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# LEAF TOTAL NITROGEN CONCENTRATION AS AN INDICATOR OF NITROGEN STATUS FOR PLANTLETS AND YOUNG PLANTS OF EUCALYPTUS CLONES

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## ABSTRACT

The use of leaf total nitrogen concentration as an indicator for nutritional diagnosis has some limitations. The objective of this study was to determine the reliability of total N concentration as an indicator of N status for eucalyptus clones, and to compare it with alternative indicators. A greenhouse experiment was carried out in a randomized complete block design in a  $2 \times 6$  factorial arrangement with plantlets of two eucalyptus clones (140 days old) and six levels of N in the nutrient solution. In addition, a field experiment was carried out in a completely randomized design in a  $2 \times 2 \times 2 \times 3$  factorial arrangement, consisting of two seasons, two regions, two young clones (approximately two years old), and three positions of crown leaf sampling. The field areas (regions) had contrasting soil physical and chemical properties, and their soil contents for total N,  $\text{NH}_4^+\text{-N}$ , and  $\text{NO}_3^-\text{-N}$  were determined in five soil layers, up to a depth of 1.0 m. We evaluated the following indicators of plant N status in roots and leaves: contents of total N,  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_3^-\text{-N}$ , and chlorophyll; N/P ratio; and chlorophyll meter readings on the leaves. Ammonium (root) and  $\text{NO}_3^-\text{-N}$  (root and leaf) efficiently predicted N requirements for eucalyptus plantlets in the greenhouse. Similarly, leaf N/P, chlorophyll values, and chlorophyll meter readings provided good results in the greenhouse. However, leaf N/P did not reflect the soil N status, and the use of the chlorophyll meter could not be generalized for different genotypes. Leaf total N concentration is not an

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ideal indicator, but it and the chlorophyll levels best represent the soil N status for young eucalyptus clones under field conditions.

**Keywords:** *Eucalyptus*, N/P ratio, nutritional diagnosis, plant analysis, soil fertility evaluation, chlorophyll meter.

## RESUMO: CONCENTRAÇÃO FOLIAR DE NITROGÊNIO TOTAL COMO INDICADOR DO STATUS DE NITROGÊNIO PARA MUDAS E PLANTAS JOVENS DE CLONES DE EUCALIPTO

O uso da concentração foliar de nitrogênio total como indicador para diagnose nutricional apresenta algumas limitações. O objetivo deste trabalho foi determinar a confiabilidade da concentração de N total como indicador do status do nutriente para clones de eucalipto e compará-la com indicadores alternativos. Conduziu-se um experimento de casa de vegetação em delineamento de blocos ao acaso, em um arranjo fatorial  $2 \times 6$ , com mudas de dois clones de eucalipto (140 dias) e seis doses de N em solução nutritiva. Também foi conduzido um experimento de campo em delineamento inteiramente casualizado, em um arranjo fatorial  $2 \times 2 \times 2 \times 3$ , com duas épocas, duas regiões, dois clones jovens (aproximadamente dois anos) e três posições de amostragem das folhas na copa. As áreas de campo (regiões) tinham contrastantes propriedades físicas e químicas do solo e suas concentrações de N total,  $\text{N-NH}_4^+$  e  $\text{N-NO}_3^-$ , foram determinadas em cinco camadas do solo, até 1 m de profundidade. Avaliaram-se como indicadores do status de N da planta, em raízes e folhas, concentrações de N total,  $\text{N-NH}_4^+$  e  $\text{N-NO}_3^-$ , clorofila, razão N/P e leituras do medidor de clorofila nas folhas. Amônio (raiz) e  $\text{N-NO}_3^-$  (raiz e folha) previram eficientemente os requerimentos de N para as mudas de eucalipto em casa de vegetação. Similarmente, N/P foliar, valores de clorofila e leituras do medidor de clorofila forneceram bons resultados em casa de vegetação. Entretanto, N/P foliar não refletiu o status de N do solo, e o uso do medidor de clorofila não pode ser generalizado para diferentes genótipos. A concentração foliar de N total não é um indicador ideal, mas essa e os níveis de clorofila melhor representam o status de N do solo para os clones jovens de eucalipto em condições de campo.

**Palavras-chave:** *Eucalyptus*, razão N/P, diagnose nutricional, análise de planta, avaliação da fertilidade do solo, medidor de clorofila.

## INTRODUCTION

The complexity of nitrogen reactions in soils hinders reliable diagnosis of N availability to plants when based only on soil analyses that may be successfully used to evaluate the availability of other nutrients (Cantarella, 2007). Plant analysis is based on the principle that the plant itself is the best indicator of N supply in the soil (Olfs et al., 2005). A desirable N indicator should be sensitive to a wide range of N supply, and predict plant growth accordingly (Rubio-Covarrubias et al., 2009; Rambo et al., 2010). Nitrate concentration ( $\text{NO}_3^-$ -N) in leaves (Rubio-Covarrubias et al., 2009), leaf total N concentration (Faria et al., 2008; Viera et al., 2013), and chlorophyll meter readings are commonly used to assess N nutrition of plants.

Total N concentration is the most widely used indicator for evaluation of plant nutritional status regarding this nutrient, and it is a routine analysis in most laboratories (Rubio-Covarrubias et al., 2009). However, the use of this indicator for nutritional diagnosis has some limitations. Studies have shown limited response of trees in terms

of leaf total N concentration under conditions of high N supply (Graciano et al., 2006; Silva et al., 2013). Total N increases rapidly in response to N fertilization when plants are deficient; however, once N demand for growth is satisfied, N concentrations increase lightly, or do not increase at all (Rubio-Covarrubias et al., 2009). In *Eucalyptus globulus* and *E. grandis*, leaf total N did not differ between responsive and nonresponsive sites (Perdomo et al., 2007). In the *Cerrado* (Brazilian tropical savanna) region of Minas Gerais, Brazil, Harrison et al. (2000) did not observe a difference in leaf total N concentrations among *E. camaldulensis*, *E. pellita*, and *E. urophylla*. Moreover, leaf total N can be insensitive to seasonal adjustment of nutrient use by plants, since seasonal fluctuations of leaf total N concentrations in N-deficient sites are not very pronounced, as reported by Knight (1988) for *E. fastigata*. Therefore, leaf total N concentration is not sensitive for detection of differences in N supply in different sites, genotypes, and seasons.

Alternative indexes of N availability attempt to overcome the limitations of leaf total N as an indicator of N status in plants. Leaf N/P ratio has

been extensively used to guide fertilization in regard to these nutrients in eucalyptus plantations in Australia and New Zealand (Perdomo et al., 2007). This ratio has also been evaluated in Brazil (Wadt and Novais, 1999; Silveira et al., 2003).

The chlorophyll meter is a nondestructive indirect method for evaluation of plant N status in real time. Madeira et al. (2009) suggested the use of these readings to monitor N concentration in *E. globulus* (up to two years old). Total N concentration is related to chlorophyll concentration in leaves (Turnbull et al., 2007). However, the relationship between chlorophyll concentrations and SPAD values is not always linear (Fontes, 2011).

Despite many attempts, there is no consensus for standardization of a better N indicator in plants, which shows a clear need for further studies to diagnose N status. Thus, the objective of this study was to evaluate total N concentration and alternative indicators of N status in eucalyptus clones under different conditions of N supply.

## MATERIAL AND METHODS

We conducted a greenhouse experiment to evaluate the response of N availability indexes to N supply in the nutrient solution, and a field experiment to evaluate how these indexes reflect differences in soil N availability.

### Greenhouse experiment

The greenhouse experiment was carried out at the Universidade Federal de Viçosa, in the municipality of Viçosa (20° 45' S, 42° 52' W), Minas Gerais, Brazil, from July to Sept. 2011. A randomized complete block design with five replicates was used in a 2 × 6 factorial arrangement consisting of plantlets of two eucalyptus clones (VM-01, *E. urophylla* × *E. camaldulenses*; and I-144, *E. urophylla*) and six levels of N in the nutrient solution (0, 0.74, 2.93, 4.39, 5.85, and 8.00 mmol L<sup>-1</sup> of NH<sub>4</sub>NO<sub>3</sub>). We used the nutrient solution of Clark (1975), which was modified to contain a NH<sub>4</sub><sup>+</sup>-N/NO<sub>3</sub><sup>-</sup>-N ratio equal to 1 (Locatelli et al., 1984) and twice the original P concentration of Clark (Caldeira et al., 1994). The nutrient solution was maintained under permanent aeration and a pH of 5.5 ± 0.05 (Locatelli et al., 1984). 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 plants cultivation. The plants were approximately 50 days old at the beginning of the experiment, and they originated from tube plantlets. The first 30 days represented the stage of plant acclimatization in the nutrient solution, and the remaining 60 days represented the exposure time of plants to

treatments. In the first 30 days of the experiment, the plants were grown in collective plastic trays (11 L), and the concentration of the nutrient solution was gradually increased week by week (25, 50, 75, and 100 % of the original concentration). Thereafter, two homogeneous plants were selected according to their height and vigor and transplanted to individual pots filled with a 6 L nutrient solution, representing the experimental unit, where they remained for 60 days, and the nutrient solution was changed weekly using deionized water.

At the end of the experiment (90 days), chlorophyll meter readings were taken (08:00 to 10:00 a.m.) by a SPAD 502 Plus device from the two youngest fully-expanded leaves of each plant, through an average of six consecutive readings. To evaluate chlorophyll concentrations, two discs (0.78 cm<sup>2</sup> each) were collected from each plant from the leaves evaluated with the chlorophyll meter. Finally, after 90 days of cultivation of plants in the nutrient solution, remaining for 60 days in the treatments, the plants were harvested. Their leaves, stems, branches, and roots were separated and then washed with deionized water and oven-dried (60 °C, for 72 h) in order to determine dry matter (DM) production. Then, the different plant tissues were separately ground in a Willey mill to prepare them for chemical analyses.

### Field experiment

The field experiment was set up in Sept. 2011 in the State of Minas Gerais in southeastern Brazil. Details of the study, such as the regions and specific agronomic practices adopted, are presented in Ferreira (2013). Planting was performed on Apr. 15, 2010 in the municipality of Pompeu (18° 53' S, 45° 02' W), MG, and on May 03, 2010 in the municipality of João Pinheiro (17° 30' S, 46° 07' W), MG, at a spacing of 3 × 2.5 m at both sites. In Pompeu, N-P-K fertilizations consisted of 360 kg ha<sup>-1</sup> (10-27-10, at planting), 240 kg ha<sup>-1</sup> (23-00-21, seven months after planting - m.a.p.), and 350 kg ha<sup>-1</sup> (23-00-21, 18 m.a.p.). For the João Pinheiro plots, 300 kg ha<sup>-1</sup> (10-27-10, at planting), 250 kg ha<sup>-1</sup> (23-00-23, seven m.a.p.), and the same quantity and formula used in Pompeu at 18 m.a.p. were applied. The areas also received 2.5 (Pompeu) and 2.0 (João Pinheiro) t ha<sup>-1</sup> of lime, 0.8 t ha<sup>-1</sup> of gypsum, and leaf fertilization of 9 L ha<sup>-1</sup> of ammonium borate (1.22 kg ha<sup>-1</sup> of B, aerial application). For the Pompeu region, 4 kg ha<sup>-1</sup> of B in the form of ulexite (10 % B) was also applied to the soil.

We used a completely randomized design with five replicates in a 2 × 2 × 2 × 3 factorial arrangement: two evaluation seasons (dry, Sept. 2011; and rainy, Feb. 2012), two regions (municipalities of Pompeu and João Pinheiro), two young eucalyptus clones (VM-01 and I-144), and three sampling positions of the crown leaf (base, middle, and tip). Rainfall recorded for the regions of Pompeu and João Pinheiro

from May 2011 to Sept. 2011 (dry season) were 14 and 0 mm, respectively; and from Oct. 2011 to Feb. 2012 (rainy season), 846 and 1,062 mm, respectively. The areas showed contrasting soil physical and chemical properties: Pompeu has a clayey soil, classified as Oxisol; and João Pinheiro has a sandy soil, classified as Entisol (Table 1) - Latossolo Vermelho-Amarelo and Neossolo Quartzarênico, respectively, by the Brazilian Soil Classification System (Embrapa, 2013).

In both regions, plots consisted of five eucalyptus rows with six plants each, with an area of 262.5 m<sup>2</sup>. The circumference at breast height (CBH) was measured at 1.3 m above the ground with a metric tape for all trees in the plots (30) and the values were recorded and averaged and transformed into diameter at breast height (DBH = CBH/π). Subsequently, five representative trees were chosen from each plot - four of them were used as references for soil sampling, and another tree was used for leaf sampling. Soil samples were collected at the 0-10, 10-20, 20-40, 40-60, and 60-100 cm depth in eight locations (four in the row and four between the row, joined into one composite sample per depth and location) of the plots. Soil samples were air dried, ground, and sieved (2 mm mesh) before laboratory analyses. As for plant sampling, the tree crowns were measured and then divided into three parts (base, middle, and tip), and leaves were sampled (8:00 to 12:00 a.m.) at mid-height of each part. We collected only fully expanded leaves, 60 leaves for each crown position, between the middle and the tip of the branches, and from two and three branches of different sides of the plant. The leaf drying and grinding procedure was the same as used for the greenhouse experiment. We extracted two discs (0.78 cm<sup>2</sup>) from the leaves for chlorophyll analysis.

### Laboratory analyses

The leaf samples (greenhouse and field experiment) were analyzed for total N, NH<sub>4</sub><sup>+</sup>-N, NO<sub>3</sub><sup>-</sup>-N, chlorophyll content, and P concentrations. Greenhouse samples were also evaluated for root total N, NH<sub>4</sub><sup>+</sup>-N, and NO<sub>3</sub><sup>-</sup>-N. Total N was analyzed after dry matter digestion in concentrated sulfuric acid, distillation with NaOH (10 mol L<sup>-1</sup>) and

boric acid 2 % and titration with HCl (0.1 mol L<sup>-1</sup>), according to the Kjeldahl method (Bremner, 1996). The mineral N forms were extracted with 1 mol L<sup>-1</sup> KCl and determined by distillation with magnesium oxide (for NH<sub>4</sub><sup>+</sup>-N) and Devarda's alloy (for NO<sub>3</sub><sup>-</sup>-N) and titration with H<sub>2</sub>SO<sub>4</sub> (0.0025 mol L<sup>-1</sup>), according to Tedesco et al. (1995). Chlorophylls (a and b) were extracted in saturated dimethylsulphoxide (DMSO) with CaCO<sub>3</sub> in a water bath (65 °C) for 1 h (greenhouse experiment), and for 2 h (field experiment); these times were defined in previous tests. Absorbance was measured at 649 and 665 nm. Chlorophylls (a and b) were calculated according to Wellburn's equations, to obtain the total chlorophyll (Wellburn, 1994). Phosphorus was extracted in HCl (0.01 mol L<sup>-1</sup>), after sample mineralization by calcination in a muffle furnace (500 °C), and quantified in ICP-OES (Inductively Coupled Plasma - Optical Emission Spectrophotometry). Its concentrations in leaves were used to calculate the leaf N/P ratio.

Soil samples were analyzed for total N (Kjeldahl's method), NH<sub>4</sub><sup>+</sup>-N (Kempers and Zweepers, 1986), and NO<sub>3</sub><sup>-</sup>-N (Yang et al., 1998), extracted with 1 mol L<sup>-1</sup> KCl (1:10 ratio); organic C (Walkley-Black); pH in water (1:2.5 ratio); P and K levels (extracted with Mehlich-1, 1:10 ratio); texture (Ruiz, 2005); and Ca<sup>2+</sup>, Mg<sup>2+</sup>, and Al<sup>3+</sup> (extracted with 1 mol L<sup>-1</sup> KCl, 1:10 ratio).

### Statistical analyses

Data were subjected to analysis of variance (F test, at 5 %) using SAS/STAT software (SAS Institute, Cary, NC, USA). We fitted equations and chose the significant model, at 5 %, with the highest R<sup>2</sup>. We estimated concentrations of leaf critical range associated with 90 and 100 % of maximum growth, derived from equations fitted to total dry matter (total DM) plant production and total N and N/P data. We also determined Pearson correlation coefficients (r) between treatment N levels and total DM or N indexes of nutritional status of the eucalyptus clones. For the field experiment, the effects of regions (soils), sampling seasons, clones, and crown position on N indexes were compared at 10 % by the F test, and means were compared by Tukey's test.

**Table 1. Soil physical and chemical properties of the field experiment in the first sampling season (dry season, Sept 2011)**

Site <sup>(1)</sup>	pH(H <sub>2</sub> O) <sup>(2)</sup>	Clay	OC <sup>(3)</sup>	P	K	Ca <sup>2+</sup>	Mg <sup>2+</sup>	Al <sup>3+</sup>	CEC <sup>(3)</sup>
		%		mg dm <sup>-3</sup>		cmol <sub>c</sub> dm <sup>-3</sup>			
Pompeu	4.85	74	1.31	5.6	28.0	0.57	0.16	0.85	1.65
João Pinheiro	4.79	18	0.66	6.4	9.6	0.16	0.16	0.51	0.85

<sup>(1)</sup> Mean values (0-100 cm layer) of samples collected from rows and between rows; <sup>(2)</sup> pH in water (1:2.5 ratio); OC: organic carbon; Walkley-Black method; P and K (extracted by Mehlich-1, 1:10 ratio); Ca<sup>2+</sup>, Mg<sup>2+</sup> and Al<sup>3+</sup> (extracted by KCl 1 mol L<sup>-1</sup>, 1:10 ratio);

<sup>(3)</sup> CEC: cation exchange capacity (CEC = Ca<sup>2+</sup> + Mg<sup>2+</sup> + K + Al<sup>3+</sup>).

## RESULTS AND DISCUSSION

### Greenhouse experiment

The N rates increased the concentrations of total N,  $\text{NH}_4^+\text{-N}$ , and  $\text{NO}_3^-\text{-N}$  in the leaves and roots of both eucalyptus clones (Figure 1). Total N in the roots did not differ between clones; however, total N in the leaves differed, and the clone VM-01 had the highest concentrations (Figures 1a and 1b). Harrison et al. (2000) reported no differences for total N concentration in the leaves and roots of *E. camaldulensis*, *E. pellita*, and *E. urophylla* at 15, 31, and 41 months of age. However, Pinto et al. (2011) observed that the clones used in the present study had different N use efficiencies [(g of total dry matter)<sup>2</sup>/mg of N in plant]. VM-01 had lower efficiency, which indicates that it produces less DM than I-144 for the same amount of N taken up. N use efficiency (DM of trunk/N content in trunk) of interspecific hybrids destined for biomass production is a desirable trait, which promotes sustainability of forest production, especially under conditions of soil nutrient scarcity (Faria et al., 2008).

Leaf total N concentration in VM-01 was  $34.93 \text{ g kg}^{-1}$  at  $2.93 \text{ mmol L}^{-1}$  of  $\text{NH}_4\text{NO}_3$ , whereas in I-144, it was  $30.73 \text{ g kg}^{-1}$  for the same  $\text{NH}_4\text{NO}_3$  rate. Critical values for total N in VM-01 ranged from  $30.60$  to  $36.54 \text{ g kg}^{-1}$ , obtained with estimated  $\text{NH}_4\text{NO}_3$  rates of  $1.63$  and  $3.80 \text{ mmol L}^{-1}$ , respectively. For I-144, leaf critical values of total N ranged from  $28.30$  to  $32.30 \text{ g kg}^{-1}$ , with rates of  $1.90$  and  $4.55 \text{ mmol L}^{-1}$ , respectively (Figure 1b). At higher N rates, leaf total N concentration tends to stabilize after it reaches a peak value, which can be attributed to greater DM of plants in these treatments and to the dilution effect. This behavior was also verified in 24-month hybrids (*E. urophylla* x *E. grandis*) (Silva et al., 2013), and in 3-month *E. grandis* (Graciano et al., 2006). To relatively high growth rate of *E. diversicolor*, in a high-N treatment, N diluted in plant tissues (Warren et al., 2000).

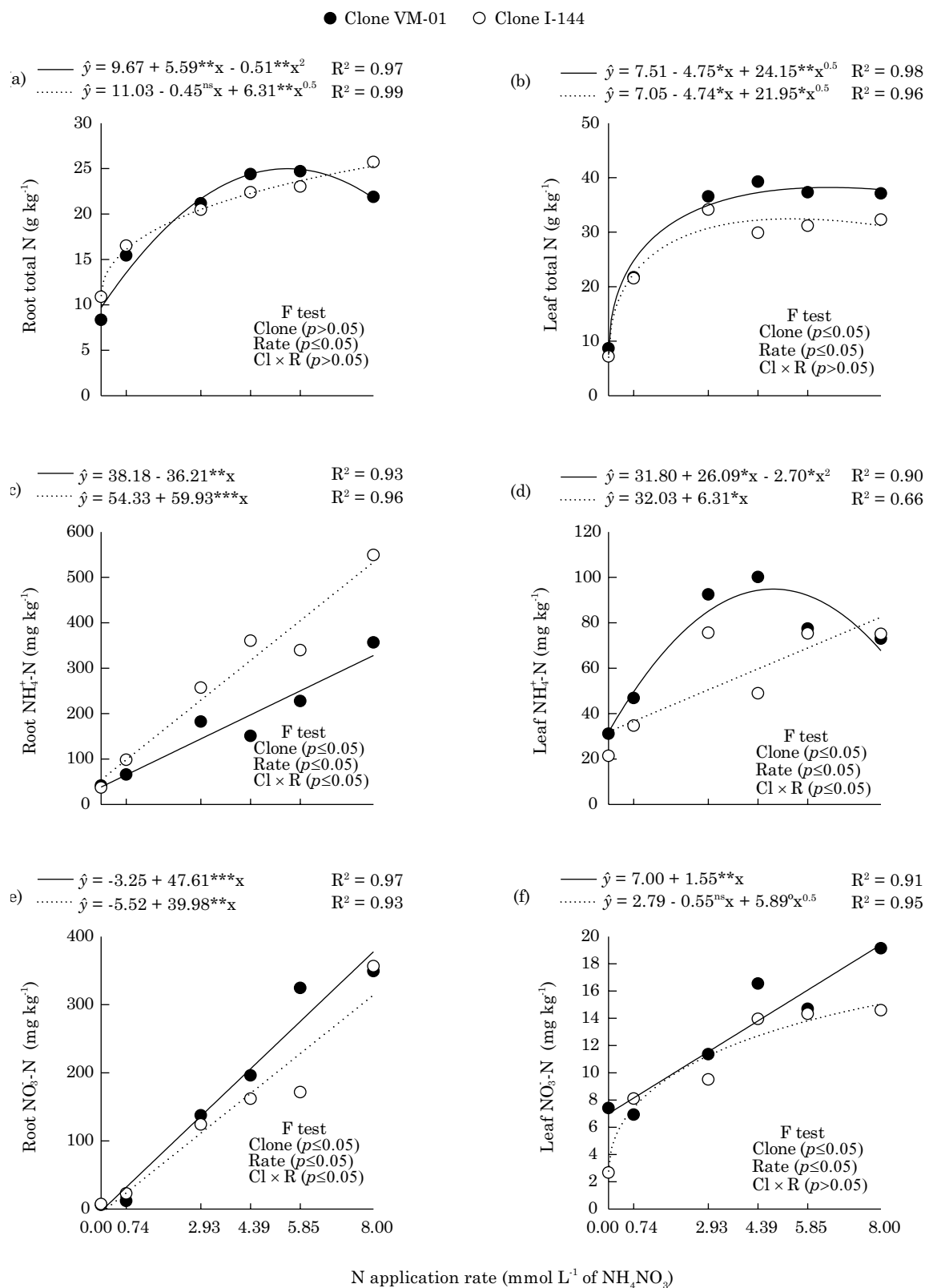
The  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  concentrations showed a linear increase in the roots of both clones, with the N rates (Figures 1c and 1e) showing a high Pearson correlation coefficient ( $r$ ) (Table 2). For total N in roots, the VM-01 clone had lower  $r$  than I-144, but, in the leaves, I-144 had a lower  $r$ , and the overall  $r$  was  $0.73$ . The overall  $r$  (both clones) was  $0.82$  for  $\text{NH}_4^+\text{-N}$ , and  $0.90$  for  $\text{NO}_3^-\text{-N}$ . For leaf  $\text{NO}_3^-\text{-N}$  concentrations, there was also good correlation (overall  $r = 0.78$ ), and a linear response for clone VM-01, and square root response for I-144 for N rates (Figure 1f). In contrast, for leaf  $\text{NH}_4^+\text{-N}$  concentrations, there was a lower  $r$  for both clones ( $0.51$  for VM-01, and  $0.64$  for I-144). A quadratic response for VM-01 and a low determination coefficient ( $R^2 = 0.66$ ) for I-144 were observed regarding  $\text{NH}_4^+\text{-N}$  in the leaves (Figure 1d). The mobile N forms showed a higher rate of leaf

accumulation than total N with an increasing N supply for nectarine (Rubio-Covarrubias et al., 2009). The authors highlight that stable N forms (total N and chlorophyll) are poor indicators under high N supply, whereas  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  concentrations were highly responsive, suggesting the usefulness of  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  concentrations as N status indicators for nectarine.

The N/P ratio in the leaf, chlorophyll level, and chlorophyll meter readings showed a similar response to N rates in the solution (Figure 2), with relatively lower  $r$  values (Table 2). There was an increase in the values of N/P ratio, chlorophyll level, and chlorophyll meter readings until reaching a peak, and then the values remained practically constant, probably due to greater DM production in plants with higher N rates (Figure 2d), as well as for total N in leaves (Figure 1b). However, I-144 had the highest values of N/P ratio in the leaf, chlorophyll level, and chlorophyll meter readings, in contrast with total N in the leaves. For N/P in the leaf, VM-01 showed a critical range from  $11.42$  to  $13.38$  for the estimated rates that provided  $90\%$  ( $1.63 \text{ mmol L}^{-1} \text{ NH}_4\text{NO}_3$ ) and  $100\%$  ( $3.80 \text{ mmol L}^{-1} \text{ NH}_4\text{NO}_3$ ) of DM production, while estimated rates of  $1.90$  and  $4.55 \text{ mmol L}^{-1} \text{ NH}_4\text{NO}_3$  were related to a critical range of  $14.70$  and  $15.90$  for I-144 (Figures 2a and 2d). These values were similar to those reported in Australia and New Zealand for *E. globulus* and *E. nitens* (Perdomo et al., 2007). The N/P ratio in the leaf increased with N fertilization in young plants of *E. grandis*, indicating that P became the limiting nutrient (Graciano et al., 2006). Wadt and Novaes (1999) analyzed the leaf N/P ratio in 18 hybrids of *E. grandis* and *E. urophylla* (80 clones) in the States of Espírito Santo and Bahia in Brazil. For *E. grandis*, the optimum N/P ratio in the leaf was defined as between  $11$  and  $18$  (Graciano et al., 2006).

For chlorophyll levels, we observed maximum values of  $51 \mu\text{g cm}^{-2}$  for VM-01 (rate of  $4.39 \text{ mmol L}^{-1} \text{ NH}_4\text{NO}_3$ ) and  $59 \mu\text{g cm}^{-2}$  for I-144 (rate of  $5.85 \text{ mmol L}^{-1} \text{ NH}_4\text{NO}_3$ ) (Figure 2b). Plants with a higher leaf total N concentration typically have higher levels of chlorophyll (Foulkes et al., 2009). Thus, for higher leaf total N concentrations (Figure 1b), there were also higher chlorophyll values. In the current study, the clone VM-01 showed high positive correlation between leaf total N concentration and chlorophyll level ( $r = 0.90$ ), chlorophyll level and chlorophyll meter readings ( $r = 0.91$ ), and leaf total N concentration and chlorophyll meter readings ( $r = 0.95$ ), whereas the clone I-144 showed low correlation between the variables ( $r = 0.63$ ,  $r = 0.65$ , and  $r = 0.59$ , respectively) (Santos et al., 2012).

Although chlorophyll meter readings represented the N rates in the solution (Figure 2c), VM-01 - which had greater leaf total N concentrations than I-144 (Figure 1b) - showed lower readings than I-144. These results indicate that it is not safe

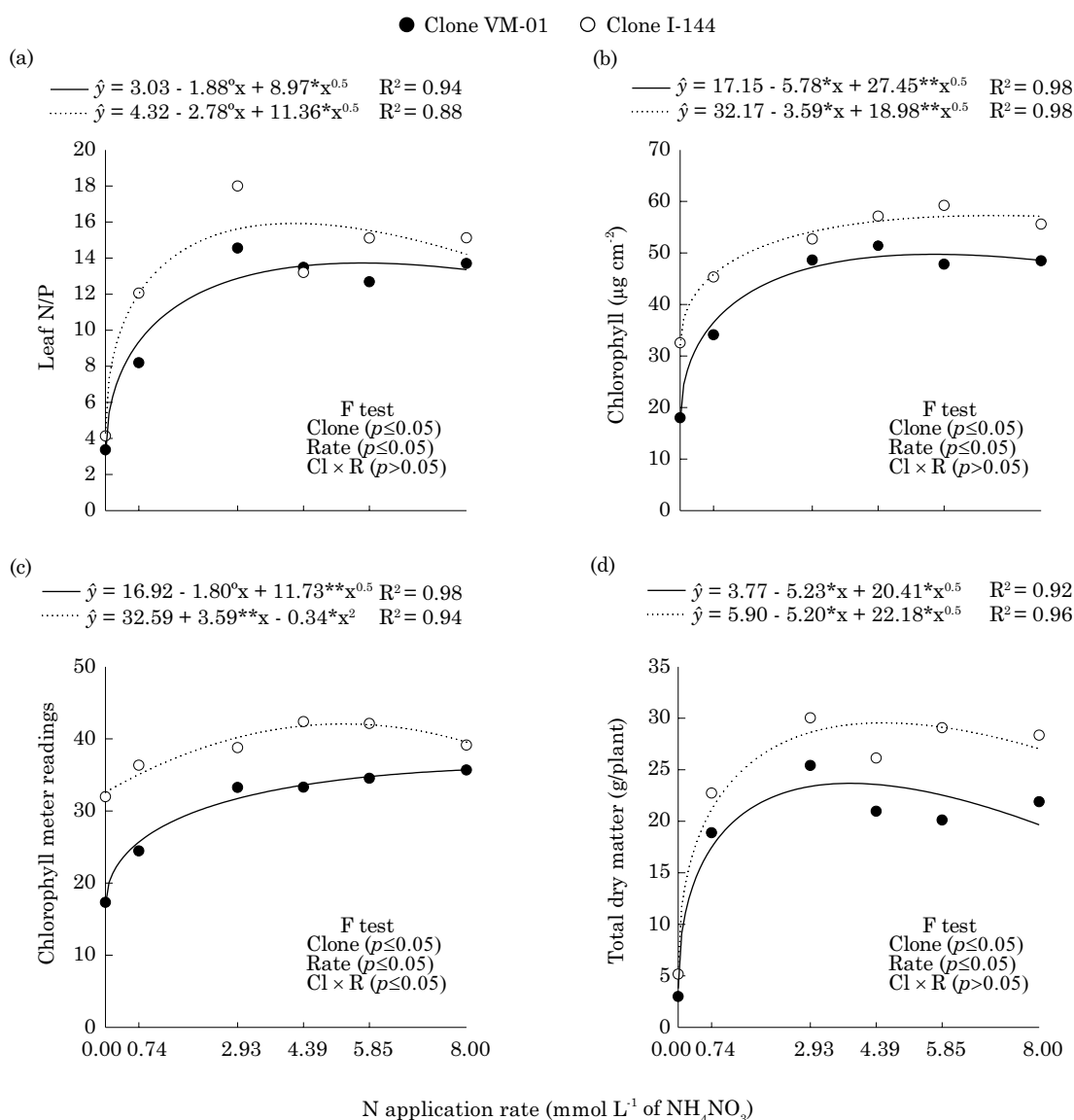


**Figure 1.** Concentrations of total N in the root (a) and in the leaf (b), NH<sub>4</sub><sup>+</sup>-N in the root (c) and in the leaf (d), and NO<sub>3</sub><sup>-</sup>-N in the root (e) and in the leaf (f) of plantlets of eucalyptus clones (140 days old) under N rates in nutrient solution in a greenhouse. ns, °, \*, \*\* and \*\*\*: not significant and significant at 10, 5, 1, and 0.1 %, respectively by the F test at 5 %. Cl (clone), and R (rate).

**Table 2. Pearson correlation coefficients among nitrogen supply (rates) and indicators of nitrogen status and total dry matter (DM) of plantlets of eucalyptus clones (140 days old) grown in nutrient solution in a greenhouse**

Clone	Total N		NH <sub>4</sub> <sup>+</sup> -N		NO <sub>3</sub> <sup>-</sup> -N		N/P	Chlorophyll	SPAD	DM
	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
Overall <sup>(1)</sup>	0.73	0.73	0.82	0.55	0.90	0.78	0.60	0.65	0.58	0.47

<sup>(1)</sup> Pearson correlation coefficient (r) considering data of both clones.



**Figure 2. Leaf N/P ratio (a), chlorophyll (b), chlorophyll meter readings (c), and total dry matter (d) of plantlets of eucalyptus clones (140 days old) under N rates in nutrient solution in a greenhouse. °, \* and \*\*: significant at 10, 5, and 1 % by the F test at 5 %. Cl (clone), and R (rate).**

to generalize information from one genotype to another. Differences between genetic materials are factors that may also influence the chlorophyll meter readings (Nielsen et al., 1995). Pinkard et al. (2006) found a species-specific relationship between chlorophyll level and the chlorophyll meter readings, which was stronger for *E. globulus* than for *E. nitens*. These differences, according to the authors, may have been a result of the differences in age between the *E. globulus* (2.5 years) and *E. nitens* (1.5 years) plants, presumably related to differences in leaf age, or also related to species differences in specific leaf area. Chlorophyll meter readings reflect the intensity of leaf green color proportional to leaf-chlorophyll concentrations (Fontes, 2011). Although N-deficient leaves are chlorotic, greater leaf total N concentrations do not always mean an increase in the green color of leaves, as observed for VM-01 and I-144. Clone VM-01 had a greater leaf total N concentration; however, it showed lower chlorophyll meter readings because of lower chlorophyll levels and, therefore, lighter green leaves. The relationship between chlorophyll concentration and chlorophyll meter readings in many species is linear, but the curvilinear nature of the relationship found for *E. globulus* and *E. nitens* suggested that chlorophyll meter readings overestimated chlorophyll at high values of chlorophyll meter readings (Pinkard et al., 2006). The non-linear shape of the relationship between chlorophyll concentration and chlorophyll meter readings agrees with effects of non-uniform distribution and multiple scattering of chlorophyll in the leaf surface that cause deviation from linearity at the two extremes (high and low) of the chlorophyll meter readings (Fontes, 2011).

Furthermore, there is not always a positive correlation between chlorophyll and N concentrations, since N is not the only constituent of the chlorophyll molecule (Dechen and Nachtigall, 2007). Thus, with the increase in N rates in the solution, N may be accumulating predominantly in other compounds instead of chlorophyll. Nitrogen consumed in excess is accumulated in plants as  $\text{NO}_3^-$  and is not incorporated into chlorophyll molecules (Fontes, 2011). Leaf total N tends to increase linearly while chlorophyll concentration tends to increase and stabilize, or even decrease, as the plant apparently has a limit for chlorophyll production (Malavolta et al., 1997). A significant relationship was found between leaf total N and chlorophyll meter readings for eucalyptus species, but the coefficient of determination was low ( $R^2 = 0.47$ ; Pinkard et al., 2006). It has been observed that eucalyptus often stores N at greater levels than the physiological requirements for photosynthesis (Close et al., 2004), so a consistent relationship may not be expected between chlorophyll meter readings and leaf total N (Pinkard et al., 2006). Therefore, although studies have indicated the chlorophyll meter readings for diagnosis of N status of eucalyptus (Madeira et al.,

2009; Ribeiro et al., 2009), this should be used with caution since chlorophyll meter readings have some limitations and they are not a specific N indicator. Pinkard et al. (2006) concluded that the chlorophyll meter (Minolta SPAD-502) is suitable for estimating chlorophyll concentrations of *E. globulus* and *E. nitens* as a 'generic' indicator of eucalyptus stress, but caution is required for it to be used to directly detect N deficiency in young crowns since there was a weak relationship between leaf total N concentration and chlorophyll meter readings for eucalyptus species.

## Field experiment

Overall, leaf total N concentrations were lower in plants of the field experiment (Figures 3a and 3b). Field plants (approximately two years old) were older than the greenhouse ones (140 days old), and leaf total N concentration tends to decrease with age (Leuning et al., 1991; Rubio-Covarrubias et al., 2009). A combination of factors leads to this, but it is mainly associated with DM accumulation in aging tissues and N translocation from old to new tissues. A reduction in nutrient concentrations in old tissues increases nutrient efficiency with age (Harrison et al., 2000). Changes in leaf concentrations of macronutrients were similar to the height growth rate of 3-year-old *E. Fastigata* (Knight, 1988). Root uptake supplied 70 % of N requirements in the first year of *Eucalyptus* ssp. growth in the State of São Paulo; and internal retranslocations in leaves supplied 30-50 % of annual N and P requirements from 2 years of age onwards (Laclau et al., 2010). Total N concentration stabilized in *E. grandis* after the first year, and this was attributed to progressive death of leaves and branches in the lower crown, when the upper tree crown began to compete for sunlight (Cromer et al., 1993).

The different soils and regions influenced leaf total N concentrations, which were greater in Pompeu (clayey soil) during the dry season (Figure 3a). Therefore, in this season, leaf total N reflected the difference between total N concentrations in the soils (Table 3). However, in the rainy season, leaf concentrations were greater in the sandy soil (João Pinheiro region) and did not effectively represent total N concentrations in the soil. In the rainy season, mineralization of soil organic matter (OM) is favored and, since the clayey soil had more OC (Table 1) and total N (Table 3), higher release of N was expected, along with greater leaf total N. The highest amounts of mineral N in the 0-20 cm soil layer were found during the rainy and hot seasons in an *E. grandis* plantation (Voigtlaender et al., 2012), confirming higher N mineralization in the soil in the rainy season. This shows the importance of standardizing the correct sampling time, which, in this case, would be the dry season for total N in the leaf. Sampling time is an important factor and

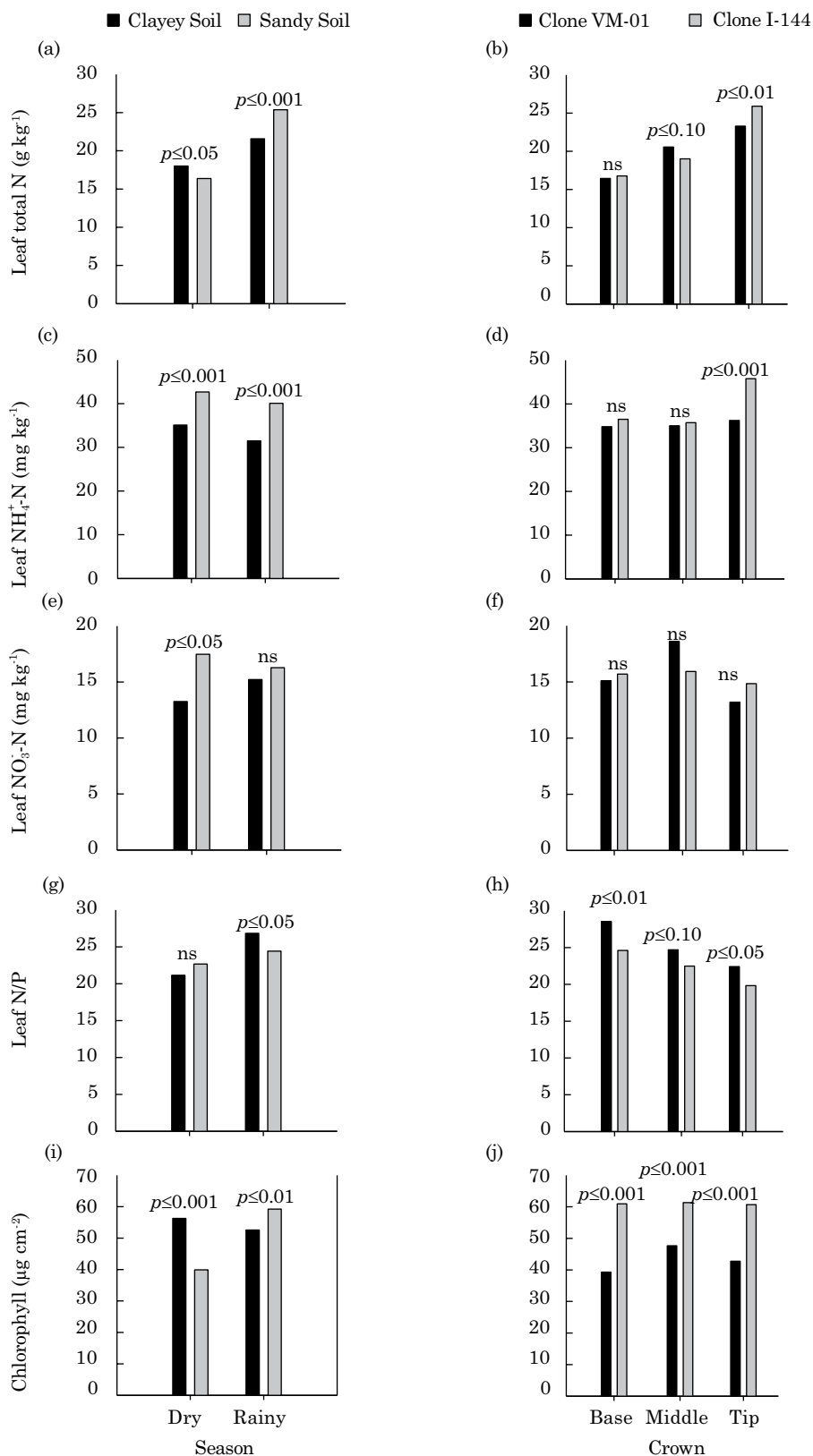


Figure 3. Leaf concentrations of total N,  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_3^-\text{-N}$ ; N/P ratio; and chlorophyll in two regions (soils) for each season (a, c, e, g, and i), and in two young clones (approximately two years old) for each position in the crown (b, d, f, h, and j) in a field experiment. ns: not significant by the F test at 10 %.

**Table 3. Concentrations of nitrogen (total, ammonium, and nitrate) in the soils under young eucalyptus clones (approximately two years old), in different regions (Pompeu and João Pinheiro, MG, Brazil) and seasons (dry and rainy), grown in a field**

Layer	Pompeu				João Pinheiro			
	VM-01		I-144		VM-01		I-144	
	Dry	Rainy	Dry	Rainy	Dry	Rainy	Dry	Rainy
cm	Total N (dag kg <sup>-1</sup> )							
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
60-100	0.06	0.05	0.08	0.06	0.02	0.01	0.03	0.03
Mean	0.08 a				0.04 b			
	NH <sub>4</sub> <sup>+</sup> -N (mg kg <sup>-1</sup> )							
0-10	40.3	30.7	37.3	32.3	26.2	23.7	20.8	27.3
10-20	30.7	27.3	30.8	27.9	22.8	24.8	18.6	26.0
20-40	25.4	27.0	21.7	27.7	18.6	22.1	19.6	36.4
40-60	24.0	32.2	23.9	24.8	20.5	19.1	16.6	34.2
60-100	25.9	31.2	22.4	24.1	20.8	18.2	17.5	24.5
Mean	28.4 a				22.9 b			
	NO <sub>3</sub> <sup>-</sup> -N (mg kg <sup>-1</sup> )							
0-10	0.6	0.5	0.9	0.5	0.7	0.5	0.7	0.4
10-20	0.5	0.4	0.6	0.6	0.6	0.5	0.6	0.6
20-40	0.5	0.3	0.5	0.4	0.6	0.5	0.5	0.3
40-60	0.6	0.4	0.6	0.4	0.6	0.3	0.6	0.3
60-100	0.6	0.3	0.9	0.6	0.9	0.5	1.1	0.5
Mean	0.52 a				0.57 a			

Mean values with the same letter in the rows are not significantly different by Tukey's test at 5 %. Mean values of samples collected from the row and between rows. Pompeu and João Pinheiro represent regions with clayey and sandy soil, respectively.

needs to be considered for leaf diagnostic purposes (Knight, 1988).

Therefore, as the response of leaf total N concentration in soils from different regions for each season established no pattern, i.e., it was greater in the region of clayey soil in the dry season but it was lower in the region of clayey soil in the rainy season (Figure 3a), total N concentrations cannot be considered an ideal indicator for N status in eucalyptus clones. The diameter at breast height (DBH) of clones responded in a similar manner between seasons, i.e., plants of both clones always showed greater DBH in the region of clayey soil, regardless of the season of evaluation (dry or rainy) (Table 4). The clones also differed in total N in the leaves, with greater values for VM-01 in the middle of the crown, as observed in the greenhouse experiment (Figure 1b). However, the I-144 had greater values at the tip of the crown (Figure 3b). Thus, there was no regularity between clones in total N concentration in different positions in the crown, in contrast with

that observed for the DBH values of the clones - I-144 always had greater DBH in both dry and rainy seasons than VM-01 (Table 4). Leaf total N concentrations of young eucalyptus plants are higher at the tip of the crown than at the base, indicating that some retranslocation of nutrients from old to young leaves occurs prior to crown closure (Leuning et al., 1991).

For *E. globulus* sites, regardless of fertilization times, leaf total N did not show a good relationship to N response in Uruguayan soils (Perdomo et al., 2007). Leaf analysis performed at different sites did not support the widespread use of total N concentration as an indicator of N deficiency in *E. nitens* (Smethurst et al., 2004). Laclau et al. (2010) suggested that biomass production affected N requirements of eucalyptus. In the current study, leaf total N did not show the same tendency as growth in DBH (Figure 3a and Table 4) and thus proved not be an ideal indicator of N supply. However, in nectarine trees (*Prunus persica*), total N concentration showed

**Table 4. Diameter at breast height (DBH) of young eucalyptus clones grown in a field under different soils (clayey and sandy) and seasons (dry and rainy)**

Clone	Dry (Sept 2011)		Mean	Rainy (Feb 2012)		Mean
	Clayey <sup>(1)</sup>	Sandy <sup>(2)</sup>		Clayey <sup>(1)</sup>	Sandy <sup>(2)</sup>	
	cm					
VM-01	7.67	5.51	6.59 B	9.23	7.93	8.58 B
I-144	8.69	5.89	7.29 A	10.69	8.53	9.61 A
Mean	8.18 a	5.70 b		9.96 a	8.23 b	

<sup>(1)</sup> Region of Pompeu (MG, Brazil); <sup>(2)</sup> Region of João Pinheiro (MG, Brazil). Mean values with different lowercase letters in the row, comparing regions (soils) in each season, and mean values with different uppercase letters in the column, comparing clones in each season, are significantly different by Tukey's test at 5 %.

the highest and most consistent association with growth and N supply (Rubio-Covarrubias et al., 2009). These divergent results indicate a need for further studies on indicators of N status in plants.

In general, total N and  $\text{NH}_4^+$ -N concentrations were higher in the upper soil layers (Table 3) because, in them, there is greater organic matter (OM) concentration, and more than 95 % of total N in the soil is found in organic forms (Cantarella, 2007). Similar values of total N were found in the soil under eucalyptus plantations in the State of São Paulo (Voigtlaender et al., 2012). In contrast, there was greater variability for  $\text{NO}_3^-$ -N concentrations in the different soil layers, probably because  $\text{NO}_3^-$ -N ions are highly mobile and subject to leaching in soils (Cantarella, 2007). Under all conditions of different regions, seasons, clones, and soil depths, there was large predominance of  $\text{NH}_4^+$ -N over  $\text{NO}_3^-$ -N concentrations (Table 3), in agreement with other studies on soils under eucalyptus plantations in Brazil (Gama-Rodrigues et al., 2005). But contrary that occurs usually in Brazilian agricultural soils under aerobic conditions (Cantarella, 2007). This is because eucalyptus is generally cultivated in acid soils with low natural fertility (Gonçalves et al., 2013), where nitrification is lower, because bacteria mainly responsible for nitrification (*Nitrosomonas* and *Nitrobacter*) are sensitive to lower pH (Moreira and Siqueira, 2002). The pH of the soils in the present study is lower than 5 (Table 1) and, according to Moreira and Siqueira (2002), nitrification is greatly reduced at pH lower than 6.0, and it is practically null in pH values lower than 4.5. Therefore, eucalyptus species preferentially take up more  $\text{NH}_4^+$ -N than  $\text{NO}_3^-$ -N (Barros and Novais, 1996). This preference for  $\text{NH}_4^+$ -N may constitute an advantage because of lower use of metabolic energy, since it does not require the action of root  $\text{NO}_3^-$ -N reductase (Grespan et al., 1998). This would be advantageous for eucalyptus, which seems to reduce  $\text{NO}_3^-$ -N further in leaves than in roots, as verified by much lower  $\text{NO}_3^-$ -N concentrations found in leaves in relation to roots in the greenhouse experiment (Figures 1e and 1f).

However, the good performance observed from  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N concentrations for plants grown in nutrient solution (greenhouse experiment; Figures 1c, 1d, 1e, and 1f) did not occur in the field (Figures 3c, 3d, 3e and 3f). Ammonium leaf concentrations were higher in the sandy soil region, in dry and rainy seasons (Figure 3c), and did not reflect the concentrations in the soil as the clayey soil had a greater  $\text{NH}_4^+$ -N concentration (Table 3), nor growth in plant diameter (Table 4). There was a difference in  $\text{NH}_4^+$ -N leaf concentrations between the clones only at the tip of the crown, with greater values for I-144 (Figure 3d); I-144 showed greater values for DBH as well (Table 4). However, in the greenhouse experiment, VM-01 had greater values of  $\text{NH}_4^+$ -N leaf concentrations (Figure 1d). For  $\text{NO}_3^-$ -N in the leaves, there was no difference in concentrations, except for soils in the dry season (Figures 3e and 3f), probably because there were no differences in  $\text{NO}_3^-$ -N concentrations in the soil (Table 3). Rubio-Covarrubias et al. (2009) suggest that the combination of both indicators (stable compounds - total N and chlorophyll, and mobile N forms -  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N) could be used to diagnose N status in a wide range of N availability in the soil.

In the field, N/P ratios in the leaf were between 20 and 29 (Figures 3g and 3h), probably as a consequence of low average concentration of P ( $<0.90 \text{ g kg}^{-1}$ ) in leaves in these plants (Dechen and Nachtigall, 2007). Silveira et al. (2003) found a much lower N/P ratio in leaves, which decreased from 3.85 to 2.50 from 55 to 97 days of evaluation for plantlets of *E. grandis*. There was no difference in N/P ratio in the leaf for sandy and clayey soils in the dry season; however, in the rainy season, we observed a higher N/P ratio in the leaf in a clayey soil region (Figure 3g). Clone VM-01 showed the highest N/P ratio in the leaf, regardless of crown position (Figure 3h), in contrast with the results observed in the greenhouse experiment (Figure 2a). The N/P ratio in the leaf increased from the lower to the upper crown from 12 to 18 in field-grown 4-year-old *E. globulus* (Turnbull et al., 2007), in contrast with the results observed in the current

study. For 3-year-old *E. Fastigata*, the N/P ratio in the leaf increased rapidly in the first two months, after N fertilization, but in the next seven months, it remained stable, and this ratio increase reflected a more favorable N and P balance (Knight, 1988). Although the analysis of N/P ratio in the leaf can be used to guide fertilization with N and P in eucalyptus (Perdomo et al., 2007), leaf analysis conducted in different sites did not support the widespread use of this ratio as an indicator of N deficiency in *E. nitens* (Smethurst et al., 2004), which was also observed in our study.

Plants grown in the field had chlorophyll values (Figures 3i and 3j) similar to those obtained in the greenhouse experiment (Figure 2b). In the dry season, plants from the clayey soil had higher chlorophyll levels; however, the values were greater in plants from the sandy soil in the rainy season (Figure 3i), similar to leaf total N concentration (Figure 3a), but different from soil total N (Table 3) and the DBH (Table 4). In the rainy season, there is more probability of OM mineralization in the soil and, since the clayey soil had higher organic C concentration (Table 1), a greater total N concentration in the soil was observed (Table 3). High and positive correlations between total N and OM ( $r = 0.90$ ) and total N and clay ( $r = 0.94$ ) were found in soils of eucalyptus plantations in the state of São Paulo (Pulito, 2009). The greater total N concentration in clayey soil, as well as other factors (water content in the soil, for example), could favor greater N uptake by plants in the rainy season, and this would reflect greater chlorophyll concentration, since N and chlorophyll concentrations in the leaf are correlated (Turnbull et al., 2007), though not always (Dechen and Nachtigall, 2007). In the rainy season, we observed higher chlorophyll levels in plants in the sandy soil, a tendency similar to total N in the leaf (Figures 3a and 3i). Thus, chlorophyll had a similar response for total N in the leaf, but a different response for total N in the soil and for growth in DBH of the plants (Table 4). In regard to differences between clones in chlorophyll levels, there was a clear difference; regardless of the crown position, clone I-144 always had greater values than VM-01 (Figure 3j), a tendency contrary to leaf total N (Figure 3b), but similar to the growth in DBH of the clones (Table 4).

## CONCLUSIONS

Concentrations of  $\text{NH}_4^+\text{-N}$  (root) and  $\text{NO}_3^-\text{-N}$  (root and leaf) show potential use in predicting N requirements of eucalyptus plantlets in the greenhouse; however, they do not effectively represent soil N levels and growth in DBH in the plants in the field.

The ratio of N/P in the leaf, chlorophyll values, and chlorophyll meter readings have similar responses to N application in the greenhouse, but the N/P ratio does not reflect the real differences in N status in the soil and the DBH of the plants, and the chlorophyll meter represents chlorophyll levels but not leaf total N, and its use cannot be generalized for different eucalyptus genotypes.

Leaf total N concentration is not an ideal indicator of N status; nevertheless, of all the indicators evaluated, leaf total N and chlorophyll levels best show the soil N status for young eucalyptus clones under field conditions.

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