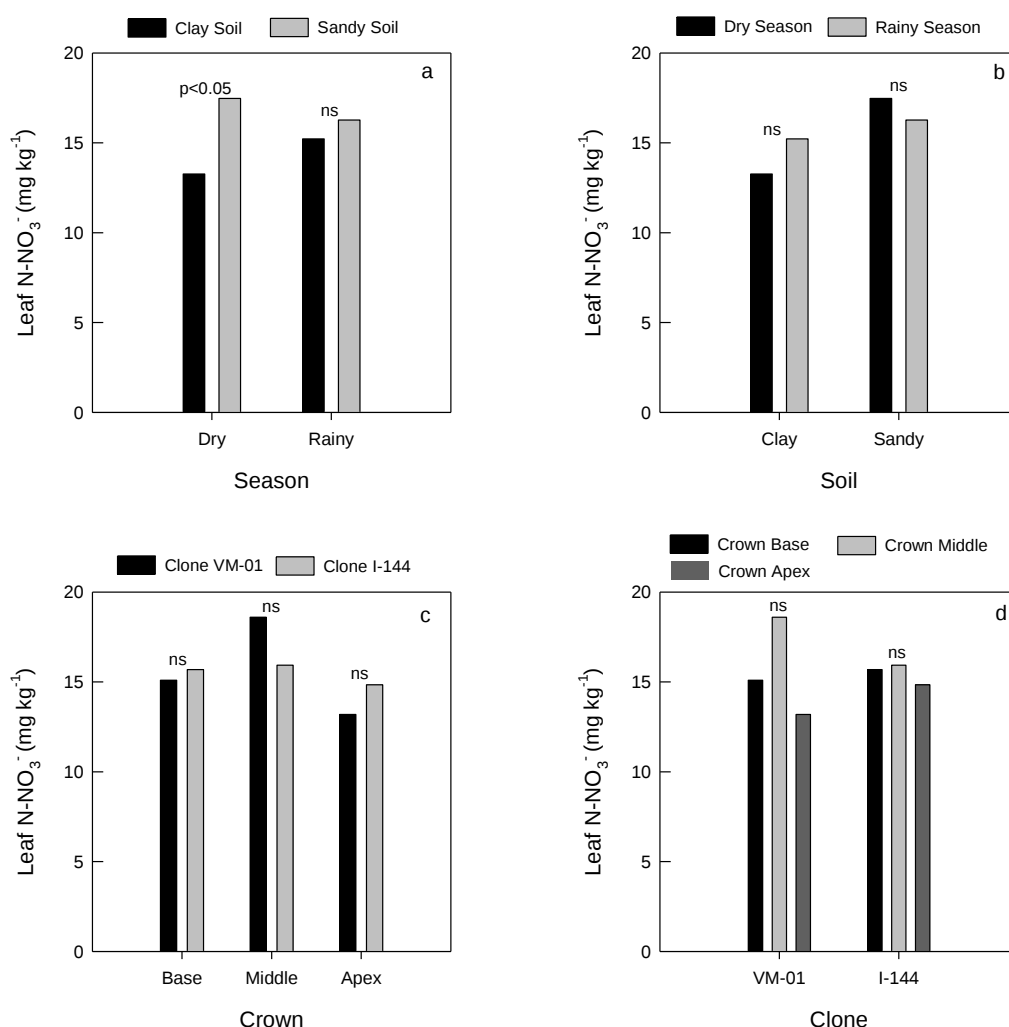


Figure 5. Leaf N-NO_3^- concentrations of eucalyptus clones in different soils for each season (a), in different seasons for each soil (b), in different clones for each position of leaves collect in the crown (c) and in different crown positions for each clone (d). ^{ns} represents not significant at 10 % by F test ($p > 0.10$).

The leaf N/P, chlorophyll and SPAD readings (Figures 6a, b and c) showed a similar behavior to N doses application in solution; with general r of 0.60, 0.65 and

0.58, respectively (Table 3). It was observed an increase in their values until reaching a peak, and then these values remained practically constant. However, the clone I-144 had the highest values these variables, unlike the leaf total N (Figure 2b). For N/P, the clone VM-01 presented a critical range from 11.42 to 13.38, related to the estimated doses of 1.63 and 3.80 mmol L⁻¹ of NH₄NO₃, respectively, while estimated doses of 1.90 and 4.55 mmol L⁻¹ of NH₄NO₃ were related to critical range of 14.70 and 15.90 for clone I-144 (Figure 6a). These values were similar to those reported in Australia and New Zealand (15; Judd et al., 1996; Knight & Nicholas, 1996) for *E. globulus* and *E. nitens*.

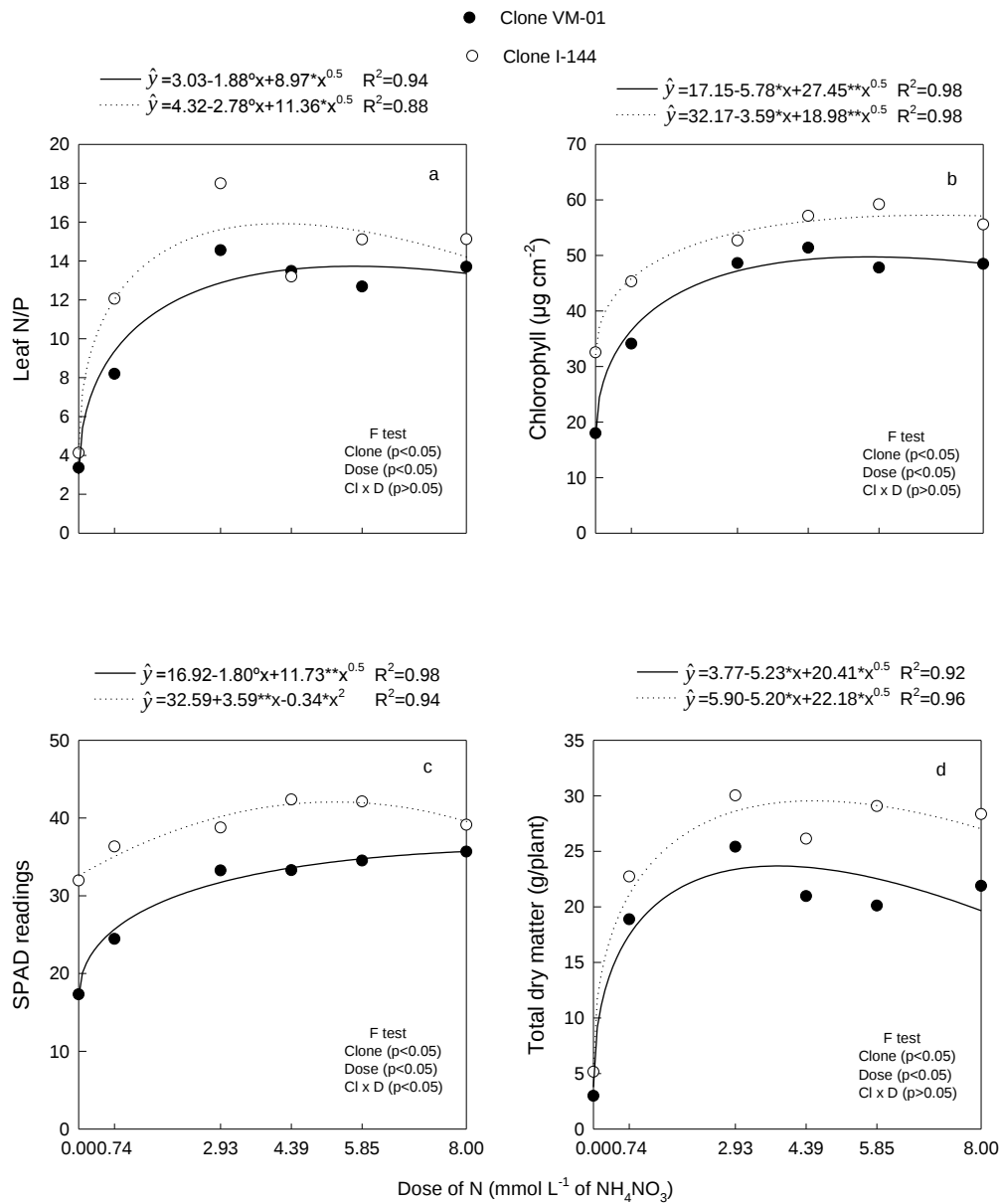


Figure 6. Leaf N/P (a), chlorophyll (b), SPAD readings (c) and total dry matter (d) of young eucalyptus clones under N doses in nutrient solution. °, * and **, represent, respectively, significant at 10, 5 and 1 % by F test (p<0.05). Cl (clone) and D (dose).

In the field, the values of leaf N/P were between 20 and 29 (Figure 7), probably as a consequence of low mean leaf concentrations of P in these plants ($< 0.90 \text{ g kg}^{-1}$, Dechen & Nachtigall, 2007). There was no difference between leaf N/P for regions of sandy and clay soils in the dry season, however in the rainy season there were higher values of this ratio in the region of clay soil (Figure 7a). For both clay and sandy soils there were higher values of leaf N/P in the rainy season (Figure 7b), as also observed for leaf total N (Figure 3b). The clone VM-01 showed the greatest N/P regardless of crown position (Figure 7c), unlike the observed in the greenhouse (Figure 6a). For the different sampling positions of leaves in the crown there was a reduction of N/P from base to apex of the trees crown for the two clones (Figure 7d) due to P leaf concentrations (data not shown) being proportionately smaller in crown base, since this nutrient is mobile in the plant (Dechen & Nachtigall, 2007). Although the analysis of leaf N/P can be used to guide fertilization with these nutrients in eucalyptus (Perdomo et al. 2007), leaf analysis conducted at different sites did not support its widespread use as an indicator of N deficiency in *E. nitens* (Smethurst et al., 2004), as was also verified in this study.

For the chlorophyll in greenhouse, were observed greater values of $51 \mu\text{g cm}^{-2}$ for clone VM-01 (dose of 4.39 mmol L^{-1} of NH_4NO_3) and of $59 \mu\text{g cm}^{-2}$ for I-144 (dose of 5.85 mmol L^{-1} of NH_4NO_3). Plants with higher N leaf concentrations typically have more chlorophyll (Foulkes et al., 2009). Thus, for higher total N leaf concentrations (Figure 2b), we verified higher chlorophyll values (Figure 6b). In the field, the plants showed similar chlorophyll values (mean of $52 \mu\text{g cm}^{-2}$; Figure 8), compared to greenhouse (Figure 6b), and the clone I-144 had higher values in all crown positions than clone VM-01 (Figure 8c), like it was also observed in the greenhouse (Figure 6b).

In dry season, the plants of clay soil region had more chlorophyll; however the chlorophyll values were greater in the plants of sandy soil region in the rainy season (Figure 8a). There was no difference in chlorophyll between the dry and rainy seasons for the clay soil region and for the sandy soil region their values were greater in the rainy season (Figure 8b). The higher chlorophyll values were found on crown middle and apex for clone VM-01 and there was no difference in these values to clone I-144 (Figure 8d). Although the SPAD readings represented the N doses in solution (Figure 6c), clone VM-01 which had greater total N leaf concentrations compared to clone I-144 (Figure 2b), showed lower readings. Rambo et al. (2010) found differences in the SPAD readings among corn hybrids and they commented that may be dangerous to generalize information obtained from one hybrid to another.

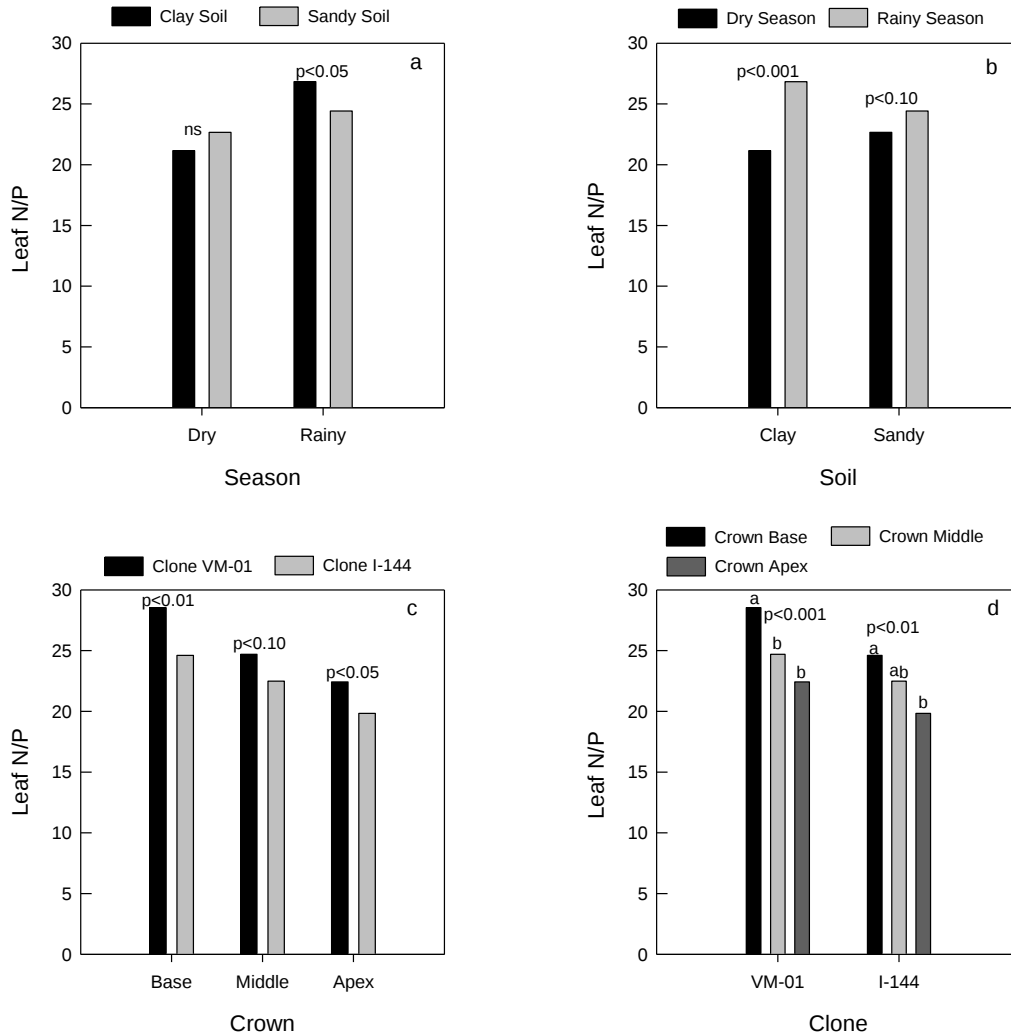


Figure 7. Leaf N/P of eucalyptus clones in different soils for each season (a), in different seasons for each soil (b), in different clones for each position of leaves collect in the crown (c) and in different crown positions for each clone (d). ^{ns} represents not significant at 10 % by F test ($p>0.10$). Means with the same letter, in each clone, are not significantly different by Tukey ($p<0.05$).

SPAD readings reflect the intensity of leaf green color (Fontes, 2011) proportional to the chlorophyll leaf concentrations. Though N deficient leaves are chlorotic (Marschner, 2012), greater total N leaf concentrations does not always mean an increase in the leaves green color, as verified when we compare the clones VM-01 and I-144. Clone VM-01 had greater total N leaf concentrations (Figure 2b); however it has showed lower SPAD readings as consequence less chlorophyll (Figure 6b) and then lighter green leaves. Also there is not always positive correlation between chlorophyll and N concentrations, since the N is not only constituent of chlorophyll molecule (Dechen & Nachtigall, 2007). So, with the increase of N doses in solution can be having predominantly N accumulation in other compounds instead of in chlorophyll. According

to Fontes (2011), in a situation of luxury consumption the N excess is accumulated in plants as NO_3^- , and still not incorporated into the chlorophyll molecules. Therefore, the SPAD use in the plant N status diagnosis should be taken with caution, since it has some limitations because it is not a specific indicator for N.

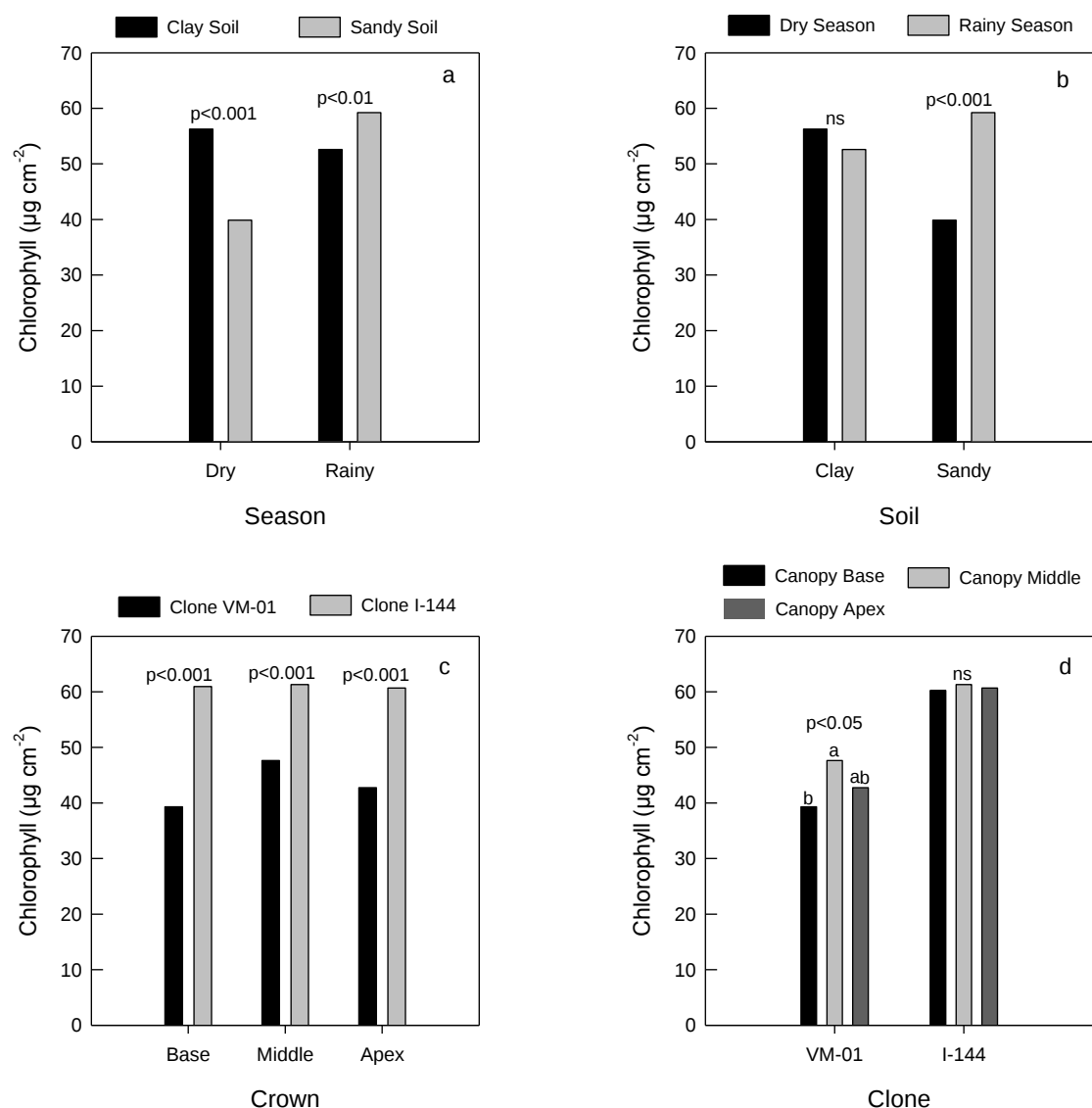


Figure 8. Chlorophyll of eucalyptus clones in different soils for each season (a), in different seasons for each soil (b), in different clones for each position of leaves collect in the crown (c) and in different crown positions for each clone (d). ^{ns} represents not significant at 10 % by F test ($p > 0.10$). Means with the same letter, in each clone, are not significantly different by Tukey ($p < 0.05$).

CONCLUSIONS

- The concentrations of N-NH_4^+ (root) and N-NO_3^- (root and leaf) showed use potential for predicting eucalyptus N requirements in greenhouse (highest correlation

coefficients- r), however, these mobile N forms (mainly N-NO₃⁻) did not represent well the soil N levels in different seasons and regions evaluated in the field;

- It was observed similar responses for leaf N/P, chlorophyll and SPAD readings to N application in greenhouse (quadratic and square root models), with highest values of r for clone VM-01, and the N/P and chlorophyll in different seasons and regions did not reflect the real differences in soil N status in the field;

- The total N leaf concentration is not good indicator of N status, because it did not have highest values of r in greenhouse. Nevertheless, of all indicators evaluated, it better represented the variations in soil N status in different seasons and regions assessed for eucalyptus clones under field conditions.

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CHAPTER 2 – Differential behavior of young eucalyptus clones in relation to nitrogen supply

Abstract- Nitrogen is a nutrient required in large amounts by plants; however, for eucalyptus the effects of its application on dry matter production are divergent. There are nutritional differences among eucalyptus species, especially with respect to plant response to N. Thus, the aim this work was to evaluate the differential behavior of eucalyptus clones in growth, mineral nutrition and gas exchanges under N doses. A greenhouse experiment was carried out using nutrient solution (Clark, 1975, modified by Locatelli et al., 1984). The treatments consisted of two eucalyptus clones (VM-01 and I-144) and six doses of N (0, 0.74, 2.93, 4.39, 5.85 and 8 mmol L⁻¹ of NH₄NO₃), arranged in a 2 x 6 factorial and laid out in randomized complete block design with five replications. After 60 days of treatment application, we assessed dry matter production, height, collar diameter and root length, gas exchanges and leaf concentration of macro and micronutrients. The gas exchanges were measured in youngest fully expanded leaves of plants using an infrared gas analyzer (IRGA) with constant radiation (1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and environment temperature and humidity. The results were submitted to analysis of variance and regression models were fit to data. Clone VM-01 was superior in height, height/collar diameter and it also had higher leaf concentration of all macronutrients and of Cu, Fe, Mo and Zn. On the other hand, clone I-144 showed greater production of total and root dry matter, root/shoot and collar diameter, moreover of greater stomatal conductance (g_s) and transpiration (E). Therefore, these two eucalyptus clones exhibited a differential behavior and clone I-144, despite lower N leaf concentration, showed, in general, better growth compared to clone VM-01, thus having highest utilization efficiency of this nutrient.

Keywords: *gas exchanges, growth, mineral nutrition, nutrient solution.*

INTRODUCTION

Brazil is one of the largest producers of cellulose and paper in the world from eucalyptus plantations (SBS, 2006). *Eucalyptus* is the most widely planted forest species in Brazil, being planted on nearly 4.9 million hectares in 2011 (ABRAF, 2012). Most eucalyptus forests in Brazil are planted in low fertility soils, frequently resulting in growth limitations due to insufficient nutrient supply (Barros & Novais, 1996; Leite et

al., 2011), and evidence of nutritional deficiencies have been documented (Silveira et al., 1995). Consequently, Brazil is the country where have been observed higher responses to mineral fertilization by eucalyptus (Pinto, 2009), with the necessity of application of high amounts of fertilizer to obtain greater productivity (Barros et al., 2004).

Nitrogen is one of the most limiting factors for plant growth and development (Majerowicz et al., 2000), being required in large amounts (Marschner, 2012). However, for eucalyptus the effects of its application on production of dry matter are positive in some cases (Smethurst et al., 2004; Jesus et al., 2012) and, in others, without positive response (Stape & Benedetti, 1997; Garategui, 2002; Pulito, 2009). Gonçalves et al. (2000) report that the intensity of the response of eucalyptus to N fertilization is of low magnitude. Also, studies (Furtini Neto et al., 1996; Santana et al., 2002) have verified nutritional differences among eucalyptus species, as plant response to N (Pinto et al., 2011). These last researchers point to out the understanding of nutrient demand and the genetic materials efficiency in converting the absorbed nutrients into biomass as an important aspect. There is wide intra and interspecific variability for eucalyptus species, mainly in features such as biomass production and growth rate (Chaperon, 1987). Changes in the root to shoot ratio in response to nutrient availability may be related to the maximization of acquisition and use of the available nutrients and carbohydrates to plant growth (Cruz et al., 1993; Majerowicz & Kerbaudy, 2002). There is a limited understanding of the mechanisms that drive the partitioning of carbohydrates and nutrients between different plant parts (Marschner et al., 1996).

The N deficiency rapidly inhibits plant growth and most species show chlorosis in older leaves, with necrosis and subsequent fall in the condition of severe deficiency (Marschner, 2012). The main effect of N low availability is the reduction of the photosynthetic rate (Hay & Porter, 2006), with reduction in chlorophyll and rubisco leaf concentration (Bungard et al., 1997). Field (1983) comments that the distribution of leaf N in plant maximizes the gain of the canopy C as a whole, since N acts as essential component of proteins, chlorophyll and thylakoids (Raven et al., 1986). The formation of new tissues of the shoot increases the N demand for protein synthesis (Majerowicz & Kerbaudy, 2002). The absorbed N is incorporated in the plant as amino acids and, with the increase of its supply, the synthesized proteins promote leaf growth, increasing the photosynthetic surface area (Dechen & Nachtigall, 2007). Hence, the dry matter accumulation is dependent on the N that the plant can absorb and built leaf area per unit

of absorbed N (Hay & Porter, 2006), existing a tight relation between leaf area index (LAI) and absorbed N (Hirel et al., 2007). There is, consequently, a close relation among N supply, leaf N distribution and leaf photosynthesis (Gasta & Lemaire, 2002).

Finally, it is expected that the eucalyptus clones have different responses in growth due to differences in nutritional demand and photosynthetic rates influenced by N supply. Therefore, this study aimed to evaluate the differential behavior of young eucalyptus clones in growth, mineral nutrition and gas exchanges under the application of N doses in nutrient solution.

MATERIAL AND METHODS

The experiment was carried out in a greenhouse at the Soil Science Department of the Federal University of Viçosa, Viçosa (20°45'14" S, 42°52'53" W), Brazil, from July to September 2011. The air temperature in the greenhouse was recorded daily and it showed minimum and maximum values of 7.9 and 33.9 °C, respectively, during the time of cultivation of plants. The first thirty days represented the stage of plant acclimatization in nutrient solution and the other sixty days represented the exposure time of plants to treatments. The nutrient solution of Clark (1975), modified to contain the $\text{N-NH}_4^+/\text{N-NO}_3^-$ equal to 1 (Locatelli et al., 1984) and the double of P concentration (Caldeira et al., 1994), was used in this study: macronutrients (mmol L^{-1} ; 1.30 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.30 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.069 KH_2PO_4 , 0.53 KCl , 0.30 K_2SO_4 and 0.069 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) and micronutrients ($\mu\text{mol L}^{-1}$; 19.0 H_3BO_3 , 7.0 $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.5 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2.0 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.086 $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ and 40.0 FeEDTA), varying only the N dose, with 2.93 mmol L^{-1} of NH_4NO_3 as reference dose.

The concentration of nutrient solution at the stage of plant acclimatization was gradually increased (25, 50, 75 and 100 % of original concentration) by each seven days. In this same stage the plants were placed in a collective plastic tray (11 L). After acclimatization, homogeneous plants were selected in relation to height and vigor and two plants were transferred to each plastic pot (6 L) composing an experimental unit. The nutrient solution was maintained under permanent aeration and changed weekly, using deionized water, and its pH was adjusted daily to 5.5 ± 0.05 (Locatelli et al., 1984) with addition of a solution of H_2SO_4 or NaOH (0.1 mol L^{-1}).

The treatments consisted of two eucalyptus clones (VM-01 and I-144) and six N doses (0, 0.74, 2.93, 4.39, 5.85 and 8 mmol L^{-1} of NH_4NO_3), arranged in a 2 x 6 factorial randomized complete block design with five replications. The clone VM-01 is

a hybrid (*Eucalyptus urophylla* vs *E. camaldulenses*) and it was used because it is considered more N demanding. On the other hand, clone I-144 is considered less N demanding, and it is a hybrid of *E. urophylla* vs *E. grandis*. The clones were provided by the forestry companies Vallourec & Mannesmann Tubes (VM-01) and Arcelor Mittal (I-144) and they were young plants derived from tube seedlings with age approximate of 50 days at the beginning of the trial.

At the end of the experimental period, plant height, collar diameter and root length were measured, and height/collar diameter was calculated. The height and root length were assessed with the use of a graduated ruler in centimeters and with a digital paquimeter were performed measures (in millimeters) of collar diameter of plants. The gas exchanges were measured in the morning period (between 7 and 11 h) in youngest fully expanded leaves of plants using an infrared gas analyzer (IRGA, Li-6400xt, Licor) with environment temperature and humidity and constant radiation ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$), in accordance with Nunes (2010) in greenhouse eucalyptus trial. The photosynthesis (A), stomatal conductance (g_s), internal (C_i) and external (C_a) CO_2 concentration ratio of leaf (C_i/C_a) and transpiration (E) were evaluated. Later, the plants were harvested, their leaves, stems, branches and roots were separated and washed with deionized water, and then oven dried (60°C) to constant weight to obtain the dry matter of their different tissues. The sum of dry matter of leaves, stem and branches represents dry matter of shoot and the total dry matter is a sum of root and shoot dry matter.

All leaves were ground (Wiley mill) and taken to the laboratory to evaluate the concentration of macro (N, P, K, Ca, Mg and S) and micronutrients (B, Cu, Fe, Mn, Mo and Zn). After mineralization of the material with sulfuric acid and heating, leaf N was determined by Kjeldahl method (Bremner, 1996). To assess the concentration of all other nutrients the samples were mineralized by calcination in muffle (gradually increasing the temperature until 500°C over 3 h) with subsequent extraction in HCl (0.01 mol L^{-1}) and determination by inductively coupled plasma optical emission spectrophotometry.

Analysis of variance (F test) were performed for all data to test the effects of clones, N doses and their interactions on measured variables by using the general linear model procedure (PROC GLM) of the SAS statistical package (SAS, 2004). Were fitted quadratic and square root regression models to describe these relations, and the significant model ($p < 0.05$) with the value of highest determination coefficient (R^2) was selected.

RESULTS AND DISCUSSION

The application of N increased shoot (Figure 1a), root (Figure 1b) and total dry matter (Figure 1d), being that the clone I-144 showed higher values. Pinto et al. (2011) observed that the clone I-144 exhibits high efficiency in N uptake and utilization, unlike clone VM-01, which presents high uptake efficiency but low utilization efficiency. The clones VM-01 and I-144 reached, respectively, maximum values of total dry matter of 25.4 and 30.0 g/plant with the dose of 2.93 mmol L⁻¹ of N, the recommended dose in the nutrient solution of Clark (1975) modified by Locatelli et al. (1984). The effects of N application on the growth of eucalyptus are positive in some cases (young plants, Jesus et al., 2012) and, in others, with no response (adult plants, Pulito, 2009). In accordance with Barros et al. (1990), N accelerates the initial growth rate of the forest, but differences among treatments are no longer significant after 30 months, as noted by Pulito (2009).

Root/shoot dry matter decreased with increasing N for the two clones studied (Figure 1c). As expected, the highest root/shoot occurred in the absence of N in solution, due to relatively lower production of shoot dry matter in this condition (Figure 1a). Additionally, in the absence of any limiting factor the roots tend to increase their growth; however it does not mean higher dry matter production of this organ (Figure 1b). Thus, under conditions of N deficiency, the root system growth is relatively increased (Marschner, 2012), shoot growth is reduced, and the root/shoot can be modified (Améziane et al., 1995), by altered allocation of carbohydrates. The increase in root/shoot under N deficiency has been correlated to shift in endogenous phytohormone levels, with an increase in abscisic acid and decrease in cytokinins (Marschner, 1995).

Eucalyptus clones presented different behavior regarding growth in height, collar diameter and height/collar diameter, whereas they did not differ ($p>0.05$) in root length (Figure 2). With increasing N doses there was an increase in height (Figure 2a) and collar diameter (Figure 2b) of the clones, while there was not difference ($p>0.05$) to height/collar diameter (Figure 2c). The N doses provided a reduction in root length to clone VM-01, however they did not influence this variable to clone I-144 (Figure 2d). The clone VM-01 showed better performance in height growth and also height/collar diameter, although the collar diameter was greater in clone I-144. Martinez et al. (1993) emphasize that the differentiated behavior, in relation to the plant growth of different

genetic materials, grown under the same condition, may indicate differences in the internal factors responsible for their nutritional efficiencies.

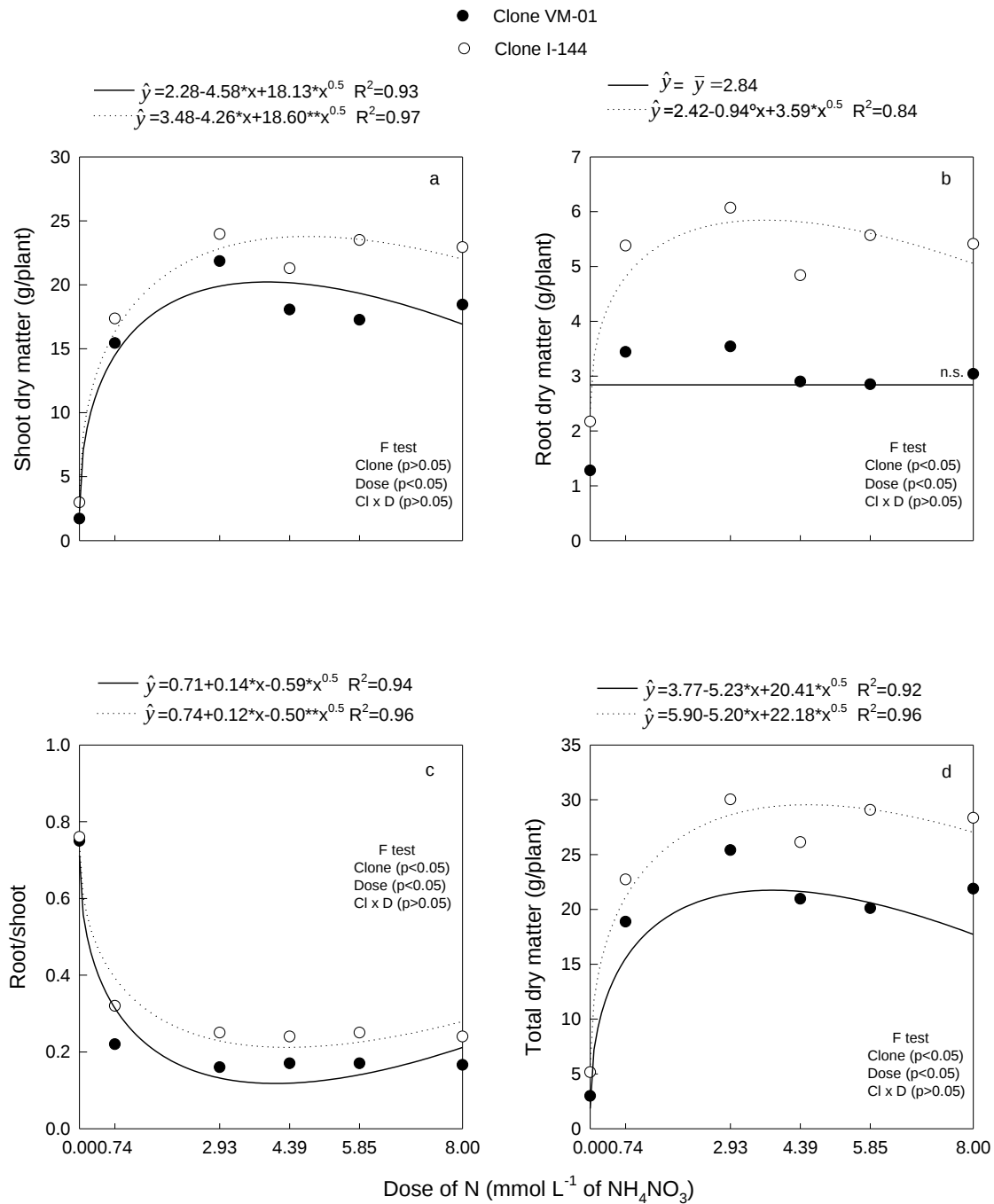


Figure 1. Shoot (a) and root (b) dry matter, root/shoot (c) and total dry matter (d) of young eucalyptus clones under N doses in nutrient solution. ^{ns}, ^o, * and **, represent, respectively, not significant and significant at 10, 5 and 1 % by F test ($p < 0.05$). Cl (clone) and D (dose).

Studies conducted by Wadt et al. (1999), regarding to variations in the nutritional status of eucalyptus under the influence of different genetic material and tree

age, demonstrated that the requirement of N is greater in the beginning of the culture cycle. In this sense, more attention should be directed to these clones with respect to N nutrition in early growth stages, mainly to the clone VM-01 that, according to Pinto et al. (2011), has lower N utilization efficiency.

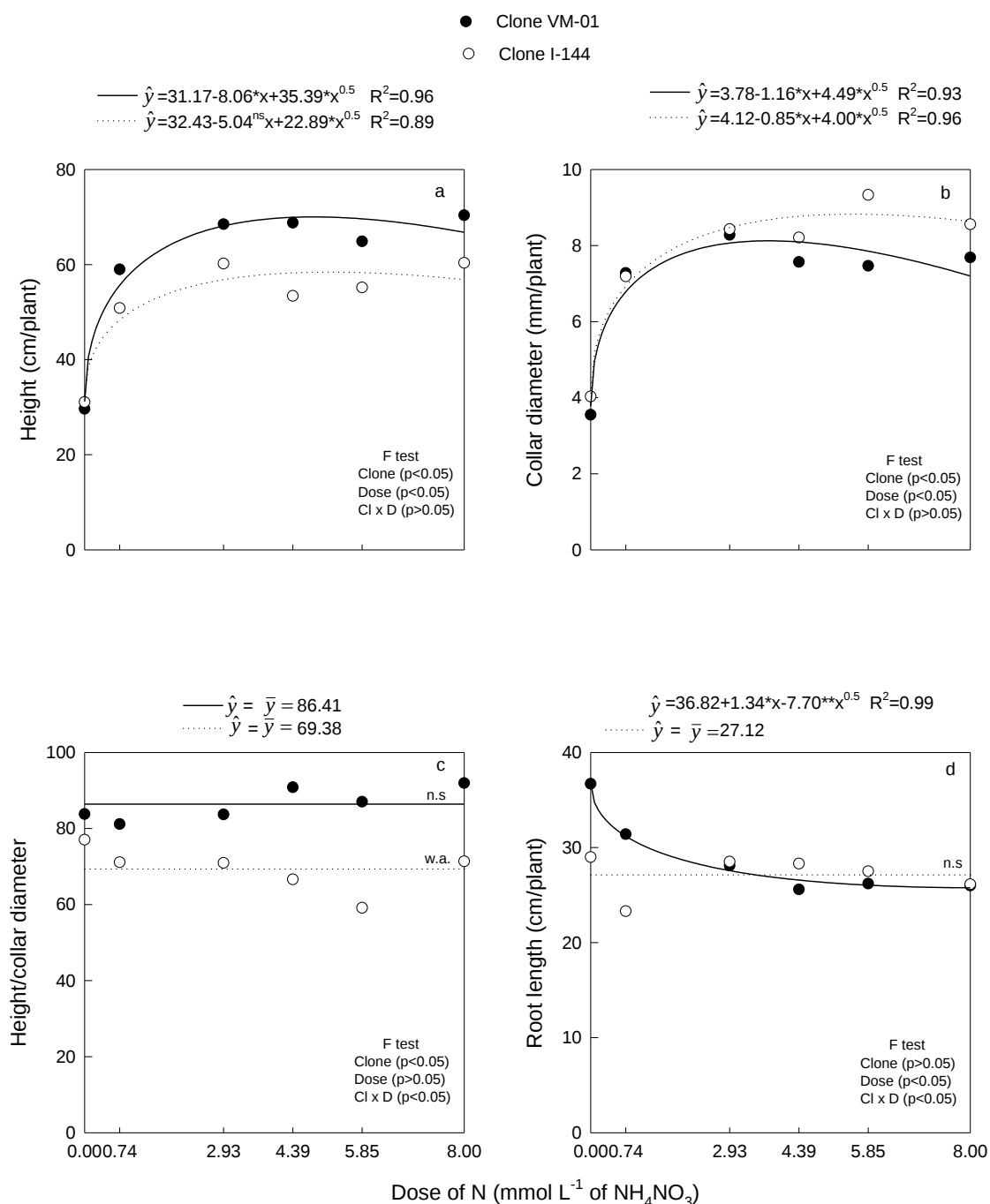


Figure 2. Height (a), collar diameter (b), height/collar diameter (c) and root length of young eucalyptus clones under N doses in nutrient solution. ^{ns}, ^{wa}, * and **, represent, respectively, not significant, without adjustment and significant at 5 and 1 % by F test (p<0.05). Cl (clone) and D (dose).

With regard to gas exchanges, there was no difference ($p>0.05$) between the clones for A (Figure 3a) and C_i/C_a (Figure 3c). It stands out that the values of A increased with increasing of N in solution (Figure 3a), while the C_i/C_a (Figure 1c) had little alteration by the application of N doses. In contrast, for g_s (Figure 3b) and E (Figure 3d), the clone VM-01 presented lowest values. To this clone, there was an increase in their values of g_s and E with increasing of N doses applied until they reached their peaks. These values were of $0.41 \mu\text{mol m}^{-2} \text{s}^{-1}$ of H_2O and $5.4 \text{ mmol m}^{-2} \text{s}^{-1}$ of H_2O , respectively. To the clone I-144, in spite of N doses have influenced these same variables, there was no adjustment (without adjustment; w.a.) for tested regression models to g_s and it was not significant (n.s.) to E . In four years old *E. globulus*, N fertilization (plus P, K and S) also stimulated 20 % increase in height, an increase in N leaf concentration and g_s , and an increase in C_i/C_a (Turnbull et al., 2007).

The positive response behavior observed of A to N application (Figure 3a) was expected, since this nutrient has a positive relation with photosynthesis (Niinemets et al., 2006). Nitrogen is part of enzymes that participate in photosynthetic processes, especially rubisco (Onoda et al., 2004), besides being present in chlorophyll molecule (Dechen & Nachtigall, 2007). For the majority of species, N deficiency induces a significant decrease in A and g_s (Hunt et al., 1985). However, the response of stomata to N deficiency is variable, occurring from increment (Ciompi et al., 1996) to decrease (Hák & Nátr, 1987; Lima et al., 2000).

Even though the clones have similar values of A (Figure 3a), VM-01 showed lower values of g_s (Figure 3b) and total dry matter (Figure 1d) compared to I-144. In general, the increase in g_s (Figure 3b), owing to the application of N, was accompanied by increase in E (Figure 3d) and A (Figure 3a), although not by C_i/C_a (Figure 3c), which resulted in gain of total dry matter (Figure 1d). Müller et al. (2012) verified that the reduction in g_s is related to the reduction in E and A in eucalyptus clones grown with B supply in nutrient solution. They reported values around $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ of CO_2 for A and between 0.4 and $0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ of H_2O for g_s , in plants cultivated in the control treatment (complete nutrient solution without water restriction). In another experiment with eucalyptus, also with addition of B in the nutrient solution, higher values of g_s were also associated with higher values of E , indicating that conditions that promote greater dry matter production also favor greater transpiration flow (Nunes, 2010). Changes in values of g_s have been described to be involved with the control of water

loss and uptake of CO₂ (C_i) to maintenance of photosynthetic rate (Taiz & Zeiger, 2004).

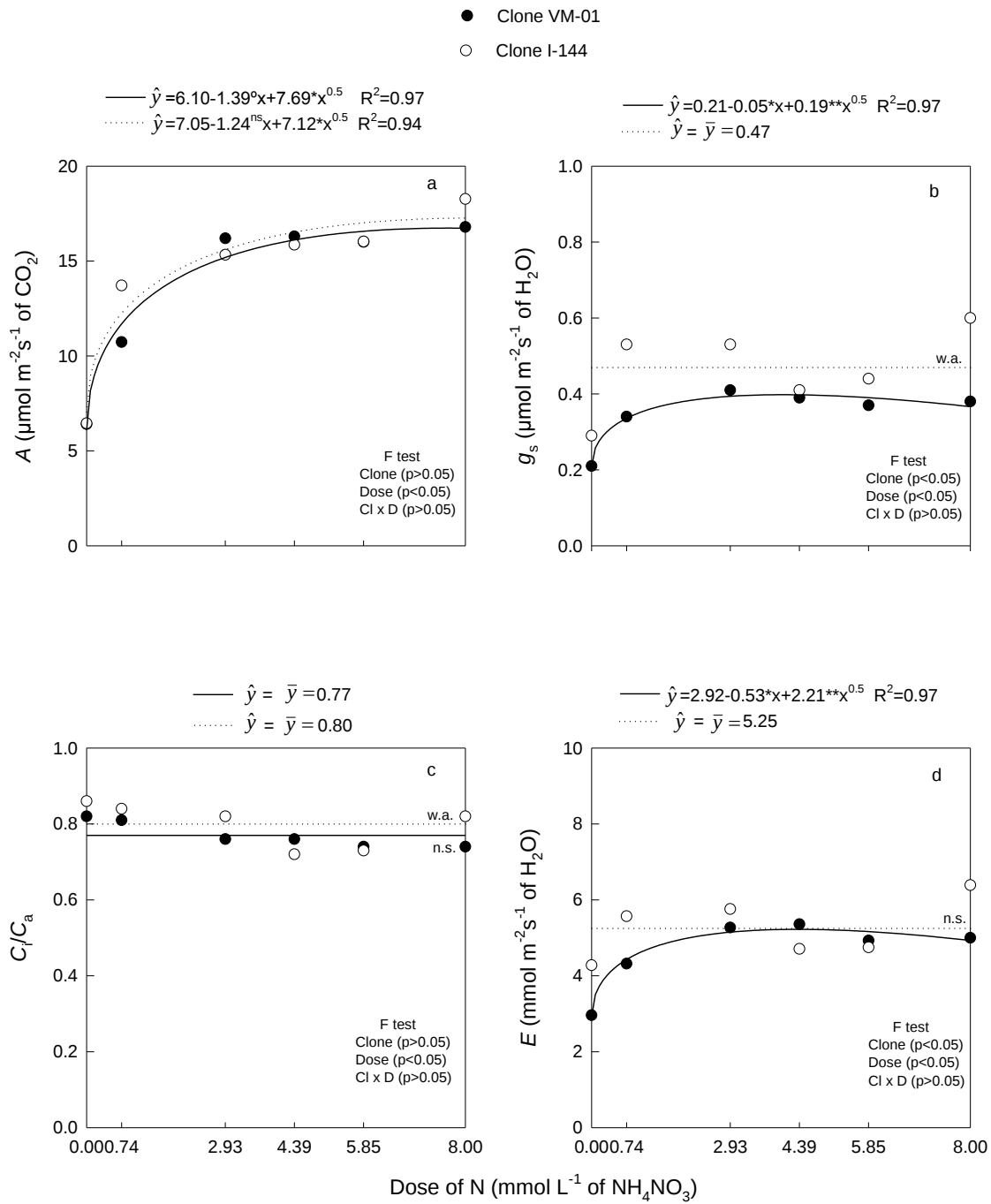


Figure 3. Photosynthesis- A (a), stomatal conductance- g_s (b), leaf internal (C_i) and external (C_a) CO₂ concentration ratio- C_i/C_a (c) and transpiration- E (d) of young eucalyptus clones under N doses in nutrient solution. ^{ns}, ^{wa}, ^o, * and **, represent, respectively, not significant, without adjustment and significant at 10, 5 and 1 % by F test (p<0.05). Cl (clone) and D (dose).

The decrease in values of g_s can restrict the availability of CO_2 , with a consequent decrease in their concentrations in the substomatal cavities and intercellular spaces (Daley et al., 1989). The g_s limitation is commonly considered the main factor in the reduction of photosynthesis (Reddy et al., 2004). However, it has been observed maintaining the internal CO_2 concentration, even with the g_s limitation (Warren et al., 2007). The small changes in C_i/C_a (Figure 3c), observed in this study, were not prejudicial to A (Figure 3a) and total dry matter (Figure 1d), perhaps because these alterations occurred in small magnitude to the point of not interfering significantly in the other variables evaluated, addition to have increased the values of g_s (Figure 3b). Mattiello et al. (2009) also observed that C_i/C_a remained practically unaltered (between 0.7 to 0.8) in eight eucalyptus clones growing in Clark's solution with added B doses, emphasizing that the substrate (CO_2) was not limiting to A .

The clones differed ($p < 0.05$) for all leaf macronutrients evaluated and clone VM-01 showed the highest values (Figure 4). Differences in nutritional efficiency among eucalyptus clones have also been observed by other authors (Neves, 2000; Lima et al., 2005; Pinto et al., 2011) and, according to Furtini Neto (1994), this fact suggests the possibility of selection and adaptation of eucalyptus genotypes to different soil conditions, specifically those with low natural fertility (Pinto et al., 2011).

The application of N resulted in an increase in their leaf concentration according to square root model (Figure 4a). The highest values of N leaf concentration found in clone VM-01 indicate its greater demand for this nutrient. In comparison to treatment without N (dose 0), applying a small amount of this nutrient (0.74 mmol L^{-1} of NH_4NO_3) was enough to elevate by approximately 2.5 times its concentration in leaves of clone VM-01, and 3 times in leaves of clone I-144. Furlani (2004) comments that plants grown in low N concentrations have light green color, characterized by generalized chlorosis, which is accentuated, especially in older leaves, because N is a mobile element within plants. In our experiment, such symptoms were observed in plants grown at doses of 0 and 0.74 mmol L^{-1} of NH_4NO_3 , from the second week of treatment application. The increase in N leaf concentration (Figure 4a) was accompanied not only by an increase in A (Figure 3a) but also by the total dry matter (Figure 1a), with the N application in solution, for both clones. However, as the clone VM-01 had higher N leaf concentration but lower total dry matter production, we can infer that it has lower utilization efficiency of N, as also verified by Pinto et al. (2011). Nitrogen deficiency causes lowest synthesis of chlorophyll and this situation does not

allow the plant using sunlight as an energy source in the photosynthetic process, thus the plant loses the ability to perform essential functions such as nutrients uptake (Dechen & Natchtigall, 2007).

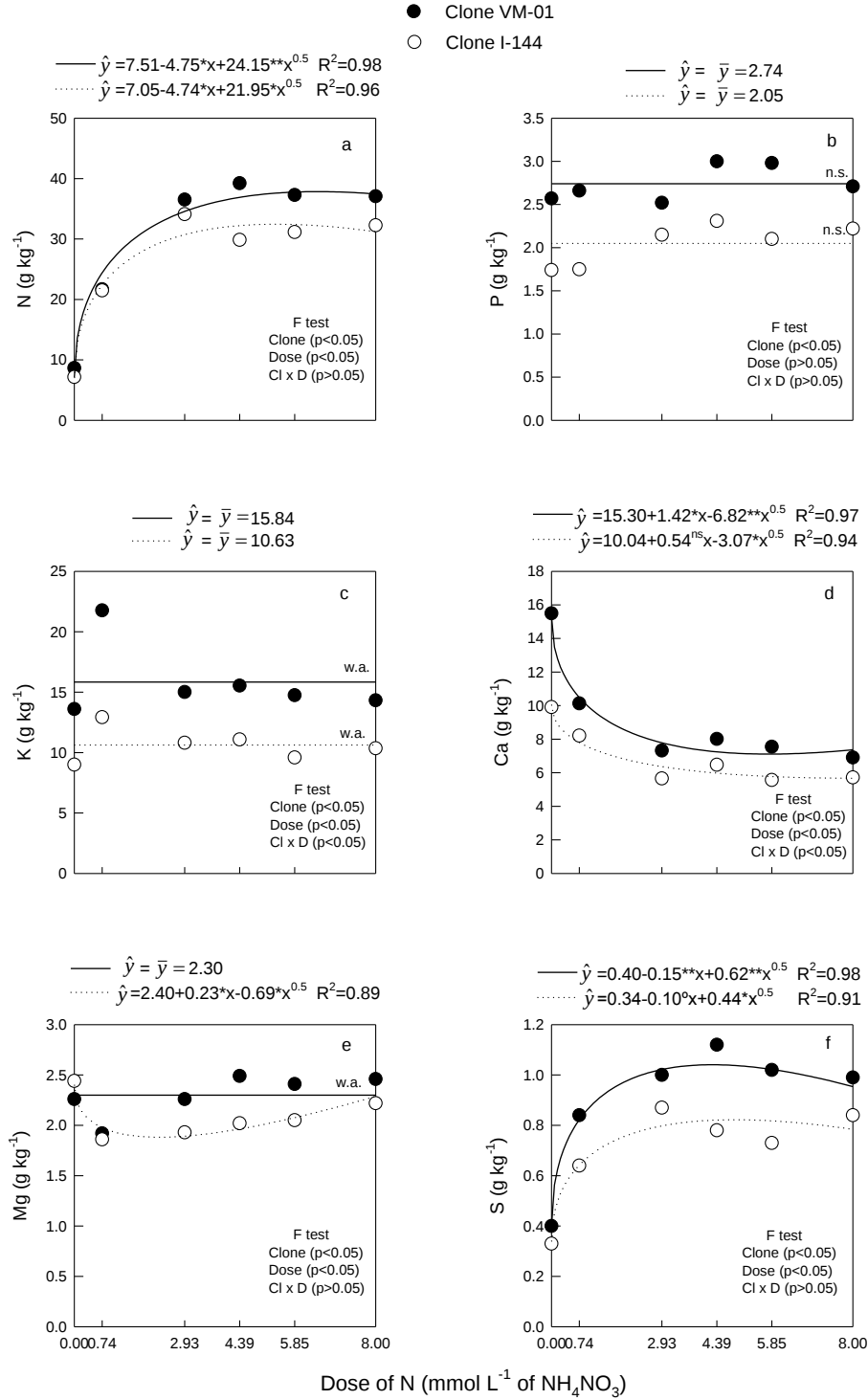


Figure 4. Concentration of N (a), P (b), K (c), Ca (d), Mg (e) and S (f) in leaves of young eucalyptus clones under N doses in nutrient solution. ^{ns}, ^{wa}, ^o, * and **, represent, respectively, not significant, without adjustment and significant at 10, 5 and 1 % by F test (p<0.05). Cl (clone) and D (dose).

Similar behavior to N leaf concentration was observed for S leaf concentration, i.e., an increase in their concentrations with increasing N doses in solution (Figure 4f). Like N, most of the S is incorporated into proteins, there being a stoichiometry between these two nutrients (Malavolta et al., 1997). The interaction between N and S seems to occur primarily in the uptake and translocation, since the greater N availability contributes to raise the S concentration in plant shoot (Barney Jr. & Bush, 1986; Alvarez V. et al., 2007), thus justifying the similar tendencies of their concentrations verified in this study. Unlike leaf N and S, Ca leaf concentrations were reduced with increasing N doses (Figure 4d). Partly, this fact could be explained by preferential uptake of N as NH_4^+ by eucalyptus (Barros & Novais, 1996). Although the clones have absorbed the two forms of mineral N (NH_4^+ and NO_3^-), with increasing N doses in solution occurred relatively greater predominance of uptake as NH_4^+ (Pereira et al., 2012) and so there was a greater competition between NH_4^+ and Ca^{2+} ions, in favor of the more-concentrated NH_4^+ . Consequently, lower amounts of Ca were absorbed and accumulated in the leaves of the clones. In bean and corn, for example, there is a reduction in the Ca concentration with increasing of NH_4^+ concentration (Souza & Fernandes, 2006). Vitti et al. (2006) report that the N as NH_4^+ , in high concentrations in the cultivation medium, reduces Ca uptake by the roots. The higher leaf Ca in the absence of N in solution may also have been an effect of Ca concentration, since the production of dry matter of plants was much lower in this situation (Figure 1).

The N doses did not influence P leaf concentration, and clone VM-01 showed the highest concentrations of this nutrient. Probably, P concentrations observed in this study were also the result of the use of double its dose as adapted by Caldeira et al. (1994) for the cultivation of eucalyptus in nutrient solution. Although K leaf concentration have been influenced by N doses in solution, there were no adjustments (w.a.) to models tested (Figure 4c). Potassium plays an important role in regulating the osmotic potential of plant cells (Marschner, 2012), contributing to the stomatal closure and opening (Dechen & Nachtigall, 2007). Thus, plants that had the lowest K concentration (dose 0 of NH_4NO_3) also had lower values of A , g_s and E (Figures 3a, b and d, respectively), thereby contributing to a lower total dry matter production (Figure 1d), since that its deficiency does not allow the stomata to open fully or close fast, causing limited control over the loss of water from plants (Dechen & Nachtigall, 2007).

It was also observed without adjustment (w.a.) behavior in Mg concentration of clone VM-01, whereas its concentration was reduced in the presence of added N in

clone I-144 (Figure 4e). In spite of, except to Mg concentration in the treatment without N, it was observed that there is an almost linear increase of its concentration with increasing of N doses to both clones, following the increase of N concentration (Figure 4a) and A (Figure 3a). This is because the Mg, along with N, plays a role as a structural component of the chlorophyll molecule and thus is essential to photosynthesis (Marschner, 2012). To clone I-144 at the dose 0 of N, there was a peak in uptake of Mg, which was fitted a square root model. In this situation there was no likely competition between the NH_4^+ and Mg^{2+} ions in solution since there was no N in solution, thus favoring the uptake of Mg. The leaf concentration of Mg may also have been favored by a concentration effect, as result of lower shoot dry matter (Figure 1a), as also occurred in Ca leaf concentration (Figure 4d).

Lastly, the N doses in solution influenced leaf concentration of all micronutrients evaluated (Figure 5). Boron concentration did not significantly fit to regression models tested, with means of 22 mg kg^{-1} to clone VM-01 and 21 mg kg^{-1} to clone I-144 (Figure 5a). Nunes (2010), working with B supply ($15 \text{ } \mu\text{mol L}^{-1}$) in nutrient solution, near the B concentration in the current study ($19 \text{ } \mu\text{mol L}^{-1}$ of B), obtained mean concentrations (new and old leaves) of 47 and 59 mg kg^{-1} of B to sensible and tolerant eucalyptus clones to water stress, respectively. Although the characteristic symptom of B deficiency is the necrosis of young leaves and terminal buds, plants deficient in B may exhibit a wide variety of symptoms, depending on species and age (Taiz & Zeiger, 2004). However, visual symptoms of B deficiency were not observed in plants of this study, perhaps the cultivation time did not allow its manifestation. There was a reduction of Mn leaf concentration with increased N dose (quadratic and square root models), being that both clones showed a minimum value of 102 mg kg^{-1} of Mn (Figure 5d). The Mn inhibits the uptake of Fe and has its uptake inhibited by Mg (Malavolta et al., 1997) so that, in general, plants with lower Mn leaf concentration also have higher Mg concentration (Figure 4e) and Fe (Figure 5c).

Leaf micronutrients Cu, Fe, Mo and Zn, were observed increase in their concentrations with application of N doses in solution (Figures 5b, c, e and f, respectively). Apart from the Cu (quadratic adjustment) and Mo (w.a.) concentrations in clone VM-01, the concentrations of these same nutrients in clone I-144 and Fe and Zn concentrations in both clones showed square root adjustment.

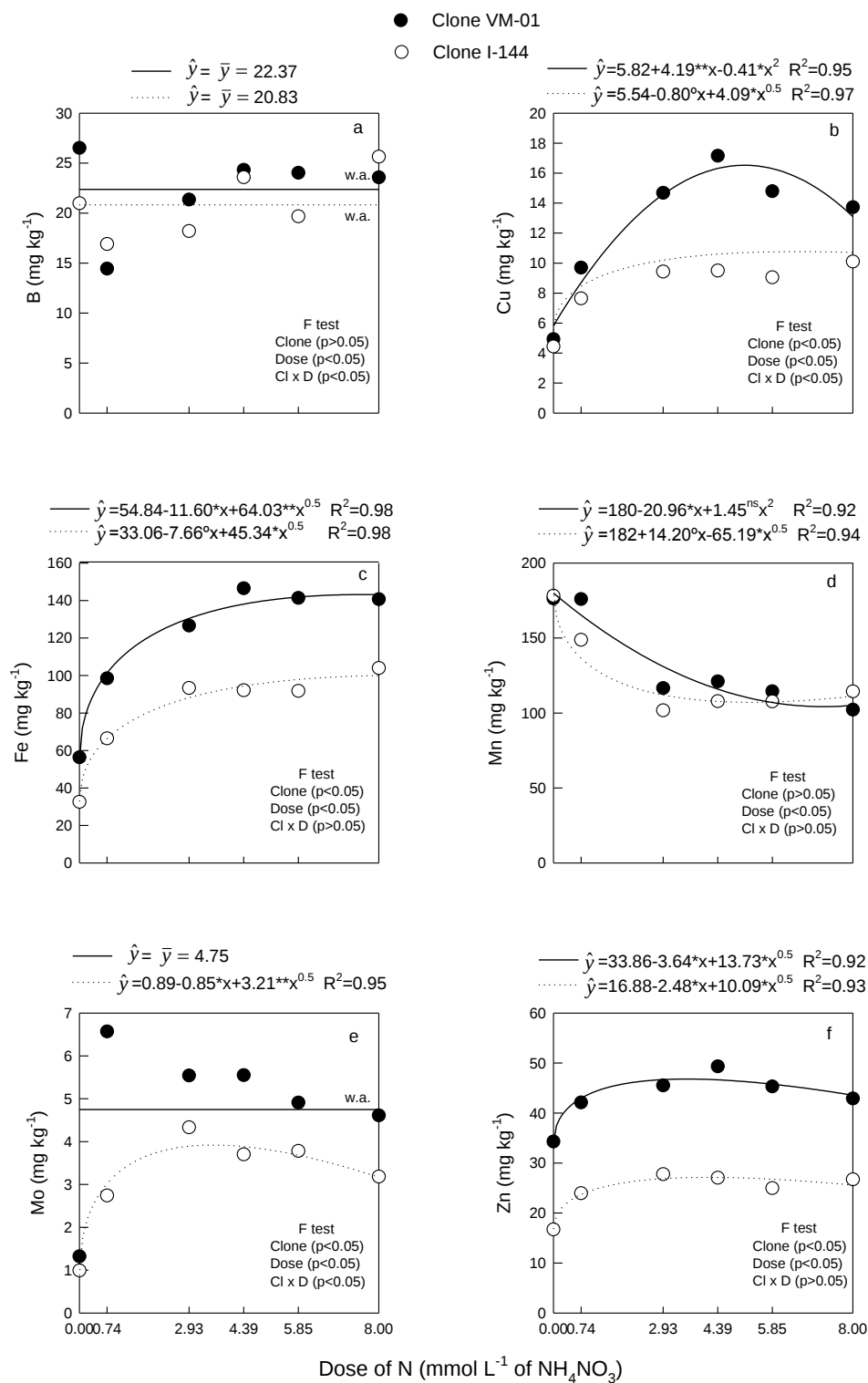


Figure 5. Concentration of B (a), Cu (b), Fe (c), Mn (d), Mo (e) and Zn (f) in leaves of young eucalyptus clones under N doses in nutrient solution. ^{ns}, ^{wa}, ^o, * and **, represent, respectively, not significant, without adjustment and significant at 10, 5 and 1 % by F test (p<0.05). Cl (clone) and D (dose).

It stands out that clone VM-01 showed higher values (p<0.05) of these four micronutrients. These results indicate that the clone VM-01 does not absorb only more N but, in general, it also absorbed more other nutrients evaluated in comparison to clone

I-144. Whereas the concentrations of Cu and Mo are above the range considered optimal for normal growth of eucalyptus (Malavolta et al., 1997), the concentrations of Fe and Zn (except for the clone VM-01) are below these ranges. One of the roles of Mo is related to the nitrate reductase, which catalyzes the reduction of NO_3^- to NO_2^- (Marschner, 2012). When there is deficiency of this micronutrient, N accumulates as NO_3^- in the plant, so that the lack of Mo has similar effect to the lack of N (Dechen & Nachtigall, 2007). By contrast, were found high concentrations of Mo, corroborating to the highest concentrations of NH_4^+ found in plants this experiment (Pereira et al., 2012). However, toxic effects of Mo were not observed in both eucalyptus clones, because, according to Dechen & Nachtigall (2007), although the plants require small amounts of this micronutrient ($<1.0 \text{ mg kg}^{-1}$ of Mo), large amounts of molybdate can be absorbed without toxic effects. While the P stimulates the uptake of Mo, it can also induce Zn deficiency in plants, because in high concentrations it decreases the uptake of this micronutrient (Malavolta et al., 1997). This may explain the very high Mo and low Zn leaf concentrations observed in this study, since it was used twice the P concentration in the nutrient solution, as adapted by Caldeira et al. (1994) to eucalyptus seedlings. Corroborating with this fact, even without adding Zn in Clark's nutrient solution, were found mean concentrations of 75 mg kg^{-1} of Zn in the seedlings shoot of *E. maculate* and *E. urophylla* (Soares et al., 2001).

CONCLUSIONS

- The clone VM-01 had greater height and height/collar diameter, while the clone I-144 showed greater production of root and total dry matter, greater root/shoot, collar diameter, and greater g_s and E ;
- The leaf concentrations of all macro and of Cu, Fe, Mo and Zn differed between eucalyptus clones, with highest values in clone VM-01;
- The eucalyptus clones VM-01 and I-144 exhibited a differential behavior in relation to N supply and the clone I-144 despite lower N leaf concentration, it showed, in general, better growth compared to clone VM-01, thus having highest utilization efficiency of this nutrient.

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CAPÍTULO 3 – Redutase do nitrato e glutamina sintetase como indicadoras do status de nitrogênio em clones de eucalipto

Resumo- Atividades enzimáticas têm sido propostas como indicadores mais sensíveis do status de nitrogênio (N) em plantas que o teor total deste nutriente, a exemplo da atividade da redutase do nitrato (RN) e da glutamina sintetase (GS). O objetivo deste trabalho foi avaliar as atividades da RN e da GS como indicadoras do status de N, em substituição ao teor foliar de N total, em clones de eucalipto cultivados em diferentes condições de N. Para tanto, desenvolveram-se um experimento em casa de vegetação e outro em campo. No primeiro caso, o experimento foi conduzido em solução nutritiva de Clark (1975) adaptada por Locatelli et al. (1984) e constituiu-se de um fatorial 2 x 6 em delineamento de blocos casualizados com cinco repetições, sendo dois clones de eucalipto (VM-01 e I-144) e seis doses de N (0; 0,74; 2,93; 4,39; 5,85 e 8 mmol L⁻¹ de NH₄NO₃). O experimento de campo foi composto por um fatorial 2 x 2 x 2, sendo duas regiões (Pompeu e João Pinheiro-MG), duas épocas de amostragem (seca e chuvosa) e dois clones de eucalipto (VM-01 e I-144) em delineamento inteiramente casualizado com cinco repetições. Foram avaliados os teores foliares de N total, proteínas totais e a atividade das enzimas RN e GS. O clone I-144 absorveu menos N (e sintetizou menos proteína), porém reduziu e assimilou mais o mesmo (maior atividade da RN e da GS), o que se converteu em maior produção de matéria seca, comparativamente ao VM-01, em casa de vegetação. A atividade das referidas enzimas foi negativamente influenciada pelo maior suprimento de N, provavelmente por um efeito inibidor tanto do NO₃⁻ (substrato da RN) como do NH₄⁺ (substrato da GS) em altas concentrações. Tanto em casa de vegetação, como em campo, as mais altas atividades da RN e GS estiveram associadas à menor disponibilidade de N no meio de cultivo das plantas. A atividade dessas enzimas apresentou também correlação negativa com o teor foliar de N total dos clones de eucalipto cultivados em casa de vegetação, com destaque para RN.

Palavras-chave: atividade enzimática, GS, N total, RN.

INTRODUÇÃO

A primeira etapa do processo de assimilação do N é a redução do NO₃⁻ a NO₂⁻ no citoplasma, pela ação da enzima redutase do NO₃⁻ (RN), com a utilização de dois elétrons transferidos pelas coenzimas NADH ou NADPH. Posteriormente, o NO₂⁻ é

translocado para os cloroplastos nas folhas e nos plastídeos nas raízes, onde é reduzido pela redutase do NO_2^- (RNi) a NH_4^+ , com a transferência de seis elétrons doados pela ferredoxina reduzida (Hay & Porter, 2006; Souza & Fernandes, 2006). Como o NH_4^+ pode ser tóxico às plantas, por ser capaz de dissipar o gradiente de prótons transmembrana, necessário ao transporte de elétrons na fotossíntese e na respiração, bem como para a captura de metabólitos nos vacúolos, as plantas o convertem a aminoácidos (Taiz & Zaiger, 2004). Dessa forma, o NH_4^+ é convertido a glutamina e glutamato no plastídeo e, ou, no cloroplasto pelo sistema das enzimas glutamina sintetase/glutamato sintase (GS/GOGAT) (Lea et al., 1990). A GS combina o NH_4^+ com o glutamato para a síntese da glutamina (Forde & Lea, 2007).

O teor foliar de N total é tradicionalmente usado na análise de rotina na maioria dos laboratórios para estimar o status de N de plantas, mas relatos indicam uma limitação de seu uso como indicador nutricional, como uma limitada resposta a alto suprimento de N em árvores (Alva et al., 1998), além de sua insensibilidade à demanda sazonal de uso do nutriente (Chapin et al., 1982). Tem sido verificado que o teor foliar de N total também não discrimina entre sítios responsivos e não responsivos para *Eucalyptus globulus* e *E. grandis* (Perdomo et al., 2007).

Assim, atividades enzimáticas têm sido propostas como indicadores mais sensíveis do status de N em plantas. Na década de 1970 já era sugerido que deficiências nutricionais em plantas poderiam ser diagnosticadas por medidas de atividade enzimática (Bar-Akiva, 1971), como também observado posteriormente (Tavdgiridze & Putkaradze, 1991; Lavres Junior et al., 2010). Martinez et al. (1999) comentam que uma das vantagens do diagnóstico metabólico é sua alta sensibilidade, já que pequena variação no conteúdo do nutriente implica alta variação no conteúdo do metabólito. No entanto, esses autores enfatizam que não há normas, nem métodos de determinação universalmente aceitos, o que indica a relevância e necessidade de mais pesquisas.

Dessa maneira, medidas da atividade da RN são usadas como ferramenta de avaliação do status nutricional de N em gramíneas tropicais (Lavres Jr. & Monteiro, 2006). A RN é citada como importante marcador bioquímico para deficiência de N (Srivastava & Singh, 2005). Esta enzima é passível de indução, sendo o aumento de sua atividade verificado após suprimento de NO_3^- às plantas e a redução da mesma em níveis baixos de luz (Souza & Fernandes, 2006). A atividade da RN na lâmina foliar recém-expandida pode constituir uma ferramenta adicional para avaliação do status de N e predizer requerimento inicial do nutriente, pois a mesma manteve correlação

positiva e significativa com o teor foliar de N total e rendimento do capim mombaça (*Panicum maximum* Jacq.) (Lavres Junior. et al., 2010). Adicionalmente, a RN é uma enzima usada com grande potencial para melhorar a qualidade da água de ambientes poluídos com NO_3^- , pela conversão deste a NO_2^- (Campbell & Campbell, 1998). Souza & Fernandes (2006) relatam que, normalmente, a atividade da RN é alta nas folhas, porém, conforme Campbell (1999), algumas plantas têm pouca ou nenhuma atividade dessa enzima neste órgão, havendo maior atividade nas raízes. Entretanto, atividade da RN nas folhas foi sempre maior que nas raízes de *E. camphora* e *E. ovata* (80 %) (Granger et al., 1994).

Souza & Fernandes (2006) citam a GS como a principal enzima na assimilação do NH_4^+ pelas plantas. A atividade da GS pode variar sob distintas condições de N (Magalhães et al., 1995). Majerowicz et al. (2002) encontraram em 30 dias de avaliação que a atividade da GS na parte aérea das plantas de *Catasetum fimbriatum* foi positivamente correlacionada com teores de proteína solúvel. Existe correlação positiva observada entre rendimento de grãos e atividade da GS (Gallais & Hirel, 2004). Em milho e trigo, a atividade da GS é representativa do status de N da planta (Hirel et al., 2007).

Diante do exposto, objetivou-se avaliar as atividades enzimáticas da RN e da GS como indicadoras do status de N, em substituição ao teor foliar de N total, em clones de eucalipto cultivados em diferentes condições de N.

MATERIAL E MÉTODOS

Este estudo constituiu-se de um experimento em casa de vegetação e outro em campo. No primeiro caso, o ensaio foi conduzido no Departamento de Solos da Universidade Federal de Viçosa, Viçosa-MG (20°45'14" S, 42°52'53" W), durante três meses (julho a setembro de 2011). Para o experimento em campo, foram utilizadas áreas de plantios comerciais de eucalipto em Minas Gerais pertencentes à empresa Vallourec & Mannesmann Tubes.

O experimento conduzido em casa de vegetação constitui-se de um fatorial 2 x 6, sendo dois clones de eucalipto (VM-01 e I-144) e seis doses de N (0; 0,74; 2,93; 4,39; 5,85 e 8 mmol L⁻¹ de NH_4NO_3), com cinco repetições em delineamento de blocos casualizados. O clone VM-01 é um híbrido de *E. urophylla* vs *E. camaldulensis* e o I-144 é um híbrido de *E. urophylla* vs *E. grandis*, sendo utilizadas mudas em tubetes com idade aproximada de 50 dias para ambos os clones. Utilizou-se solução nutritiva de

Clark (1975) adaptada por Locatelli et al. (1984) para manter a relação $N-NH_4^+/N-NO_3^-$ de 1 e o dobro da concentração de P (Caldeira et al., 1994). A solução nutritiva foi mantida com aeração permanente e seu pH foi ajustado diariamente a 5,5 ($\pm 0,05$) com soluções ($0,1 \text{ mol L}^{-1}$) de NaOH ou H_2SO_4 , sendo feita a sua troca semanalmente. O primeiro mês de condução deste experimento representou a fase de aclimação das mudas na solução nutritiva, onde as plantas permaneceram em bandejas coletivas (11 L) em que aumentaram-se, gradualmente, as concentrações da solução a cada sete dias (25, 50, 75 e 100 % da concentração original). Transcorrido esse período, foram selecionadas plantas homogêneas quanto à altura e ao vigor e foram transplantadas duas por vaso (6 L), representando assim uma unidade experimental, onde permaneceram por dois meses com a aplicação dos tratamentos. Ao final do período experimental, coletaram-se folhas no terço médio de cada planta, sendo feita a pesagem e envolvendo as mesmas em papel Al e colocando-as em N líquido até serem armazenadas em freezer (-80°C) no laboratório e liofilizadas para as análises de proteína e atividades enzimáticas (RN e GS). Posteriormente, as plantas foram colhidas (separando-as em raízes, caule, galhos e folhas), lavadas em água desionizada e secas em estufa de circulação forçada de ar ($60^\circ \text{C}/72 \text{ h}$) para a avaliação da matéria seca. Em seguida, as folhas foram passadas em moinho Wiley para a determinação dos teores de N total.

Para o experimento em campo, utilizou-se um fatorial $2 \times 2 \times 2$ com cinco repetições em delineamento inteiramente casualizado, sendo duas regiões (municípios de Pompeu e João Pinheiro), duas épocas de amostragem (seca e chuvosa) e dois clones de eucalipto (VM-01 e I-144). Os solos da região de Pompeu ($18^\circ 53' \text{ S}$ e $45^\circ 02' \text{ O}$) e João Pinheiro ($17^\circ 30' \text{ S}$ e $46^\circ 07' \text{ O}$) apresentaram, respectivamente, na camada de 0 a 1 m de profundidade valores médios de 4,85 e 4,79 de pH (em água), 2,25 e 1,14 dag kg^{-1} de matéria orgânica (Walkley-Black), 5,55 e 6,37 mg dm^{-3} de P e 28 e 10 mg dm^{-3} de K (Mehlich 1) e 74 e 18 % de argila (Ruiz, 2005). O solo da região de Pompeu (argiloso) apresentou 0,08 dag kg^{-1} de N total (método Kjeldahl), enquanto na região de João Pinheiro o solo estava com 0,04 dag kg^{-1} de N total na referida camada. A amostragem da época seca foi realizada em set./2011 e fev./2012 representou a amostragem da época chuvosa, sendo as mesmas realizadas no final de cada época. Os clones de eucalipto estavam, no momento da primeira amostragem (set./2011), com idade aproximada de 17 meses em ambas as regiões. A parcela útil foi composta de 30 plantas (cinco linhas com seis plantas cada) em 225 m^2 , de acordo com o espaçamento de plantio de $3 \times 2,5 \text{ m}$ ($1333 \text{ plantas ha}^{-1}$). Em Pompeu, as adubações de N-P-K constaram-se de 360 kg ha^{-1}

(10-27-10, no plantio), 240 kg ha⁻¹ (23-00-21, sete meses após o plantio- m.a.p.) e 350 kg ha⁻¹ (23-00-21, 18 m.a.p.). Para as parcelas de João Pinheiro, aplicaram-se 300 kg ha⁻¹ (10-27-10, no plantio), 250 kg ha⁻¹ (23-00-23, sete m.a.p.) e aos 18 m.a.p. a mesma quantidade e fórmula utilizadas em Pompeu. As áreas também receberam 2,5 (Pompeu) e 2,0 (João Pinheiro) t ha⁻¹ de calcário, 0,8 t ha⁻¹ de gesso e adubação foliar de 9 L ha⁻¹ de borato de amônio quelatado (1,22 kg ha⁻¹ de B, aplicação aérea). Para a região de Pompeu, também foi realizada aplicação no solo de 4 kg ha⁻¹ de B na forma de ulexita (10 % de B).

Foram medidas as circunferências à altura do peito (CAP-1,30 m) de todas as árvores de cada parcela (30 plantas), realizando-se o cálculo da média e do desvio padrão. Posteriormente, foram escolhidas cinco árvores representativas dentro desse intervalo. Dessas cinco árvores, uma foi utilizada para a coleta de folhas. Essas foram coletadas em número de 60/planta, na parte mediana da copa, do meio para as pontas dos galhos (somente folhas completamente desenvolvidas), de dois a três galhos de diferentes lados da planta, das 8 as 12 h. Para a amostragem de fev/2012, foi escolhida outra árvore para a coleta das folhas em cada parcela, visto que poderia haver interferência da amostragem anterior (set/2011), pois as plantas tiveram muitos galhos danificados. O procedimento de armazenagem das folhas para a análise de proteína e atividades enzimáticas e de secagem e moagem para análise dos teores de N total foi o mesmo utilizado para o material de casa de vegetação.

Análises de laboratório

Analizaram-se os teores foliares de N total pelo método Kjeldahl (Bremner, 1996), após mineralização com ácido sulfúrico e aquecimento das amostras. Para as análises de proteínas totais e as atividades enzimáticas *in vitro* da RN (EC 1.6.6.1.) e GS (EC 6.3.1.2), extratos foram obtidos pela maceração de $\approx 0,2$ g de folhas (sem a nervura principal) em N líquido e homogeneizados em meio (2 mL) com tampão tris-HCl 0,1 mmol L⁻¹, pH 8,1, NiSO₄ 4 mmol L⁻¹ e glutathione reduzida 20 mmol L⁻¹ (Cambraia et al., 1989), mais fluoreto de fenilmetilsulfonil-PMSF (1 mmol L⁻¹) e 0,2 g de polivinilpolipirrolidona (PVPP). Os extratos brutos foram filtrados em quatro camadas de gaze e centrifugados (15.000 g/15 min. a 4 °C), usando-se o sobrenadante para as respectivas determinações. Para a quantificação de proteínas totais (Bradford, 1976) foram utilizados 30 μ L do extrato, completando-se o volume para 500 μ L com água, e 2,5 mL da solução de Bradford. As leituras das amostras (em duplicata) foram feitas em

absorvância de 595 nm e os teores de proteínas totais foram determinados de acordo com a curva de calibração com albumina de soro bovino (BSA).

Para a avaliação da atividade da RN, alíquotas de 0,3 mL do extrato enzimático foram adicionadas a 1,7 mL de um meio de reação constituído de tampão tris-HCl (100 mmol L⁻¹), pH 7,5, KNO₃ (20 mmol L⁻¹), NADH (0,1 µmol L⁻¹) e triton X-100 (15 mmol L⁻¹), sendo a mistura incubada a 30 °C por 5 min. com agitação. Após este tempo, a reação foi interrompida pela adição de 2 mL de sulfanilamida 1 % (v/v) em mistura com N-naftil etilenodiamino 0,01 % (v/v) em HCl 1,0 mol L⁻¹. O nitrito produzido foi determinado por espectrofotometria a 540 nm. Os teores de NO₂⁻ nas amostras foram estimados com base em curva de calibração preparada com padrões de NO₂⁻ e a atividade enzimática da RN foi expressa em µmol de NO₂⁻ h⁻¹ mg⁻¹ de proteína.

No caso da determinação da atividade da GS, 0,1 mL do extrato enzimático foi adicionado a um meio de reação (0,9 mL) que continha tris-HCl (100 mmol L⁻¹), pH 7,5, 2- mercaptoetanol (10 mmol L⁻¹), MgSO₄.7H₂O (20 mmol L⁻¹), NH₂OH.HCl (15 mmol L⁻¹), ATP (10 mmol L⁻¹) e L-glutamato (50 mmol L⁻¹). Esse meio foi incubado a 30 °C/5 min. com agitação e após a reação foi paralisada pela adição de 1 mL da solução de cloreto férrico (0,3 mol L⁻¹ de FeCl₃, 0,67 mol L⁻¹ de HCl e 0,2 mol L⁻¹ de ácido tricloroacético) e as amostras foram centrifugadas (15.000 g/7 min. a 4 °C). Foi realizada a leitura em espectrofotômetro (540 nm) para determinar a formação do quelato Fe-L-glutamil- γ- hidroxamato (GHA). A atividade da GS foi expressa em µmol de GHA h⁻¹ mg⁻¹ de proteína. Durante todos os procedimentos as amostras foram mantidas em gelo e protegidas da luz até o momento das leituras.

Análises estatísticas

Os resultados foram submetidos à análise de variância (teste F) e quando o efeito das fontes de variação e, ou, interação entre as mesmas nas variáveis respostas foi significativo (p<0,05) realizaram-se ajustes de equações de regressão (experimento de casa de vegetação). Também foram feitas análises de correlação (coeficiente de Pearson- r) dos teores de proteína e atividade da RN e da GS com o suprimento de N (doses) e o teor foliar de N total. Para verificação dos efeitos dos tratamentos nas variáveis do experimento em campo, adotou-se o teste F com significância de 10 % (p<0,10). Para tanto, utilizou-se o programa estatístico SAS (2004) e o software Sigmaplot para confecção dos gráficos.

RESULTADOS E DISCUSSÃO

Houve aumento dos teores protéicos foliares do clone VM-01 com o aumento das doses de N em solução, atingindo um pico nas doses intermediárias, conforme modelo quadrático (Figura 1a). Houve baixa correlação ($r=0,38$) entre esses teores e as doses de N em solução para os dois clones (Tabela 1). Apesar de modelos diferentes (quadrático e raiz quadrático), os teores de proteína tiveram tendência similar aos teores foliares de N total (Figura 2a), com destaque para o clone VM-01 ($r=0,70$), e também aos de produção de matéria seca das plantas (Figura 2b). Como o N absorvido é incorporado na planta como aminoácidos e, com o incremento do seu fornecimento, as proteínas sintetizadas promovem o crescimento foliar, aumentando a área fotossintética (Dechen & Nachtigall, 2007), há maior produção de matéria seca da planta. Para o clone I-144 não houve diferença ($p>0,05$) entre os teores foliares de proteína, sendo estes menores quando comparados aos do clone VM-01 (Figura 1a).

Geralmente verifica-se aumento na atividade da RN com o aumento do suprimento de N na forma nítrica, com o teor foliar de N total ou com a produtividade em diferentes espécies vegetais, como em capim Mombaça (Lavres Junior et al., 2010), mandioca (Cruz et al., 2004), café (DaMatta et al., 1999; Reis et al., 2007), espécies herbáceas (Granger et al., 1994) e até mesmo em *E. grandis*, *E. regnans* e *E. obliqua* (Caldeira et al., 1994; Adams & Attiwill, 1982). Contrariamente, a atividade da RN nas folhas de ambos os clones avaliados foi maior na ausência de N em solução e diminuiu com o aumento das doses aplicadas deste nutriente, de acordo com modelo raiz quadrático (Figura 1b). Assim, houve correlação inversa entre a atividade da RN e o suprimento de N ($r=-0,49$) e o teor foliar de N total ($r=-0,70$) (Tabela 1). Kumar & Singh (2002) também observaram em híbridos de milho durante junho a setembro mais alta atividade da RN com baixo acúmulo de N. A atividade da RN é regulada por vários fatores ambientais e intracelulares, como luz, concentração de compostos nitrogenados, CO_2 , Mo, Fe, fitohormônios e metabólitos de C (Lea & Leegood, 1995). A luz tem efeito que pode ser direto, ativando a enzima, ou indireto, pela fotossíntese, fornecendo energia para assimilação do NO_3^- (Smirnoff et al., 1984). Ainda, segundo estes últimos autores, a atividade dessa enzima também é influenciada pela concentração de NH_4^+ .

Tanto a síntese como a atividade da RN são induzidas pela presença do substrato (Adams & Attiwill, 1982; Somers et al., 1983), assim uma redução na absorção do NO_3^- provocaria diminuição na atividade da enzima. Contudo, até folhas de mandioca deficientes em N, em que não foram detectáveis teores foliares de NO_3^- , apresentaram

atividade da RN (Cruz et al., 2004). De acordo com Campbell (1999), o NO_3^- recém absorvido e transportado para a parte aérea parece ser mais determinante para induzir a atividade da RN que o pool de NO_3^- que está principalmente armazenado no vacúolo, em vez de no citoplasma, onde ocorre a primeira fase de redução do NO_3^- .

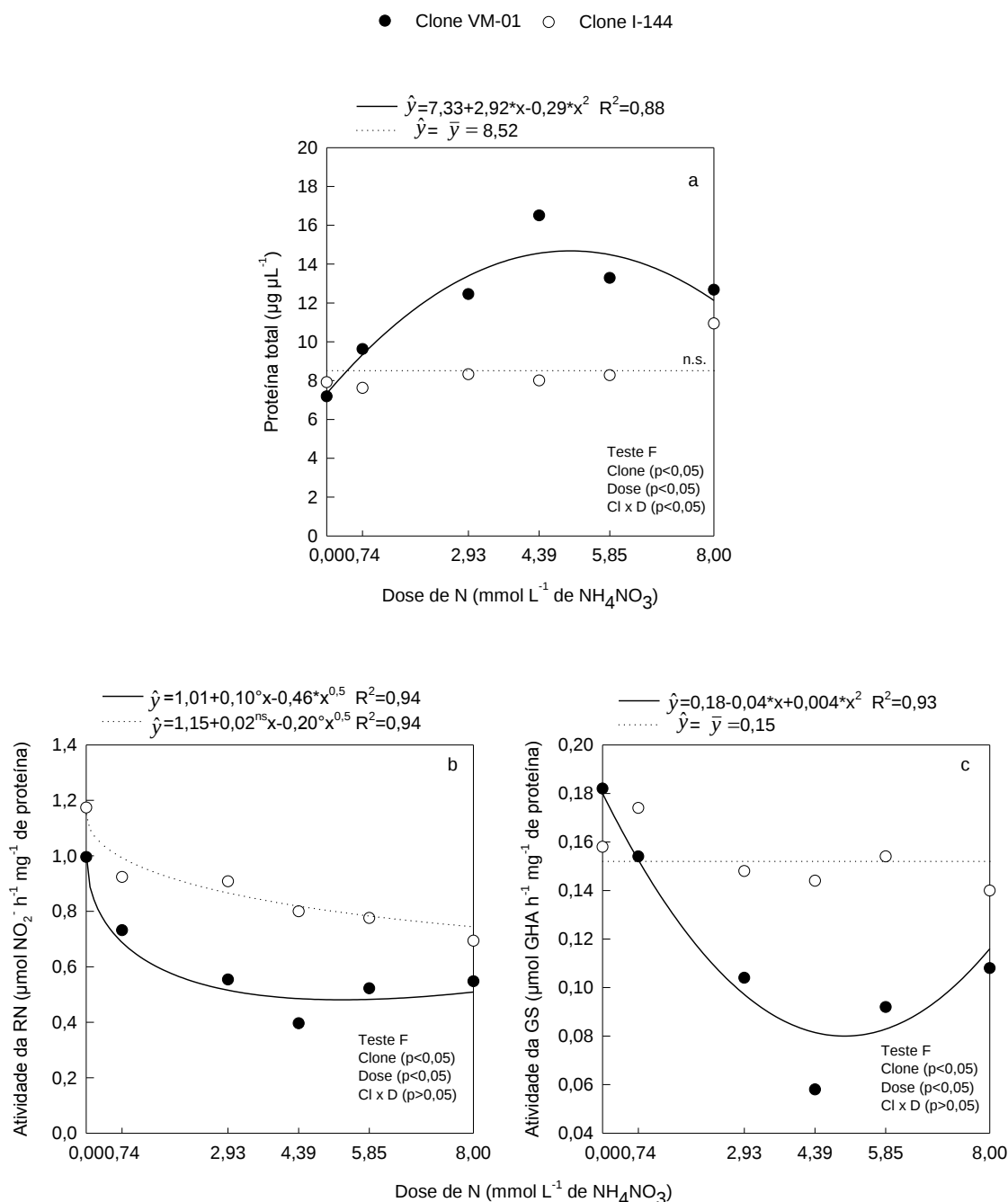


Figura 1. Teores de proteína total (a) e atividades da redutase do NO_3^- -RN (b) e da glutamina sintetase-GS (c) em folhas de clones jovens de eucalipto cultivados com doses de N em solução nutritiva. ^{ns}, ° e *, representam, respectivamente, não significativo e significativo a 10 e 5 % pelo teste F ($p<0,05$). Cl (clone) and D (dose).

Tabela 1. Coeficiente de correlação de Pearson (r) de suprimento de N (doses) e teor foliar de N total com proteína total e atividade das enzimas redutase do nitrato (RN) e glutamina sintetase (GS) em clones de eucalipto crescidos em solução nutritiva

Clone	Proteína		RN		GS	
	vs		vs		vs	
	Dose de N	Teor de N	Dose de N	Teor de N	Dose de N	Teor de N
	-----r-----					
VM-01	0,52	0,70	-0,58	-0,79	-0,50	-0,64
I-144	0,32	0,24	-0,53	-0,62	-0,18	-0,19
Geral*	0,38	0,54	-0,49	-0,70	-0,34	-0,48

* Coeficiente de correlação de Pearson (r) considerando os dados de ambos os clones.

O rápido transporte de NO_3^- para fora do citoplasma explica as quedas de atividade da RN quando o suprimento externo deste ânion é diminuído, mesmo quando o teor total de NO_3^- na planta é elevado (Fernandes & Souza, 2006). Queiroz et al. (1993) relatam que o influxo de NO_3^- desempenha um papel mais relevante na indução da atividade da RN que o NO_3^- previamente armazenado no vacúolo. Foi verificado decréscimo na atividade da RN em mudas de pupunheira (*Bactris gasipaes*) em altas concentrações de NO_3^- no meio externo, indicando efeito deletério do excesso de substrato sobre a atividade da enzima (Oliveira et al., 2005). A adição de concentrações mais altas de NO_3^- também reduziu a atividade da RN em algas (Awasthi, 2005; Chow et al., 2007).

Com relação aos teores de NH_4^+ , altos valores podem ter efeito retroinibidor na atividade desta enzima (Delu-Filho, 1994). Esse autor verificou que no período da tarde houve baixa atividade da RN nas raízes de seringueira e atribuiu tal fato a uma possível retroinibição da enzima causada pelo acúmulo de NH_4^+ neste órgão, consequente da redução na translocação deste cátion para a parte aérea provocada pela diminuição do fluxo transpiratório, decorrente do maior fechamento estomático das plantas. Dessa forma, com o aumento das concentrações de NH_4^+ em solução, pelas maiores doses de N aplicadas, promovendo maiores teores foliares na forma amoniacal (capítulo 1), houve redução na atividade da RN. Isto se torna relevante, principalmente para espécies

de eucalipto que, em geral, apresentam absorção preferencial (Barros & Novais, 1996) e altos teores de NH_4^+ (capítulo 1).

Corroborando com tal observação, em raízes de seringueira foi verificado que o NH_4^+ aparentemente teve forte efeito repressor na atividade da RN, mesmo em plantas que foram cultivadas em doses de NO_3^- próximas do ótimo (6 mmol L^{-1} de NO_3^- e 2 mmol L^{-1} de NH_4^+) (Lemos et al., 1999). De acordo com Redinbaugh & Campbell (1993), essa repressão da atividade da RN nas raízes pode ser devida ao acúmulo de NH_4^+ ou de outros compostos nitrogenados, que estariam inibindo a síntese ou a atividade dessa enzima (Lewis et al., 1982).

A enzima RN é uma flavoproteína com alto peso molecular formada por duas subunidades idênticas, com três grupos FAD, heme e um cofator molibdênio (coMo) (Chow et al., 2007). Assim, os altos teores de Mo verificados nas folhas dos clones cultivados nas maiores doses de N (capítulo 2) poderiam também ter influenciado negativamente a atividade da enzima, mesmo sabendo que a deficiência deste nutriente reduz a sua atividade (Smirnoff et al., 1984). O K também tem ação importante na ativação das enzimas de assimilação de N quando o NH_4^+ está em concentrações tóxicas nos tecidos vegetais (Souza & Fernandes, 2006).

E, por último, um outro fator que também pode estar influenciando, embora em menor grau, a maior atividade da RN nas menores doses de N aplicadas, é que a mesma está expressa em função dos teores de proteínas. Isto porque, pelo menos para o clone VM-01, houve menores teores protéicos nas plantas cultivadas nas menores doses de N (Figura 1a), em função dos menores teores foliares de N total (Figura 2a), fazendo com que houvesse aumento na relação (menor valor do denominador) contribuindo assim para maior valor da atividade.

Também, como hipótese, a verificação de maior atividade da RN nas menores doses de N poderia indicar que plantas com menores teores foliares de N total apresentariam maior eficiência desta enzima, como, a princípio, um comportamento de compensação nesta condição. Este argumento é reforçado ao se comparar a atividade da RN dos dois clones, em que o clone I-144 que possui menores teores foliares de N total foi justamente o que apresentou as maiores atividades desta enzima e produção de matéria seca (Figuras 1a e 2). Dessa forma, a atividade da RN apresentou tendência inversa tanto ao N da solução (doses) quanto aos teores foliares totais deste nutriente (Tabela 1).

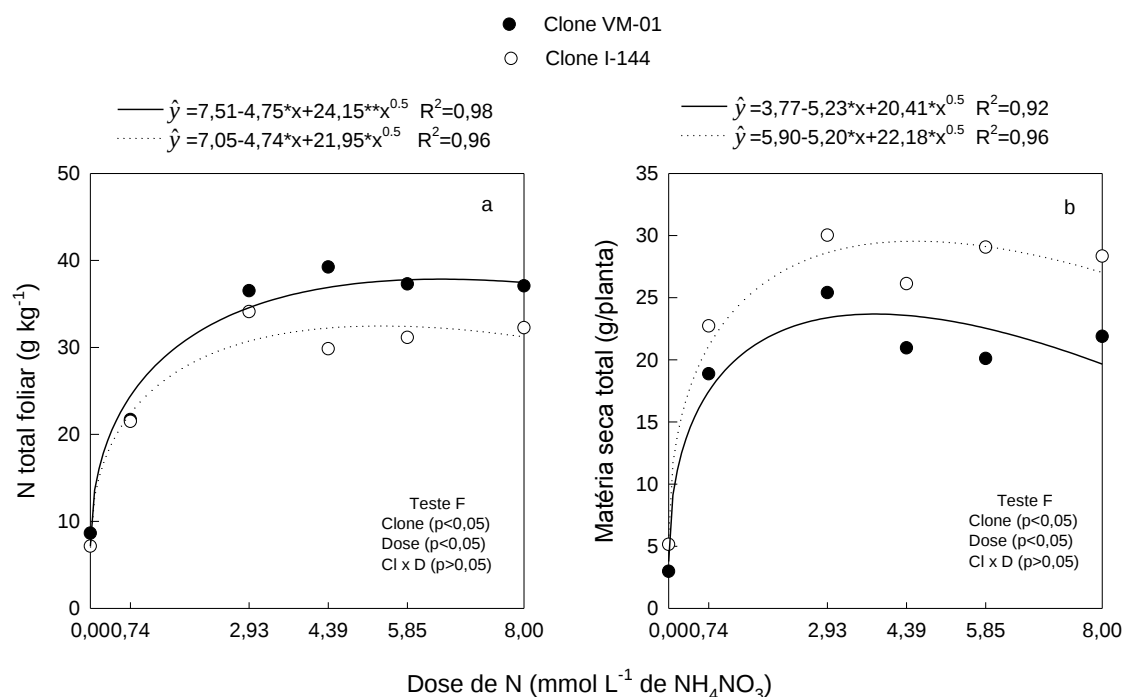


Figura 2. Teores foliares de N total (a) e matéria seca total (b) de clones jovens de eucalipto cultivados com doses de N em solução nutritiva.* e **, representam, respectivamente, significativo a 5 e 1 % pelo teste F (p<0,05). Cl (clone) and D (dose).

Para a atividade da GS do clone VM-01 também houve maiores valores nas menores doses de N, diminuindo com o aumento das mesmas, seguindo o modelo quadrático (Figura 1c). Este fato poderia ser explicado pelo maior fornecimento de NH₄⁺ como substrato para GS, como consequência da maior redução do N nessas condições. No entanto, deve-se salientar que o NH₄⁺ nos vegetais não é formado apenas pela redução do NO₃⁻, mas também por outros processos, como pela biosíntese de lignina, catabolismo de aminoácidos, quebra de proteínas em tecido senescente e também pela fotorrespiração (Marschner, 2012), além da sua absorção direta pelas plantas. Quantidade de NH₄⁺ produzido durante a descarboxilação da glicina para produção de serina sob condições fotorrespiratórias chega a ser até dez vezes maior que a quantidade produzida durante a redução do NO₃⁻ (Lea & Blackwell, 1992). Menores atividades da GS são resultado de um efeito regulatório que atua em condições de baixo fornecimento de C e, ou, N para a planta, mantendo a enzima na sua forma inativa, como alternativa para conservação de ATP (Delu-Filho, 1994). O NO₃⁻ exerceu efeito positivo sobre a atividade da GS em folhas de seringueira (Delu-Filho, 1994), como também verificado por Purcino (1992) com cultivares de milho sobre a concentração da GS. Entretanto, o aumento da concentração de NH₄⁺ na solução nutritiva contribuiu para

reprimir a atividade da GS nas raízes de seringueira (Lemos et al., 1999). Os teores foliares de NH_4^+ dos clones de eucalipto do presente estudo tiveram aumento linear com o incremento das doses de N (capítulo 1). Dessa maneira, nas maiores doses de N, os mais altos teores foliares deste cátion podem ter influenciado negativamente a atividade da enzima, sugerindo um efeito inibitório sobre a mesma.

Para o clone I-144, não houve diferença ($p < 0,05$) para a atividade da GS, como também verificado para os seus teores protéicos (Figuras 1a e c). A GS catalisa a conversão do glutamato em glutamina, com a utilização de NH_4^+ , ATP e um cátion divalente (Mifflin et al., 1981), promovendo assim a síntese do aminoácido. Por outro lado, este clone apresentou, de forma geral, maiores atividades da GS em relação ao clone VM-01, o que não refletiu em maiores teores de proteína. Enfatiza-se que a GS representa apenas de 1 a 2 % do total de proteínas solúveis em órgãos capazes de assimilar o NH_4^+ (Hungria et al., 1992). Assim, maiores atividades da GS não necessariamente significam maiores teores de proteínas, como verificado na comparação dos dois clones, visto que existem inúmeras outras enzimas e fatores envolvidos na síntese protéica das plantas. A exemplo da atividade da RN, porém com menores valores, observou-se correlação inversa da atividade da GS com o suprimento de N em solução ($r = -0,34$) e com os teores foliares de N total ($-0,50$) (Tabela 1).

Para os clones cultivados em campo, foi verificado maior teor total de proteína foliar no clone VM-01 (Figura 3a), a exemplo do observado no experimento de casa de vegetação (Figura 1a), ocorrendo menores valores na primeira condição em que eram plantas mais velhas (aproximadamente 17 meses) e cultivadas em campo. Quando se compararam as plantas das regiões de solo arenoso e argiloso em cada época de avaliação (seca e chuvosa), os seus teores de proteína foliar não diferiram ($p > 0,10$, Figura 3b), mesmo o solo argiloso possuindo o dobro do teor de N total. Destaca-se que não somente a presença de maior teor de N no solo significará sempre maior absorção do nutriente e síntese de proteínas pelas plantas, visto que estes processos são dependentes de vários fatores, como disponibilidade de C, energia, atividade de várias enzimas e até condições climáticas como temperatura e chuva.

Por outro lado, tanto no solo argiloso quanto no arenoso, a época chuvosa contribuiu para maiores teores de proteína total foliar (Figura 3c). Maior decomposição da matéria orgânica do solo, pela maior atividade microbiana resultante de uma maior umidade nos solos nesta época, em função de maiores precipitações pluviais, pode ter

favorecido uma maior absorção de N (capítulo 1) pelas plantas e assim contribuir para uma maior síntese protéica.

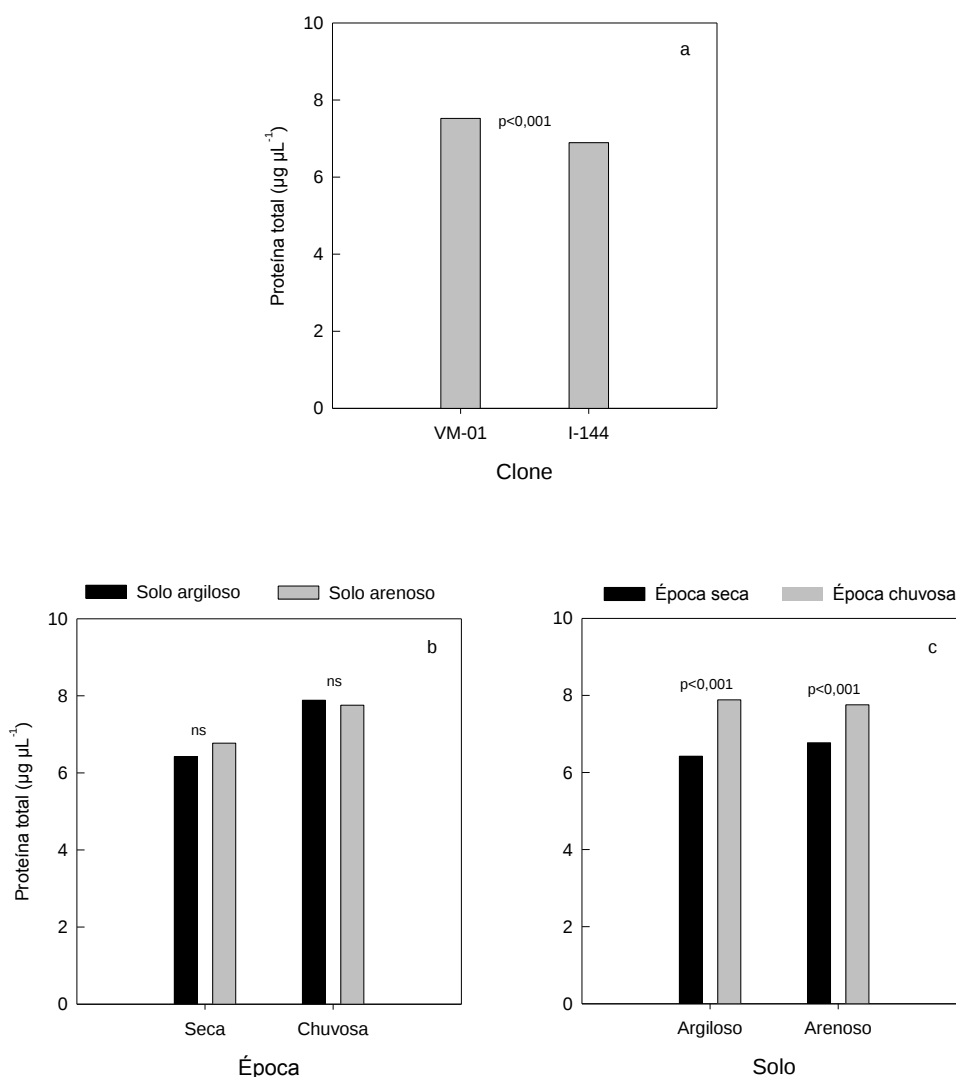


Figura 3. Teores de proteína total foliar comparando clones de eucalipto (a), regiões de diferentes solos para cada época de amostragem (b) ou diferentes épocas para cada solo (c). ^{ns} representa não significativo a 10 % pelo teste F ($p > 0,10$).

A atividade da RN verificada nos clones de eucalipto cultivados no campo (média de $0,90 \mu\text{mol de NO}_2^- \text{ h}^{-1} \text{ mg}^{-1}$ de proteína, Figura 4a) é considerada moderada (Campos, 2009). A atividade da RN é drasticamente influenciada em condições de elevada acidez (Towsend, 1970), podendo este fato ser um indicativo de menores atividades da RN em eucalipto, visto que esta espécie, de maneira geral, está associada a solos ácidos (Gama-Rodrigues et al., 2005), onde também o processo de nitrificação é reduzido (Moreira & Siqueira, 2002). Os clones não diferiram ($p > 0,10$) quanto à atividade da RN foliar (Figura 4a), sendo, portanto, neste caso sua atividade insensível

para detectar as diferenças no status de N entre os clones, visto que eles diferenciaram nos teores foliares de N total (capítulo 1). Porém, este fato poderia ser vantajoso, já que permitiria a utilização de um indicador de status de N para esta espécie, independentemente do clone avaliado. Martinez et al. (1999) apontam que a dificuldade na aplicação de métodos enzimáticos vem a ser o fato de que a variação na atividade de determinada enzima é influenciada por outros fatores que não somente o nutriente em estudo. A atividade da RN foi maior nas plantas cultivadas em região de solo arenoso na época chuvosa, em relação as de solo argiloso, não havendo diferenças ($p>0,10$) na época da seca (Figura 4b), contrariamente ao teor de N total do solo que foi maior no solo argiloso. Este fato indica novamente, como observado em casa de vegetação (Figura 1b), a maior atividade da enzima em plantas cultivadas em ambientes de menor disponibilidade de N.

Corroborando para esse indicativo, também observou-se maior atividade da RN na amostragem realizada na época da seca para as plantas de ambas as regiões, em que novamente os teores de N total do solo eram menores (Tabela 3- capítulo 1, análise estatística não apresentada). Granger et al. (1994) observaram que a atividade da RN variou grandemente tanto entre espécies de plantas como entre épocas de amostragem, sendo que espécies herbáceas geralmente têm maior atividade que lenhosas. Foi observada variação da atividade da RN conforme a idade da planta (Carelli et al., 1990), com redução da atividade, tanto nas raízes como nas folhas, com o aumento da idade de pupunheira (*Bactris gasipaes*) (Oliveira et al., 2005). Dessa maneira, a atividade da referida enzima avaliada na segunda amostragem (época chuvosa) poderia também ter sido influenciada pela maior idade das plantas.

A atividade da RN pode apresentar flutuações sazonais em resposta a fatores internos e ambientais (Beevers & Hageman, 1969), a exemplo da ausência de atividade da enzima verificada em folhas de café acompanhada pelo declínio da temperatura (abaixo de 12,5 °C) registrado no inverno (DaMatta et al., 1999). A atividade da RN é também fortemente influenciada pela disponibilidade de água no solo (Oliveira et al., 2005). Casarino (2009) também verificou maiores valores de atividade da RN em meses mais chuvosos em espécies arbóreas. A autora sugere que a redução na atividade da enzima no período seco se deve à baixa disponibilidade de NO_3^- em solução, uma vez que a umidade do solo é mais baixa. A diminuição da transpiração causa menor influxo de NO_3^- , podendo influenciar a atividade da enzima (Plhak, 2003). A síntese da RN pode ser inibida mesmo com breve período de balanço hídrico negativo (Hsiao, 1973).

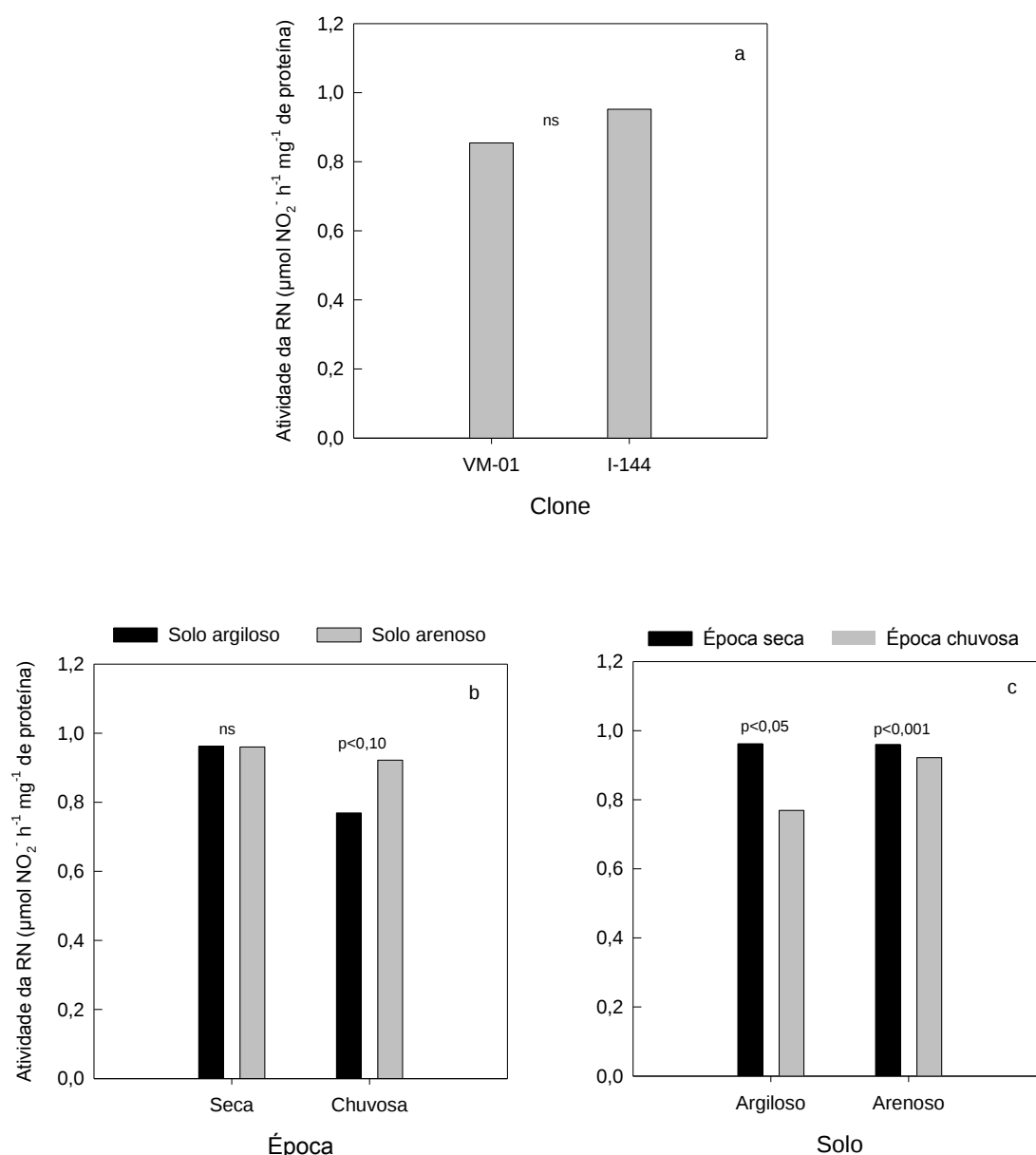


Figura 4. Atividade da redutase do nitrato (RN) foliar comparando clones de eucalipto (a), regiões de diferentes solos para cada época de amostragem (b) ou diferentes épocas para cada solo (c). ^{ns} representa não significativo a 10 % pelo teste F ($p > 0,10$).

Reis et al. (2007) observaram resposta positiva da atividade da RN às doses de N de fevereiro a março e atribuíram tal fato à maior precipitação pluvial nesse período, uma vez que a sua atividade também pode ser regulada pela água. Esta última, além de outros fatores, estimula a proteína fosfatase, que desfosforila vários resíduos de serina na proteína RN, promovendo a ativação da enzima (Taiz & Zeiger, 2004). Resultados sugeriram forte relação entre a redução da atividade da RN e do potencial de água nas folhas, podendo estar relacionado à maior transpiração no período de maior intensidade luminosa do dia (Oliveira et al., 2005). Maior atividade da RN foliar em cafeeiro em

janeiro foi atribuída à maior intensidade de luz que ocorre nesta época, por possuir maior comprimento do dia, aumentando a taxa fotossintética dessa espécie (Reis et al., 2007), sendo parte da produção de energia utilizada para ativar a RN (Queiroz et al., 1993). Ainda assim, mesmo com todas essas considerações a favor de maiores atividades da RN em períodos de chuva e com maior intensidade luminosa, a atividade dessa enzima dos clones foi contrariamente maior no período seco, até então sem uma justificativa conhecida para tal fato. Talvez, os menores teores de proteína verificados nas plantas na época da seca (Figura 3c), tenham elevado, em maior extensão, os valores da atividade da enzima, visto que os resultados são expressos com base em proteína e as leituras da atividade enzimática tenham sido similares, neste caso.

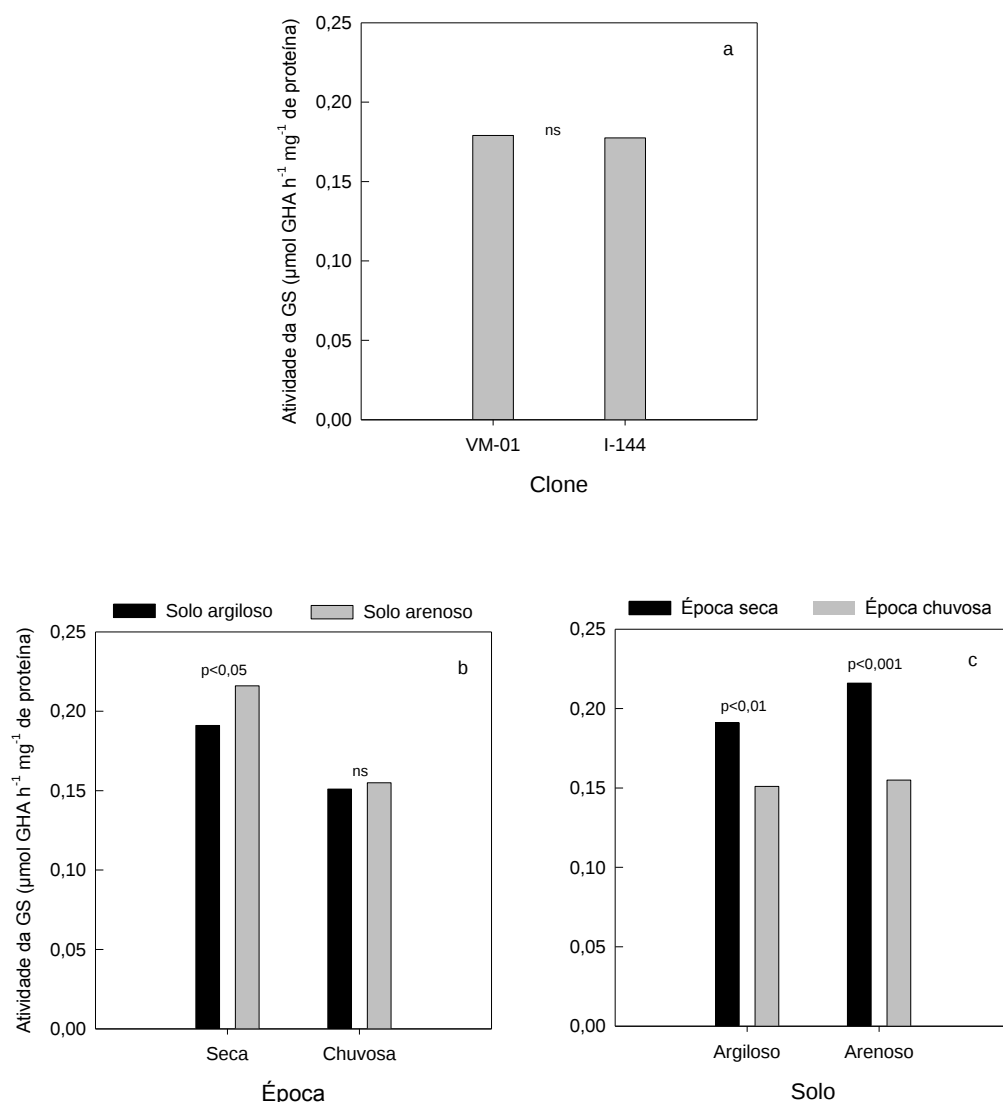


Figura 5. Atividade da glutamina sintetase (GS) foliar comparando clones de eucalipto (a), regiões de diferentes solos para cada época de amostragem (b) ou diferentes épocas para cada solo (c). ^{ns} representa não significativo a 10 % pelo teste F ($p > 0,10$).

Também como observado para a atividade da RN, não houve diferenças ($p>0,10$) para a atividade da GS entre os clones (Figura 5a). As plantas no solo arenoso, onde havia menor teor de N total do solo e da planta (capítulo 1), apresentaram a maior atividade da GS na época seca, não existindo diferenças ($p>0,10$) na época chuvosa (Figura 5b). E, ao se comparar a atividade desta enzima entre as duas épocas de amostragem verificam-se maiores valores na seca para ambas as regiões de solo argiloso e arenoso (Figura 5c). Assim, de forma geral, observa-se comportamento similar entre as atividades da RN e da GS, indicando de certa forma influência de uma sobre a outra nas rotas de assimilação do N pelas plantas, visto que o produto da RN (NO_2^-) é convertido em NH_4^+ pela ação da redutase do nitrito (RNi) e este, em última análise, será substrato para a incorporação do N em glutamina pela GS. Baixas concentrações de GS foram correlacionados com a baixa capacidade para redução do NO_3^- foliar (Stewart et al., 1990). Em síntese, de modo geral, e como consequência, a atividade dessas duas enzimas avaliadas apresentaram tendências similares no tocante à influência de diferentes condições de disponibilidade de N aos clones de eucalipto.

CONCLUSÕES

- O clone I-144 absorveu menos N (e sintetizou menos proteína), porém reduziu e assimilou mais o mesmo (maior atividade da RN e da GS), o que se converteu em maior produção de matéria seca, comparativamente ao VM-01, em casa de vegetação;
- As atividades da RN e da GS foram negativamente influenciadas pelo maior suprimento de N, provavelmente por um efeito inibidor tanto do NO_3^- (substrato da RN) como do NH_4^+ (substrato da GS) em altas concentrações;
- Tanto em casa de vegetação, como em campo, as mais altas atividades da RN e GS estiveram associadas à menor disponibilidade de N no meio de cultivo das plantas. A atividade dessas enzimas apresentou também correlação negativa com o teor foliar de N total dos clones de eucalipto cultivados em casa de vegetação, com destaque para RN.

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FINAL REMARKS

The increase of N availability in solution resulted in increase of its mineral forms (N-NH_4^+ and N-NO_3^-), its total concentration, which were accompanied by an increase in the concentration of protein, chlorophyll, stomatal conductance, transpiration and photosynthesis in the two eucalyptus clones. As consequence, there was also an increase in the production of dry matter (root, shoot and total) of both clones, which indicates synergism between the metabolisms of N and C, given the need of C skeletons for N assimilation. In general, total N, N-NH_4^+ , N-NO_3^- , leaf N/P and chlorophyll did not reflect the real soils N status in the field. Nevertheless, the leaf total N concentration was considered, although not ideal, the indicator that best represented the N availability in the different conditions (seasons, regions and clones). There were greater SPAD readings (greenhouse) for clone I-144, which absorbed less N, thus not allowing generalization of its use for different genetic materials. Although the clone I-144 absorbed less N (and synthesized less protein), it proportionately reduced and assimilated more N, which resulted in greater dry matter production, compared to VM-01, in greenhouse. The N insufficiency in the cultivation medium of plants induced higher activities of the enzymes nitrate reductase (NR) and glutamine synthetase (GS), which indicated, in principle, greater enzymatic efficiency in this condition and also a possible inhibiting effect of NO_3^- (substrate of NR) and NH_4^+ (substrate of GS) in high concentrations, in greenhouse and in the field. Activity of these enzymes also showed a negative correlation with leaf total N concentration of the clones in greenhouse, mainly for NR. It is emphasized that, unfortunately, these issues did not allow elucidating, in a concrete way, why of the low positive response of eucalyptus to N. However, we believe that this study has contributed to better understanding of the N assimilation in eucalyptus, and also that it will be an incentive to guide research on this topic. Maybe, factors as soil organic matter and presence of endophytic diazotrophic bacteria, for example, among others, can bring best explanations for the fact in question. Evaluation of other compounds as indicators such as internal cycling of amino acids may also help eucalyptus nutrition programs.