

Nitrate reductase (NR) and glutamine synthetase (GS) can be used as indicators of nitrogen status in eucalyptus clones

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Abstract

Nitrate reductase (NR) (EC 1.6.6.1.) activity and glutamine synthetase (GS) (EC 6.3.1.2) activity, have been proposed as more sensitive indicators of nitrogen (N) status in plants than total N content. The aim of this study was to assess the activities of NR and GS as indicators of the status of N in eucalyptus clones grown under different conditions of N supply. For this purpose, experiments were carried out in both greenhouse and field conditions. In the greenhouse, the experiment was conducted in a nutrient solution and consisted of a 2 × 6 factorial arrangement, with two eucalyptus clones (VM-01 and I-144) and six application rates of N (0, 0.74, 2.93, 4.39, 5.85, and 8 mmol L⁻¹ of NH₄NO₃). The field experiment was composed of a 2 × 2 × 2 factorial arrangement, with two regions (Pompeu and João Pinheiro, MG State, Brazil), two sampling periods (dry season and rainy season), and two eucalyptus clones (VM-01 and I-144). We evaluated the total N and total protein contents and enzymatic activities of NR and GS in the leaves. In the greenhouse, the clone I-144 took up less N, inducing less protein synthesis; however, it reduced and assimilated more N (greater NR and GS activity), which resulted in greater dry matter production compared to VM-01. Enzyme activity was negatively affected by greater N supply (r = -0.58 for NR and r = -0.50 for GS), probably through an inhibitory effect from both NO₃⁻ (substrate of NR) and NH₄⁺ (substrate of GS) at high concentrations. In the greenhouse, the activity of these enzymes also showed negative correlation with the leaf content of total N (r = -0.79 for NR and r = -0.64 for GS) of the eucalyptus clones.

Keywords: Enzyme activity; forest nutrition; leaf diagnosis; N metabolism; total N.

Abbreviations: ATP_adenosine triphosphate; AsnA_asparagine synthetase; C_carbon; CBH_circumference at breast height (1.30 m); FAD_flavin adenine dinucleotide; g_gravity force; GHA_Fe-L-glutamyl-γ-hydroxamate; GS_glutamine synthetase; I-144 and VM-01_eucalyptus clones; m.a.p._months after planting; min_minute; Moco_molybdenum cofactor; N_nitrogen; NH₄⁺_ammonium, NiR_nitrite reductase; NO₂⁻_nitrite; NO₃⁻_nitrate; NR_Nitrate reductase; PMSF_phenylmethylsulfonyl fluoride; PVPP_polyvinylpyrrolidone; r_Pearson correlation coefficient; S_south; W_west.

Introduction

Leaf content of total nitrogen (N) has traditionally been used to estimate the status of N in plants; however, there are limits to its use as a nutritional indicator, such as low response to high N supply in trees (Alva et al., 1998), as well as its insensitivity to seasonal demand in use of the nutrient (Chapin et al., 1982). It has been observed that total N content in the leaf tissues also does not differentiate between responsive and unresponsive sites for *Eucalyptus globulus* and *E. grandis* (Perdomo et al., 2007). Thus, enzymatic activities have been proposed as more sensitive indicators for the status of N in plants. In the 1970s, it had already been suggested that nutritional deficiencies in plants could be diagnosed through measurement of enzymatic activity (Bar-Akiva, 1971), and this was also observed in later studies (Tavdgiridze and Putkaradze, 1991; Lavres Jr et al., 2010). One of the advantages of the metabolic diagnosis is its high sensitivity since a small variation in nutrient content leads to a high variation in the metabolite content (Martinez et al., 1999). Nevertheless, these authors emphasize that there are

no standards or universally accepted methods of determination, which indicates the relevance and necessity of discovering a novel protocol. Nitrate reductase (NR) is cited as an important biochemical marker for N deficiency (Srivastava and Singh, 2005), and measurements of its activity are used as a tool for evaluation of the nutritional status of N in tropical grasses (Lavres Jr and Monteiro, 2006). The activity of NR in the recently expanded leaf blade had a positive and significant correlation with total N content in the leaf and yield of mombaça grass (*Panicum maximum* Jacq.) (Lavres Jr et al., 2010). In addition, NR is an enzyme with great potential for improving water quality of environments polluted with NO₃⁻, through converting it to NO₂⁻ (Campbell and Campbell, 1998). Normally NR activity is high in the leaves (Souza and Fernandes, 2006); however, according to Campbell (1999), some plants have little or no activity of this enzyme in the leaves, but they have greater activity in the roots. Activity of NR in the leaves was greater (80 %) than in the roots of *E. camphora* and *E. ovata*

(Granger et al., 1994). This enzyme exhibits an inductive character; an increase in its activity is seen after supplying NO_3^- to plants, and a decrease in its activity at low light levels (Souza and Fernandes, 2006). Souza and Fernandes (2006) cite glutamine synthetase (GS) as the main enzyme in assimilation of NH_4^+ by plants. The activity of GS may vary under different conditions of N (Magalhães et al., 1995). The activity of GS in shoots of *Catsetum fimbriatum* plants was positively correlated with soluble protein contents (Majerowicz and Kerbaudy, 2002). There is a positive correlation between grain yield and GS activity (Gallais and Hirel, 2004). In corn and wheat, GS activity is representative of the N status of the plant (Hirel et al., 2007). In light of the above, the aim of this study was to evaluate the enzymatic activities of NR and GS as indicators of N status as a substitution for total N content in the leaf in eucalyptus clones grown under different conditions of N supply. Although enzymatic activities were already proposed in the past century as more sensitive indicators for N status in plants, the activities of NR and GS have still not been assessed for this purpose in eucalyptus clones.

Results and Discussion

Greenhouse experiment

There was an increase in leaf protein contents of the clone VM-01 with the increase in the application rates of N in solution (Fig 1a), reaching a peak at the intermediate rates, according to the quadratic model (Fig 1a). There was low correlation ($r = 0.38$) between protein contents and the application rates of N in solution for the two clones (Table 1). In spite of different models (quadratic and square root), the protein contents had a tendency similar to total N contents in the leaf (Fig 1b) for the clone VM-01 ($r = 0.70$), and also to dry matter production of plants (Fig 1e). As part of the N taken up is incorporated into the plant as amino acids, and the synthesized proteins promote leaf growth with an increase in its supply, increasing the area of photosynthesis (Dechen and Nachtigall, 2007), there is greater dry matter production of the plant. For clone I-144, there was no difference between the leaf contents of protein, which were less than those of clone VM-01 (Fig 1a), corroborating with its lower total N contents in the leaf (Fig 1b). An increase in NR activity is generally seen with the increase in N supply in nitric form, with the increase in total N content in the leaf, or with the increase in yield of different plant species, as in Mombuca grass (Lavres Jr et al., 2010), manioc (Cruz et al., 2004), coffee (DaMatta et al., 1999; Reis et al., 2007), and herb species (Granger et al., 1994), and even in *E. grandis*, *E. regnans*, and *E. obliqua* (Adams and Attiwill, 1982; Caldeira et al., 1994). In contrast, NR activity in the leaves of both clones assessed was greater in the absence of N in solution and decreased with the increase in the rates applied of this nutrient, according to the square root model (Fig 1c). Thus, there was an inverse correlation between NR activity and the N supplied ($r = -0.49$) and the total N content in the leaf ($r = -0.70$) (Table 1). Kumar and Singh (2002) also observed greater NR activity with low accumulation of N in corn hybrids during the months of June to September. The NR activity is regulated by various environmental and intracellular factors, such as light, concentration of N compounds, CO_2 , Mo, Fe, plant hormones, and C metabolites (Lea and Leegood, 1995). Light has an effect which may be direct, activating the enzyme, or indirect, by photosynthesis, supplying energy for assimilation of NO_3^- (Smirnov et al., 1984). Moreover, according to the last authors mentioned, the

activity of this enzyme is also affected by the concentration of NH_4^+ . Both the synthesis and the activity of NR are induced by the presence of the substrate (Adams and Attiwill, 1982; Somers et al., 1983); therefore, a reduction in uptake of NO_3^- would lead to a reduction in the activity of this enzyme. Nevertheless, manioc leaves deficient in N, in which leaf contents of NO_3^- were not detectable, showed NR activity (Cruz et al., 2004). According to Campbell (1999), the NO_3^- newly taken up and transported to plant shoot appears to be a more determinant factor for induction of NR activity than the NO_3^- stored in the vacuole, since the first phase of reduction of NO_3^- occurs in the cytoplasm. Rapid transport of NO_3^- to outside of the cytoplasm explains the declines in NR activity when the external supply of this anion is reduced, even when the total content of NO_3^- in the plant is high (Fernandes and Souza, 2006). The influx of NO_3^- to the cytosol performs a more relevant role in induction of NR activity than the NO_3^- stored in the vacuole (Queiroz et al., 1993). A decrease was observed in NR activity in peach palm (*Bactris gasipaes*) seedlings at high concentrations of NO_3^- in the external medium, indicating a negative effect from excess of substrate on enzyme activity (Oliveira et al., 2005). The addition of higher concentrations of NO_3^- also reduced NR activity in algae (Chow et al., 2007). In relation to NH_4^+ , high contents may have an inhibitory effect on NR activity (Delu-Filho, 1994). This author observed that in the afternoon, there was low NR activity in the roots of the rubber tree and attributed this fact to possible retroinhibition of the enzyme, caused by the accumulation of NH_4^+ in this organ. The high concentration of NH_4^+ in the roots results from low translocation of this cation to plant shoot, brought about by a decrease in transpiration flux, arising from greater stomatal closing of the plants. Thus, the increase in the concentration of NH_4^+ in nutrient solution led to greater leaf content of NH_4^+ (Ferreira, 2013), with reduction in NR activity. This becomes relevant especially for eucalyptus species, which, in general, have preferential uptake of N-NH_4^+ (Barros and Novais, 1996) and high contents of this cation in their tissues (Ferreira, 2013). In rubber tree roots the NH_4^+ had a strong repressive effect on NR activity, even in plants that were grown at near optimal NO_3^- application rates (6 mmol L^{-1} of NO_3^- and 2 mmol L^{-1} of NH_4^+) (Lemos et al., 1999). According to Redinbaugh and Campbell (1993), this repression of NR activity in the roots may be due to the accumulation of NH_4^+ or of other N compounds that would be inhibiting the synthesis or the activity of this enzyme (Lewis et al., 1982). The enzyme NR is a high molecular weight flavoprotein formed of two identical subunits, with three FAD groups, heme and a molybdenum cofactor (Moco) (Chow et al., 2007). Thus, the high Mo contents seen in the leaves of the clones grown under the highest application rates of N (Ferreira, 2013) may also have had a negative effect on enzyme activity, even knowing that the Mo deficiency reduces its activity (Smirnov et al., 1984). Potassium also plays an important role in activation of the enzymes of N assimilation when NH_4^+ is at toxic levels in plant tissues (Souza and Fernandes, 2006). Another factor that may also have had an effect, although to a lesser degree, on greater NR activity at the lower rates of N application is that the NR activity was expressed as a function of protein contents. That is because, at least for the clone VM-01, there were lower protein contents in the plants grown at the lower application rates of N (Fig 1a) as a result of the lower total N contents in the leaf (Fig 1b), which made for an increase in the ratio, thus contributing to the greater value of its activity.

Greater NR activity at lower application rates of N could indicate that plants with lower leaf contents of total N would

Table 1. Pearson correlation coefficients (r) of N supply (rates) and total N content in the leaf with total protein, nitrate reductase (NR) activity and glutamine synthetase (GS) activity in eucalyptus clones grown in nutrient solution.

Clone	Protein		NR		GS	
	Rate of N	Total N	Rate of N	Total N	Rate of N	Total N
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
General*	0.38	0.54	-0.49	-0.70	-0.34	-0.48

* Pearson correlation coefficient (r) considering the values of both clones

● Clone VM-01 ○ Clone I-144

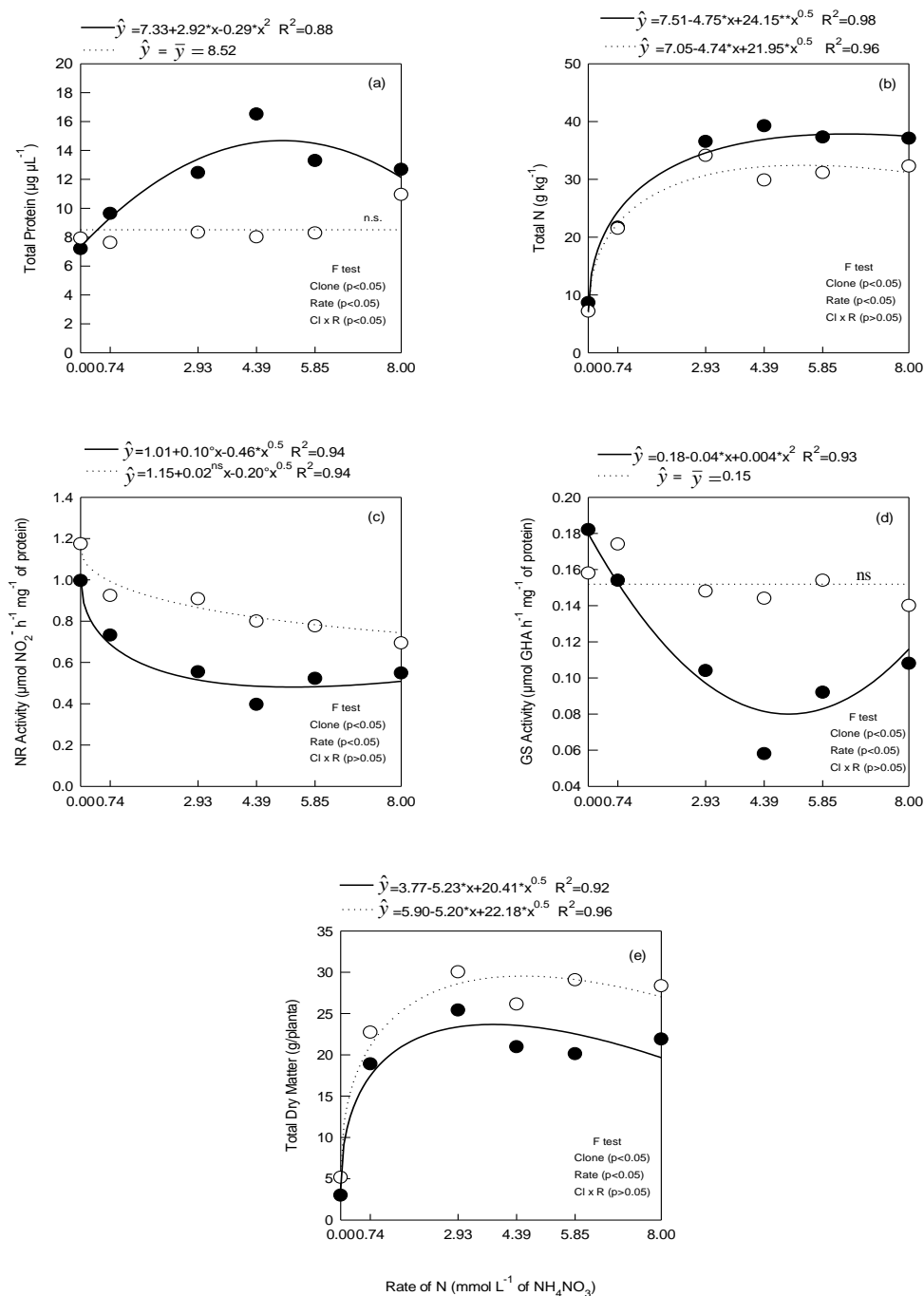


Fig 1. Total protein (a), total N (b), nitrate reductase (NR) activity (c) and glutamine synthetase (GS) activity (d) in the leaves, and total dry matter (e) of young eucalyptus clones under N rates in nutrient solution. ^{ns}, °, * and ** indicate not significant, significant at 10, 5 and 1 %, by the F test, at 5 % probability. Cl (clone) and R (rate)

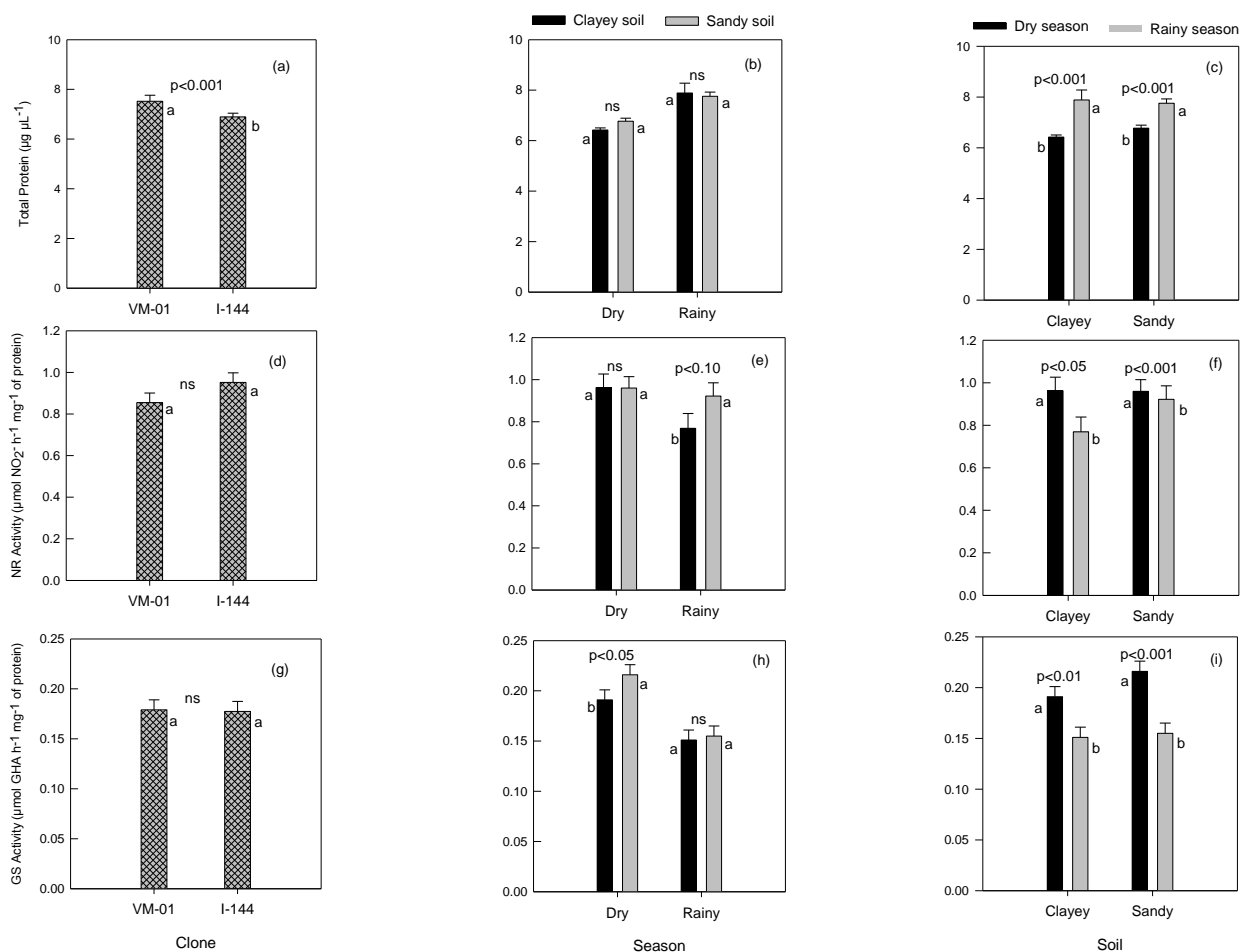


Fig 2. Leaf contents of total protein comparing eucalyptus clones (a), regions of different soils by each sampling season (b) or different seasons by each soil (c), leaf nitrate reductase (NR) activity comparing eucalyptus clones (d), regions of different soils by each sampling season (e) or different seasons by each soil (f) and leaf glutamine synthetase (GS) activity comparing eucalyptus clones (g), regions of different soils by each sampling season (h) or different seasons by each soil (i). ns No significant at 10 % by the F test ($p > 0.10$). Letters different in each set of two columns are different at 5 % by Tukey's test. Bars at the top of columns represent standard error (SE).

exhibit greater efficiency of this enzyme, indicating compensation in this condition. This argument is strengthened upon comparing the NR activity of the two clones with I-144 being that which showed the lowest total N contents in the leaf and, in contrast, greater activities of this enzyme and greater dry matter production (Fig 1b, 1c, 1e). That way, NR activity showed an inverse response both to N in the solution (application rates) and the total contents of this nutrient in the leaf (Table 1). For GS activity of the clone VM-01, greater values were also observed at lower N application rates, values decreasing with an increase in the rates, following the quadratic model (Fig 1d). This could be explained by greater supply of NH_4^+ as a substrate for GS as a consequence of greater reduction of N under these conditions (greater NR activity). Nevertheless, it should be noted that NH_4^+ in plants is not formed only through reduction of NO_3^- but also by other processes, such as lignin biosynthesis, catabolism of amino acids, breakdown of proteins in senescent tissue, and photorespiration (Marschner, 2012), as well as by direct uptake by plants. The amount of NH_4^+ produced during decarboxylation of glycine for production of serine under photorespiratory conditions is up to ten times greater than the amount produced during reduction of NO_3^- (Lea and Blackwell, 1992). Low GS activity results from a regulatory effect that acts under

conditions of low supply of C and, or, N to the plant, keeping the enzyme in its inactive form, as an alternative for conservation of ATP (Delu-Filho, 1994). Nitrate had a positive effect on GS activity on rubber leaves (Delu-Filho, 1994) and on corn cultivars (Purcino, 1992). Nevertheless, the increase in concentration of NH_4^+ in the nutrient solution contributed to repressing GS activity in the roots of the rubber tree (Lemos et al., 1999). The leaf contents of NH_4^+ of the eucalyptus clones of the present study had a linear increase with the increase in N application rates (Ferreira, 2013). Thus, with the increase in N application rates, the high leaf contents of NH_4^+ may have negatively affected GS activity, suggesting an inhibitory effect on it. For the clone I-144, there was no difference in GS activity, as there was likewise no difference for protein contents (Fig 1a, 1d). The GS enzyme catalyzes the conversion of glutamate into glutamine through the use of NH_4^+ , ATP and a divalent cation (Mifflin et al., 1981), thus promoting synthesis of the amino acid. In contrast, this clone generally showed greater GS activities in relation to the clone VM-01, which was not reflected in greater protein contents. It is noteworthy that GS represents just 1 to 2 % of total soluble proteins in organs capable of assimilating NH_4^+ (Hungria et al., 1992). Thus, greater GS activities do not necessarily mean greater protein

contents, as seen in comparison of the two clones, since there are innumerable other enzymes and factors involved in protein synthesis in plants. As in NR activity, an inverse correlation was observed between GS activity and the N supplied in solution ($r = -0.34$) and the total N contents in the leaf (-0.48) (Table 1).

Field experiment

For clones grown in the field, greater leaf content of total protein was observed in clone VM-01 (Fig 2a), like that observed in the experiment in the greenhouse (Fig 1a). Lower leaf contents of total protein were observed under field conditions, in which there were older plants (approximately 17 months of age) in comparison to plants grown in the greenhouse (Fig 1a, 2a). When comparing plants from regions of sandy soil and clayey soil in each period of assessment (dry and rainy season), leaf protein contents did not differ (Fig 2b), despite the clayey soil having double the total N content (Ferreira, 2013). It should be noted that, by itself, the presence of greater N content in the soil will not always mean greater uptake of the nutrient, and protein synthesis by plants, since these processes are dependent on various factors, such as availability of C, energy, enzyme activity, and even climate conditions like temperature and rain. In both clayey soil and sandy soil, the rainy season contributed to greater contents of leaf protein (Fig 2c). Greater decomposition of organic matter in the soil through greater microbial activity as a result of greater moisture in the soils in this season from greater rainfall may have favored greater availability and uptake of N by plants (Ferreira, 2013), contributing to greater protein synthesis. The NR activity seen in eucalyptus clones grown in the field (mean value of $0.90 \mu\text{mol of NO}_2^- \text{ h}^{-1} \text{ mg}^{-1}$ of protein, Fig 2d, 2e, 2f) is considered moderate (Campos, 2009). The NR activity is drastically affected by high acidity conditions (Towsend, 1970), and this may be an indication of lower NR activities in eucalyptus since this species, in general, is associated with acid soils (Gama-Rodrigues et al., 2005), where, moreover, the nitrification process is reduced (Moreira and Siqueira, 2002). The clones did not differ in regard to NR leaf activity in the field (Fig 2d). In this case, NR activity was not sensitive to detect differences in N status among the clones since they differed in total N contents in the leaf (Fig 1b). However, this fact could be advantageous since it would allow the use of an indicator of N status for the species, regardless of the clone assessed. The difficulty in application of enzymatic methods results from the fact that variation in the activity of the determined enzyme is affected by factors other than simply the nutrient under study (Martinez et al., 1999). The NR activity was greater in plants grown in a region of sandy soil in the rainy season in relation to plants in clayey soil, there being no differences in the dry season (Fig 2e), in contrast with the total N content in the soil, which was greater in the clayey soil (Ferreira, 2013). This fact once more indicates, just as observed in the greenhouse (Fig 1c), greater enzyme activity in plants grown in environments of lower N availability. Also leading to this conclusion, greater NR activity was likewise observed in sampling undertaken in the dry season for the plants from both soils (Fig 2f), in which, once more, the total N contents of the soil were lower (Ferreira, 2013). NR activity varied widely both among plant species and among sampling periods, and herbaceous species generally exhibited greater activity than woody species (Granger et al., 1994). The NR activity varied according to the age of the coffee plant (Carelli et al., 1990) and there was reduction in its activity, in both roots and leaves, with

increased age of peach palm (*Bactris gasipaes*) (Oliveira et al., 2005). Thus, NR activity assessed in the second sampling (rainy season) may also have been reduced by greater age of the plants. The NR activity may exhibit seasonal fluctuations in response to internal and environmental factors (Beevers and Hageman, 1969), as in the absence of activity of this enzyme seen in coffee leaves along with a drop in temperature (below $12.5 \text{ }^\circ\text{C}$) recorded in the winter (DaMatta et al., 1999). The NR activity is also strongly affected by water availability in the soil (Oliveira et al., 2005). Casarino (2009) observed greater NR activity in tree species in rainier months and suggested that the reduction in enzyme activity in the dry season is due to low availability of NO_3^- in solution since soil moisture is lower. Decrease in transpiration causes lower influx of NO_3^- , which may affect NR activity (Plhak, 2003). NR synthesis may be inhibited even with a brief period of negative water balance (Hsiao, 1973). Reis et al. (2007) observed a positive response of NR activity to N application from February to March and attributed this to greater rainfall in this period, since its activity may also be regulated by soil moisture. Better soil moisture status, in addition to other factors, stimulates the protein phosphatase, which dephosphorylates various residues of serine in the NR protein, promoting the activation of NR (Taiz and Zeiger, 2004). Results suggested a strong relationship between reduction of NR activity and of water potential in the leaves, which may be related to greater transpiration in the period of greater light intensity (Oliveira et al., 2005). Greater leaf NR activity in coffee plants in January was attributed to the greater light intensity which occurs in this season since the days are longer, increasing the photosynthetic rate of this species (Reis et al., 2007), with part of the energy production being used to activate NR (Queiroz et al., 1993). Even with all these considerations in favor of greater NR activities in periods of rain and with greater light intensity, leaf activity of this enzyme in the clones was, on the contrary, higher in the dry season, and thus without a known justification for such fact. Perhaps the lower leaf contents of protein seen in plants in the dry season (Fig 2c) raised the values of NR activity since the results were expressed based on protein contents and the readings of enzymatic activity were similar.

Moreover, as observed for NR activity, there were no differences for GS activity among the clones (Fig 2g). The plants from sandy soil, where there was lower content of total N in the soil and in the plant (Ferreira, 2013), exhibited greater GS activity in the dry season, there being no differences in the rainy season (Fig 2h). According to Invers et al. (2004), some studies show that GS activity correlates positively with N availability (Pregnall et al., 1987; Kraemer et al., 1997), but others report that its activity does not follow the conditions of external availability of N and is, above all, controlled by internal requirements (Finneman and Schjoerring, 1998; Thompson and Valiela, 1999). Invers et al. (2004) only found an increase in *in vivo* GS activity in *Posidonia oceanica* plants fertilized in the period of greatest demand for N (maximum lengthening of the leaf). In transgenic *Brassica napus* L., the effect of gene expression of asparagine synthetase (AsnA, E.C. 6.3.1.1) of *Escherichia coli* on the growth of these plants may be more pronounced under conditions limiting GS activity (Seiffert et al., 2004).

Upon comparing the GS activity between the two sampling periods, greater values are seen in the dry season for both the clayey soil and sandy soil regions (Fig 2i). Thus, in a general way, a similar response is observed between the NR and GS activities, in a certain way indicating the effect of one upon the another in the assimilation pathways of N by the plants since the product of NR (NO_2^-) is converted into NH_4^+ by the

action of nitrite reductase (NiR) and that, in the final analysis, will be the substrate for incorporation of N in glutamine by GS. Low concentrations of GS were correlated with low capacity for reduction of leaf NO_3^- (Stewart et al., 1990). In short, generally and consequently, the activity of these two enzymes assessed exhibited similar tendencies in regard to the effect of different conditions of N supply to eucalyptus clones.

Materials and Methods

Description of study sites

One experiment was conducted in a greenhouse and another in the field. In the first, the trial was undertaken in the Soil Department of the Federal University of Viçosa, Viçosa-MG (20°45'14" S, 42°52'53" W), Brazil, over three months (July to September 2011). For the field experiment, commercial eucalyptus areas in the municipalities of Pompeu and João Pinheiro (state of Minas Gerais, Brazil) were used, belonging to the company Vallourec & Mannesmann Tubes. The soils of the Pompeu region (18°53' S, 45°02' W) and João Pinheiro region (17°30' S, 46°07' W) contained mean values of 4.85 and 4.79 of pH (in water), 2.25 and 1.14 dag kg^{-1} of organic matter (Walkley-Black), 5.55 and 6.37 mg dm^{-3} of P, and 28 and 10 mg dm^{-3} of K (Mehlich 1), and 74 and 18 % clay (Ruiz, 2005) in the 0-1 m depth layer, respectively. The soil of the Pompeu region (clayey) contained 0.08 dag kg^{-1} of total N (Kjeldahl method), while in the region of João Pinheiro, the soil had 0.04 dag kg^{-1} of total N in the layer indicated. Pompeu and João Pinheiro have soils classified, respectively, as Latossolo Vermelho-Amarelo and Neossolo Quartzarênico by the Brazilian Soil Classification System (Embrapa, 2013).

Treatments and experimental procedure

The experiment conducted in the greenhouse consisted of a 2 × 6 factorial arrangement, with two eucalyptus clones (VM-01 and I-144) and six application rates of N (0, 0.74, 2.93, 4.39, 5.85, and 8 mmol L^{-1} of NH_4NO_3), with five replications, in a randomized block design. The clone VM-01 is a hybrid of *E. urophylla* x *E. camaldulensis* and I-144 is an *E. urophylla*. Seedlings in tubes at an age of around 50 days were used for both clones. The nutrient solution of Clark (1975), adapted by Locatelli et al. (1984), was used to maintain the N- NH_4^+ /N- NO_3^- ratio at 1, with double the concentration of P (Caldeira et al., 1994). The nutrient solution was kept permanently aerated and its pH was adjusted daily to 5.5 (± 0.05) with solutions (0.1 mol L^{-1}) of NaOH or H_2SO_4 , and it was changed weekly. The first month of carrying out this experiment represented the acclimation phase of the seedlings in the nutrient solution, where the plants remained in collective trays (11 L) and the concentration of the solution was gradually increased by each seven days (25, 50, 75 and 100 % of the original concentration). With the passing of this period, homogeneous plants for height and vigor were selected, and two of these plants were transplanted in each pot (6 L), thus representing an experimental unit, where they remained for two months with the application of the treatments. At the end of the experimental period, leaves were collected from the middle third of each plant; they were weighed and then wrapped in aluminum foil and placed in liquid N until being stored in a freezer (-80 °C) in the laboratory for analyses of protein and enzymatic activity (NR and GS). At this same time, the plants were harvested (separating them into roots, stem, branches

and leaves), washed in deionized water, and dried in an air circulation oven (60 °C/72 h) for assessment of dry matter. The leaves were then ground in a Wiley mill for later determination of total N contents. For the experiment in the field, a 2 × 2 × 2 factorial arrangement with five replicates was used in a completely randomized design, consisting of two regions (Pompeu- clayey soil and João Pinheiro- sandy soil), two sampling periods (dry and rainy season), and two eucalyptus clones (VM-01 and I-144). Sampling from the dry season was carried out in September 2011 and sampling from the rainy season in February 2012; both were undertaken at the end of each season. The eucalyptus clones were approximately 17 months of age in both regions at the time of the first sampling (September 2011). The useful area of the plot was composed of 30 plants (five rows with six plants each) in 225 m^2 according to the plant spacing of 3 x 2.5 m (1,333 plants ha^{-1}). In Pompeu, fertilizations of N-P-K consisted of 360 kg ha^{-1} (10-27-10, at planting), 240 kg ha^{-1} (23-00-21, seven months after planting - m.a.p.) and 350 kg ha^{-1} (23-00-21, 18 m.a.p.). For the João Pinheiro plots, 300 kg ha^{-1} (10-27-10, at planting), 250 kg ha^{-1} (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^{-1} of lime, 0.8 t ha^{-1} of gypsum, and leaf fertilization of 9 L ha^{-1} of ammonium borate (1.22 kg ha^{-1} of B, aerial application). For the Pompeu region, 4 kg ha^{-1} of B in the form of ulexite (10 % B) was also applied in the soil. The circumferences at breast height (CBH – 1.30 m) of all the trees in each plot (30 plants) were measured, calculating the mean value and standard deviation, and choosing five representative trees within this interval. Of these, one tree was used for leaf sampling. Leaves were collected in the amount of 60/plant in the middle part of the canopy, from the middle to the tip of the branches (only completely developed leaves) from two to three branches at different sides of the plant, from 8:00 a.m. to 12:00 noon. For the sampling of February 2012, another tree was chosen for collection of the leaves in each plot since there may have been interference from the previous sampling (September 2011) because the plants had many damaged branches. The procedure for storage of leaves for analysis of protein and enzymatic activity and for drying and grinding for analysis of total N contents was the same used for material from the greenhouse.

Laboratory analyses

Total N contents in the leaf were analyzed by the Kjeldahl method (Bremner, 1996) after mineralization with sulfuric acid and heating of the samples. For analyses of total protein and enzymatic activities *in vitro* of NR (EC 1.6.6.1.) and GS (EC 6.3.1.2), extracts were obtained according to Cambraia et al. (1989) and with modifications (1 mmol L^{-1} phenylmethylsulfonyl fluoride-PMSF) and 0.2 g of polyvinylpyrrolidone-PVPP) in order to optimize the activity these enzymes in eucalyptus leaves. The crude extracts were filtered in four layers of gauze and centrifuged (15,000 g at 4 °C for 15 min.), using the supernatant for the respective determinations. The quantification of total protein was performed according to Bradford (1976). For assessment of NR activity (Radin, 1974; Cambraia et al., 1989) and GS activity (Elliott, 1953) some modifications (volume and concentration of the reaction medium) were used, described in detail in Ferreira (2013). The enzymatic activity of NR was expressed in $\mu\text{mol of NO}_2^- \text{h}^{-1} \text{mg}^{-1}$ of protein and GS activity was expressed in $\mu\text{mol of GHA h}^{-1} \text{mg}^{-1}$ of protein.

Throughout all the procedures, the samples were kept in ice and protected from light up to the time of readings.

Statistical analyses

The results were subjected to analysis of variance (F test), and when the effect of the sources of variation and, or, interaction between them in the response variables was significant ($p \leq 0.05$), regression equations were fitted (greenhouse experiment). Correlation analyses were also made (Pearson coefficient - r) of the protein contents and NR and GS activity, with the N supply (application rates) and the leaf content of total N. To verify the effects of the treatments on the variables in the field experiment, the F test was adopted with 10 % significance ($p \leq 0.10$) and means were compared by Tukey's test. The SAS (2004) statistical program was used for that purpose, and the Sigmaplot software for creating figures.

Conclusion

In the greenhouse, the clone I-144 took up less N, inducing less protein synthesis; however, it reduced and assimilated more this nutrient (greater activities of the enzymes NR and GS), which was converted into greater dry matter production compared to VM-01; The NR and GS activities were negatively affected by greater supply of N, probably through an inhibitory effect both from NO_3^- (substrate of NR) and from NH_4^+ (substrate of GS) at high concentrations; Both in the greenhouse and in the field, the greatest NR and GS activities were associated with lower availability of N in the growing medium of the plants. The activity of these enzymes also exhibited negative correlation with the total N content in the leaf of the eucalyptus clones grown in the greenhouse.

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