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Nitrogen supply affects root and shoot amino acid composition in Eucalyptus clones

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Abstract

Changes in amino acid composition are frequently observed in plants under various conditions of stress, such as nutrient deficiencies. Hence, amino acids have been proposed as more sensitive indicators of N status than total N concentration. The goal of this study was to evaluate alterations in amino acid composition of young eucalyptus clones as a result of variable N supply. An experiment was carried out in a greenhouse with two Eucalyptus clones (VM-01 and I-144) and six N application rates (0, 0.74, 2.93, 4.39, 5.85, and 8 mmol L^{-1} of NH_4NO_3) grown in a nutrient solution in a randomized block design. Amino acid concentrations varied greatly as a function of N supply and depended on the organ (root or leaf) and the genotype evaluated. Roots showed greater number of amino acids than leaves (17 and 14, respectively), probably because of a higher amino acids synthesis or translocation to the roots. For both clones, N deficiency induced a significant decrease in proline, arginine, and methionine concentration in roots and a decrease in tyrosine, alanine, threonine, and methionine in leaves. These decreases were also associated with lower total N concentration and total dry matter of the eucalyptus clones. In conclusion, N supply affects amino acid composition, and the amino acids listed above are likely to be more sensitive indicators of N status than total N in eucalyptus clones.

Keywords: Leaf diagnosis; N metabolism; nutrient solution; plant nutrition; total N.
Abbreviations: Al_aluminum; approx. (~)_approximately; C_carbon; DON_dissolved organic nitrogen; GS_glutamine synthetase; HPLC_high performance liquid chromatography; min_minute; N_nitrogen; NH_4^+_ammonium, NO_3^-_nitrate; rpm_revolutions per minute; vs_versus.

Introduction

Ammonium (NH_4^+) is the main form of mineral N in most soils where eucalyptus trees grow (Turnbull et al., 1996), and it is suggested that this species prefers N-NH_4^+ to N-NO_3^- (Garnett et al., 2003). However, uptake of the glycine amino acid by *Eucalyptus obliqua* has also been observed at rates even higher than uptake rates of N-NO_3^- (Warren and Adams, 2006). Positive and negative effects of amino acid uptake on plant growth have also been observed (Forde and Lea, 2007). Absorbed amino acids are sources of C skeletons for plant metabolism (Majerowicz and Kerbauy, 2002), but providing amino acids as the sole N source for plants often leads to strong inhibition of N-NO_3^- uptake (Muller and Touraine, 1992). The N-NH_4^+ taken up is incorporated into glutamine and glutamate in the plastid or in the chloroplast by glutamine synthetase and glutamate synthase enzyme system (GS/GOGAT) (Ireland and Lea, 1999). Once assimilated into glutamine and glutamate, N is incorporated in other amino acids through transamination reactions, mediated by the action of aminotransferases (AT), with the participation of the phosphate pyridoxal cofactor (Vitamin B_6) (Forde and Lea, 2007). In the assimilation phase, the N-NH_4^+ incorporated in free amino acids is subjected to a strong feedback as a consequence of photorespiratory activity due to the immediate need for re-assimilation into glutamine and glutamate (Hirel and Lea, 2001). Nitrogen is often translocated in the phloem as amino acids or amides (Fernandes and Souza, 2006), and amino acid cycling between shoot and roots of cereal crops has been proposed as a signal that controls N uptake by the roots, mediated by shoot demand (Larsson et al., 1991). Studies have also shown that glutamine was the most effective amino acid for regulating N uptake in barley (Miller et al., 2008). Under conditions of stress, metabolism can be diverted to N storage by more economical pathways in terms of C consumption, in which arginine would be preferred because it has an N:C ratio of 4:5, whereas this ratio is only 2:5 for glutamine (Ferreira, 1986). Free amino acid concentration in tissues of fertilized *Posidonia oceanica* increased significantly, suggesting an N storage and translocation function for these compounds (Invers et al., 2004). A decrease in amino acid concentration was followed by an increase in leaf carbohydrate concentration in tobacco (Paul and Driscoll,

1997) and an increase in root biomass of chamomile (Kovácik et al., 2006) under N deficiency.

The accumulation of certain amino acids is frequently observed in plants subjected to various stress conditions (Mjza-Basso et al., 1986), such as accumulation of gamma amino butyric acid (Bown et al., 2006), arginine (Lea et al., 2007), ornithine (Alcázar et al., 2006), and proline (Munns, 2005). Proline accumulated in plant tissues following exposure to environmental stress and increased hardiness due to its role as a cryoprotectant and osmoregulant (Srivastava and Singh, 2005). Small increases in leaf glutamate were found under conditions of greater N supply (Geiger et al., 1999). An increase in the amount of amino acids in *Artemisia* species at higher altitude (3600 m) was attributed to an adaptational metabolic modification (Nautiyal, 1984). Amino acid concentration was also successfully used to classify genotypes of *E. globulus* by degree of resistance to cold (Castillo et al., 2010). Nutrient deficiency has been linked to alteration in the composition of the soluble N fraction in plants (Ferreira, 1986; Vergutz et al., 2012), with little variation in total N concentration (Hewitt and Smith, 1975). Arginine accumulation has been characterized as a consequence of P deficiency in plants (Achituv and Bar-Achiva, 1978). However, findings of Ferreira (1986) with *E. grandis* suggested that accumulation of this amino acid is mainly associated with a decrease in leaf protein S concentrations, which would have negatively altered NO_3^- uptake and assimilation, resulting in the appearance of chlorosis, an indication of N deficiency. Paul and Driscoll (1997) described the role of carbohydrates in signaling N deficiency through source and sink imbalance and showed that chlorosis develops following removal of the sink, causing depletion in total N, proline, arginine, and serine concentrations (Schaffer et al., 1986). Evaluation of total N concentration in the leaves is currently still the most used tool for verifying possible N deficiencies in plants (Araújo, 2007), but leaf total N.can be insensitive to N supply as in eucalyptus plantations (Gomes, 2009). Then, in citrus, changes in free amino acids have shown greater promise than total N in understanding N metabolism (Calot et al., 1988). Total-leaf-N concentration is less useful as a stress indicator because nitrogenous compounds such as amino acids can increase under stress without any change in total N (Warren et al., 2000). Nitrogen compounds such as arginine in peach roots (Taylor and Van den Ende, 1969) and amino acids in beech leaves (Balsberg, 1992) have been proposed as more sensitive indicators of N fertilization than total N concentration in perennial species (Rubio-Covarrubias et al., 2009). The actual concentrations of free amino acids in plants reflect the steady state among protein synthesis, proteolysis, and transport processes to and from the organs involved (Mjza-Basso et al., 1986). To date, we are unaware of any studies using amino acid profiles as indicators of N status in eucalyptus clones. Thus, the goal of this study was to evaluate alterations in the amino acid composition of young eucalyptus clones as a consequence of variable N supply in the nutrient solution.

Results and Discussion

Root amino acids

Different nitrogen availabilities substantially altered concentrations of some amino acids in eucalyptus roots but not others (Table 1). Only the amino acids serine, valine isoleucine, and aspartate in the roots of clones were not influenced by N availability ($p>0.05$, Table 1). Changes in levels of some amino acid are less expressive under the conditions of macronutrient deficiencies than micronutrient deficiencies (Fischella et al., 1977). However, the concentrations of alanine (82 % for VM-01 and 60 % for I-144), threonine (43 % for VM-01 and 57 % for I-144), and glutamine (73 % for VM-01 and 87 % for I-144) decreased much in the roots of both eucalyptus clones in the absence of N compared to the control treatment (2.93 mmol L^{-1} of NH_4NO_3, Table 1). This reduction in some amino acid concentrations was larger than the reduction we observed for total N concentration in the roots (61 % for VM-01 and 47 % for I-144; Fig 1a). The magnitude of changes illustrates the greater sensitivity of these amino acids in detecting plant N deficiency. In previous studies of *E. grandis* roots, alanine was the amino acid most sensitive to the absence of P and S, with a greater predominance of glutamine (more than 90 % of the amino acid fraction) in its exudates (Ferreira, 1986). Glutamine (plus glutamate) serves to translocate organic N from sources to sinks (Campbell, 1999), and thus its smaller concentrations in the absence of N (Table 1) were associated with lower total N root concentration and total dry matter (Fig 1a, 1c). In the roots of fertilized *Posidonia oceanica* plants, glutamine was the most abundant amino acid (Invers et al., 2004). Nitrogen supply significantly altered the concentration of all other amino acids evaluated in the roots (Fig 2 and 3). There was an increase in proline concentration according to the quadratic root (for VM-01) and quadratic model (for I-144) (Fig 2a). Leucine concentration had no adjustment for the models tested (without adjustment- w.a.; Fig 2b). A significant linear increase in lysine concentration was observed in VM-01 but not in I-144 (Fig 2c). For arginine, there was quadratic behavior for both clones (Fig 2d). There is evidence that proline is the largest component of N flow in both xylem and phloem (Brugiere et al., 1999). Among the free amino acids, proline is most abundantly present, and arginine shows wide variation and a tendency to decrease when free amino acid concentrations are low (Srivastava and Singh, 2005). Proline may also be accumulated in stressed trees (Vance and Zaerr, 1990) as temperature increases (Durzan, 1995). Arginine has a high N:C ratio (2:3) and together with asparagine (1:2) acts as a compound for greater N storage in higher plants (Forde and Lea, 2007). These amino acids are predominant as forms for N transport and storage in apple trees (Sircelj et al., 1999). Storage proteins are extremely rich in arginine, the amino acid with the highest N concentration (Canovas et al., 2007). Thus, in the nutrient solution without N (rate 0), there was nearly a complete reduction (~100 %) in arginine concentrations in the roots of both clones (Fig 2d), which indicates the sensitivity of arginine for representing N deficiency. When arginine N is mobilized for normal leaf growth, the most immediate metabolic product is omithine, which is recycled back to arginine or converted to proline and urea (Durzan, 1995). It has been proposed that arginine could reflect the N status of trees better than other parameters used in herbaceous plants (Edfast et al., 1996), and thus its levels in plants have been used as an indicator of N status in peach (Taylor and Van den Ende, 1969), grape (Kliewer and Cook, 1974), and pistachio (Durzan, 1995). In citrus, a reduction in arginine concentration is used to establish N deficiency (Srivastava and Singh, 2005), as was observed in this study (Fig 2d). In *E. grandis* leaves, Ferreira (1986) observed accumulation of arginine with the isolated supply of N, P, and S. Asparagine also appears to be a sensitive indicator of N availability in roots (Fig 3a). Asparagine was influenced by N rates in both clones, with its concentration fitted to the quadratic model for VM-01, but for I-144, there was no significance for tested models (Fig 3a). In oilseed rape leaves, Seifferta et al. (2004) also found an increase in asparagine

Table 1. Amino acids in the roots of eucalyptus clones not statistically (F test, p>0.05) influenced by interaction between N rates in the nutrient solution and clones.

Clone	Rate of N (mmol L^{-1} of NH_4NO_3)					
	0	0.74	2.93	4.39	5.85	8.00
	----------Amino acids (%)*----------					
			Alanine[a]			
VM-01	18	52	100	77	95	101
I-144	40	105	100	140	124	93
			Serine[b]			
VM-01	112	136	100	135	114	112
I-144	81	90	100	68	79	60
			Valine[c]			
VM-01	87	93	100	232	111	112
I-144	97	61	100	98	155	61
			Threonine[a]			
VM-01	57	102	100	140	127	102
I-144	43	107	100	75	111	73
			Isoleucine[b]			
VM-01	83	82	100	152	145	144
I-144	30	45	100	74	60	52
			Aspartate[b]			
VM-01	85	205	100	165	180	243
I-144	37	56	100	34	40	59
			Glutamine[a]			
VM-01	27	62	100	153	100	105
I-144	13	20	100	132	92	70

* % in relation to control rate (2.93 mmol L^{-1} of NH_4NO_3). [a, b]Influenced statistically (F test, p≤0.05) only by N rates and only by clones, respectively. [c]Not statistically (F test, p>0.05) influenced nor by N rates or by clones.

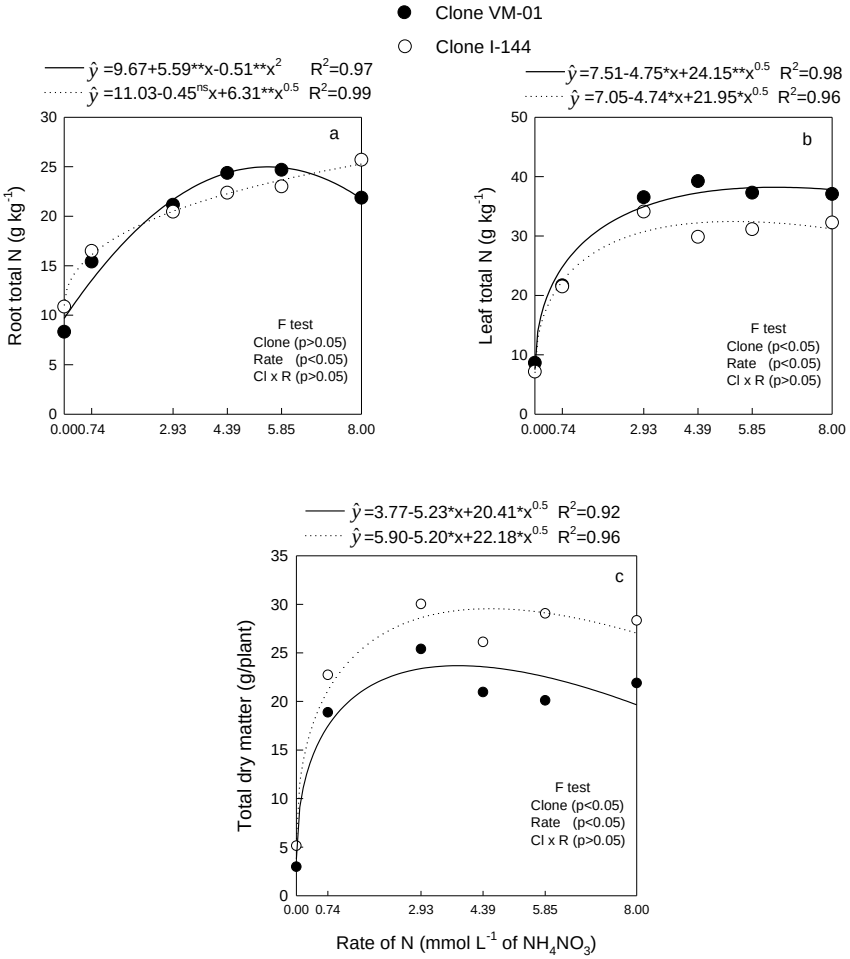

Fig 1. Concentrations of total N in roots (a) and in leaves (b) and total dry matter (c) of young eucalyptus clones under N rates in a nutrient solution. [ns], *, and ** indicate, respectively, not significant, significant at 5 and 1 % by the F test (p<0.05). Cl (clone) and R (rate). Values (%) are in relation to the control rate (2.93 mmol L^{-1} of NH_4NO_3).

Table 2. Amino acids in the leaves of eucalyptus clones not statistically (F test, p>0.05) influenced by interaction between N rates in the nutrient solution and clones.

	Rate of N (mmol L^{-1} of NH_4NO_3)					
Clone	0	0.74	2.93	4.39	5.85	8.00
	----------Amino acids (%)*----------					
	Valine[a]					
VM-01	90	87	100	103	94	168
I-144	103	89	100	111	98	105
	Leucine[b]					
VM-01	50	82	100	138	130	165
I-144	38	84	100	131	162	145
	Isoleucine[b]					
VM-01	55	66	100	108	154	168
I-144	30	90	100	117	150	131
	Phenylalanine[b]					
VM-01	80	104	100	99	194	201
I-144	32	108	100	147	206	188

* % in relation to control rate (2.93 mmol L^{-1} of NH_4NO_3). [a]Not statistically (F test, p>0.05) influenced nor by N rates or by clones. [b]Influenced statistically (F test, p≤0.05) only by N rates.

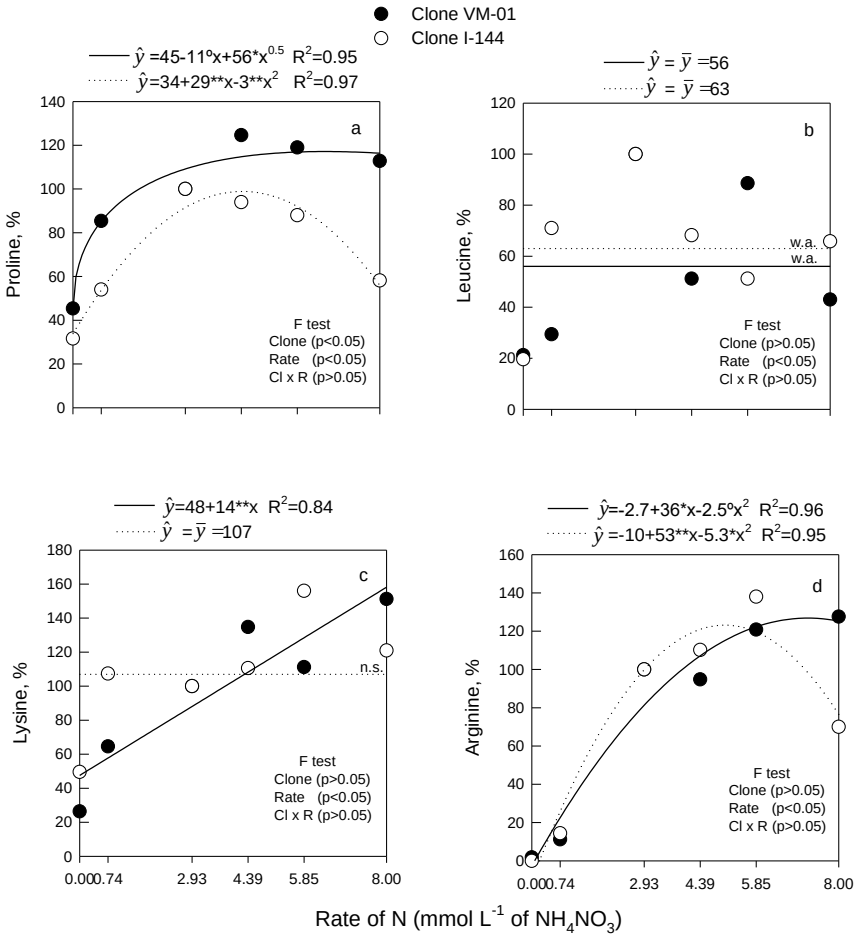

Fig 2. Amino acids proline (a), leucine (b), lysine (c), and arginine (d) in roots of eucalyptus clones under N rates in the nutrient solution. n.s., °, *, and ** indicate, respectively, not significant, significant at 10, 5, and 1 % by the F test (p<0.05). Cl (clone), R (rate) and w.a. (without adjustment). Values (%) are in relation to the control rate (2.93 mmol L^{-1} of NH_4NO_3).

concentration with an increase in the N supply. Asparagine is a key metabolite for N transport in plants (Lea et al., 2007) and is a more efficient N carrier than glutamine because of its higher N:C ratio (0.5 vs 0.4) (Seifferta et al., 2004; Canovas et al., 2007). It is the predominant constituent of free amino acids found in *Citrus unshiu* fruits, which showed a direct relation to leaf N status (Kato, 1983). *A. comosus* (bromelia) accumulated mainly this amino acid when exposed to N (Endres and Mercier, 2001). Glutamate concentrations were statistically unchanged with N growth conditions for both eucalyptus clones (Fig 3b). Forde and Lea (2007) suggested that at different stages of growth there may be great variation in the level of soluble amino acids, but glutamate varies less than other amino acids, particularly glutamine. For clone VM-01, the greater glutamine (Table 1) and glutamate (Fig 3b) concentrations in plants grown at a higher N rate are in accordance with greater NH_4^+ concentrations (Ferreira et al., 2015a). High NH_4^+ concentration can be toxic to plants (Marschner, 2012) and thus it is incorporated into glutamine and glutamate (Ireland and Lea, 1999). Endres and Mercier (2001) found higher concentrations of free amino acids in bromelia genotypes when N was supplied as NH_4^+ (50 mmol L^{-1} of N), and they suggested that this result could reflect a strategy by these plants to avoid toxic effects. Methionine concentrations increased linearly for both clones with an increase in N supply (Fig 3c). Methionine was one of the six dominant amino acids in *E. blakelyi* leaves (Journet and Cochrane, 1978). In our study, N rates in clone I-144 did not have an effect on phenylalanine, tyrosine, and tryptophan concentrations, whereas for VM-01, these amino acids were influenced by N application (Fig 3d, 3e, 3f). For VM-01, there was no adjustment for phenylalanine and tryptophan, and an increase in the N rates led to a linear increase in tyrosine concentration. For *E. globulus* (12 months old) under water deficit, the phenylalanine concentration in phloem sap increased at the expense of glutamine, suggesting a change in resource partitioning within the free amino acid pool (Merchant et al., 2010). A decrease in tyrosine concentration was also found in chamomile leaf (*Matricaria chamomile*) exposed to N deficiency (Kovácik et al., 2006).

Shoot amino acids

In leaves of our clones, only the concentration of valine was statistically unaffected by N rates (Table 2). Merchant et al. (2010) noted changes in amino acid concentration in the phloem sap, but not in leaf concentrations in *E. globulus*, and they concluded that leaf amino acids were poor predictors of water stress. This fact indicates that under those conditions, the amino acid concentration in the phloem sap was more sensitive to drought than amino acid concentration in the leaf. However, considering both clones together, the concentrations of leucine, isoleucine, and phenylalanine were statistically affected by N rates (Table 2). There was an effect of N application on the concentration of all other amino acids evaluated in the leaves of the clones (Fig 4 and 5). The N taken up is incorporated into the plant as amino acids, with an increase in the N supply; the synthesized proteins promote leaf growth by increasing the photosynthetic area (Dechen and Nachtigall, 2007). In general, with an increase in the N supply, there was an increase in the concentration of most of the amino acids in the leaves (Fig 4 and 5). This fact was associated with a higher total N concentration (Fig 1b) and greater protein synthesis (Ferreira et al., 2015b) and photosynthetic rate (Ferreira et al., 2015c), which resulted in an increase in the total dry matter production of the clones (Fig 1c) since the amino acids taken up are sources of C skeletons for plant metabolism (Majerowicz and Kerbauy, 2002). The smaller concentrations in leaf amino acids of the plants grown at lower N rates (0 and 0.74 mmol L^{-1} of NH_4NO_3) were also observed through visual symptoms of generalized chlorosis, since total N concentration (Fig 1b) and chlorophyll levels were both lower (Ferreira et al., 2015a). Chlorosis is developed following the removal of the sink, causing depletion in total N, proline, arginine, and serine (Schaffer et al., 1986). For aspartate, N rates did not alter concentrations in the clone VM-01, whereas for I-144 there was a linear increase in aspartate concentration (Fig 4a). Aspartate is generated by transamination from glutamate (Canovas et al., 2007) and there is a major metabolic demand for it in the cytosol as a substrate for asparagine biosynthesis (Azevedo et al., 2006). Glutamine concentrations were influenced by the N supply for both clones, with a linear increase in I-144, but for VM-01 there was no adjustment (Fig 4b). Glutamine synthetase (GS) combines NH_4^+ with glutamate for synthesis of glutamine (Forde and Lea, 2007). Thus, in general, the highest levels of glutamine (Fig 4b) were associated with higher levels of NH_4^+ (Ferreira et al., 2015a) and glutamate (Fig 4c), but not associated with GS activity in the leaves of the clones (Ferreira et al., 2015b). Seifferta et al. (2004) also found that glutamine concentrations increased significantly in oilseed rape leaves with an increase in N supply. Glutamine represented up to 50 % of the total amino-N pool in the phloem sap of *E. globulus* (Merchant et al., 2010). Ferreira (1986) observed a substantial reduction in leaf glutamine concentration of *E. grandis* when the nutrients N, P, and S were supplied in an isolated manner to the root system. Sircelj et al. (1999) found an increase in glutamine concentrations in leaves of apple cultivars under drought stress. Glutamate concentration was not significantly altered by N rates for VM-01 but it increased linearly for I-144 (Fig 4c). Reduction in glutamate concentration has been quite effective in establishing N deficiency in citrus (Srivastava and Singh, 2005). Glutamate is one of the most abundant amino acids in the soil as it is a component of dissolved organic N (DON) and a constituent of root exudates (Paynel et al., 2001). There was large predominance (approx. 40 %) of glutamate in oilseed rape leaves, especially at the lower N application rate (Seifferta et al., 2004). Glutamate is also a direct precursor of proline (Mjza-Basso et al., 1986). For tyrosine concentrations of the current study, a linear increase was found for both clones with the increase in N rates (Fig 4d), and thus it well represented the supply of this nutrient for the clones. Our results also showed an increase in alanine concentrations for both clones with increasing N supply (Fig 5a). Other researchers have also found alanine to be a sensitive indicator of N supply. Castillo et al. (2010), studying the classification of 28 genotypes of *E. globulus* under cold stress conditions, found the highest correlation among percent of foliar damage and the amino acids tyrosine, arginine, and alanine. In water-stressed *E. tereticornis*, Marsh and Adams (1995) also found significant increases in alanine concentrations (plus glutamate). Alanine synthesis is known to play a key role in the response to hypoxia or anoxia (Ricoult et al., 2006). In our study, serine and proline concentrations in leaves showed no significant changes for VM-01 but a linear increase for I-144 with an increase in the N supply (Fig 5b, 5c). In other studies, an increase in the concentration of leaf proline was a useful biochemical marker associated with N deficiency in citrus (Srivastava and Singh, 2005). After cold treatment, an eight- to ten-fold increase was observed in proline levels with a decrease in glutamate in *Nothofagus dombeyi* leaves (Mjza-Basso et al., 1986). Proline was one of the six dominant

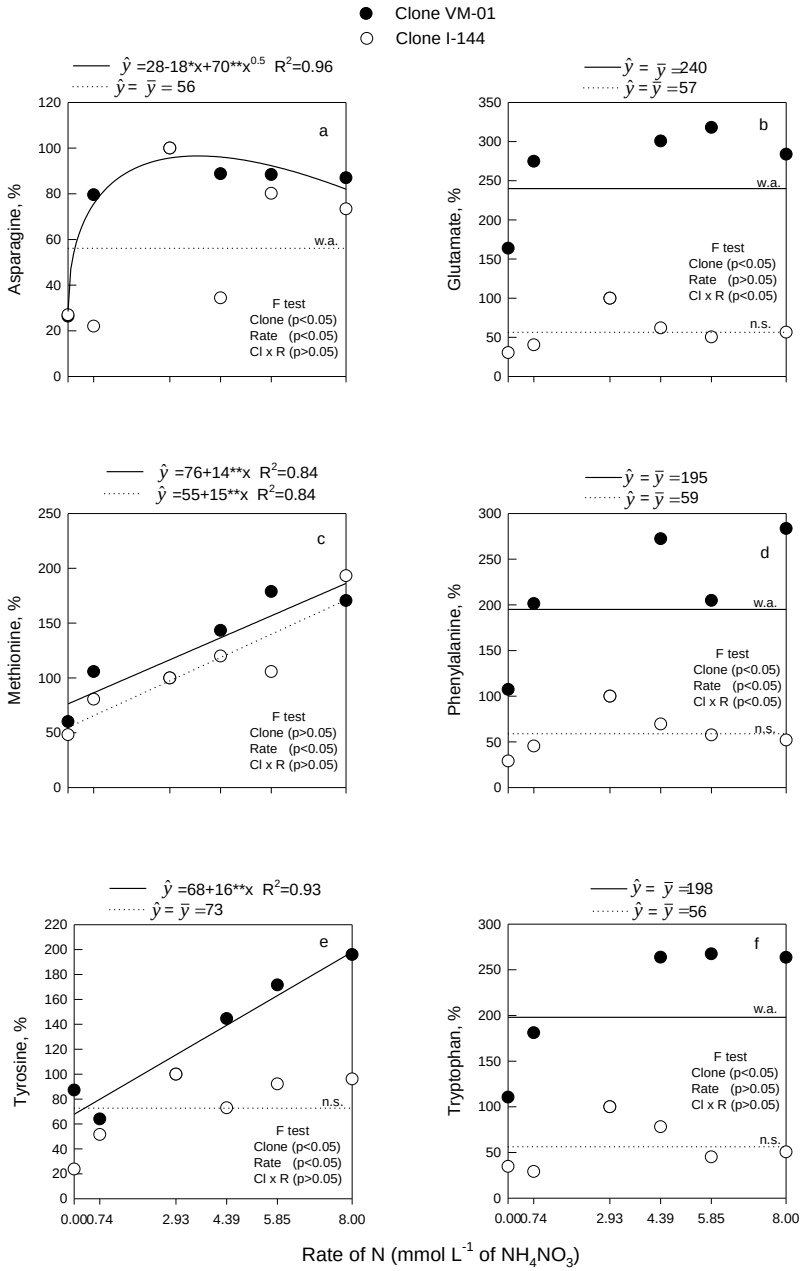

Fig 3. Amino acids asparagine (a), glutamate (b), methionine (c), phenylalanine (d), tyrosine (e), and tryptophan (f) in roots of eucalyptus clones under N rates in the nutrient solution. $^{n.s.}$, * and ** indicate, respectively, not significant, significant at 5 and 1 % by the F test ($p<0.05$). Cl (clone), R (rate) and w.a. (without adjustment). Values (%) are in relation to the control rate (2.93 mmol L^{-1} of NH_4NO_3).

amino acids in *E. blakelyi* leaves (Journet and Cochrane, 1978), and an increase in its concentration was seen in leaves of apple cultivars under drought stress (Sircelj et al., 1999). For serine (plus threonine), Marsh and Adams (1995) observed a significant increase in its concentration in water-stressed *E. tereticorni*. Threonine concentrations observed in our study decreased with lower N availability in both clones (Fig 5d). A decrease in serine and threonine levels was found in chamomile leaf (*Matricaria chamomile*) exposed to N deficiency (Kovácik et al., 2006). Finally, for methionine and tryptophan in clone VM-01, there was a linear increase with an increase in the N supply; in contrast, for I-144 a quadratic adjustment was observed for the first amino acid and no adjustment for the second one (Fig 5e, 5f). In summary, the composition of some amino acids in both roots and shoot was significantly affected by different rates of N supply in eucalyptus clones. In fact amino acid concentrations were sometimes more sensitive indicators than total N. This result suggests that they may be useful early indicators of N status in eucalyptus clones, a topic that warrants additional future research.

Materials and Methods

Plant materials

We used fifty day old seedlings (at the beginning of the trial) of eucalyptus clones (VM-01 and I-144) coming from tubes. Clone VM-01 is a hybrid of *E. camaldulensis* and *E. urophylla*, and I-144 is an *E. urophylla* alone.

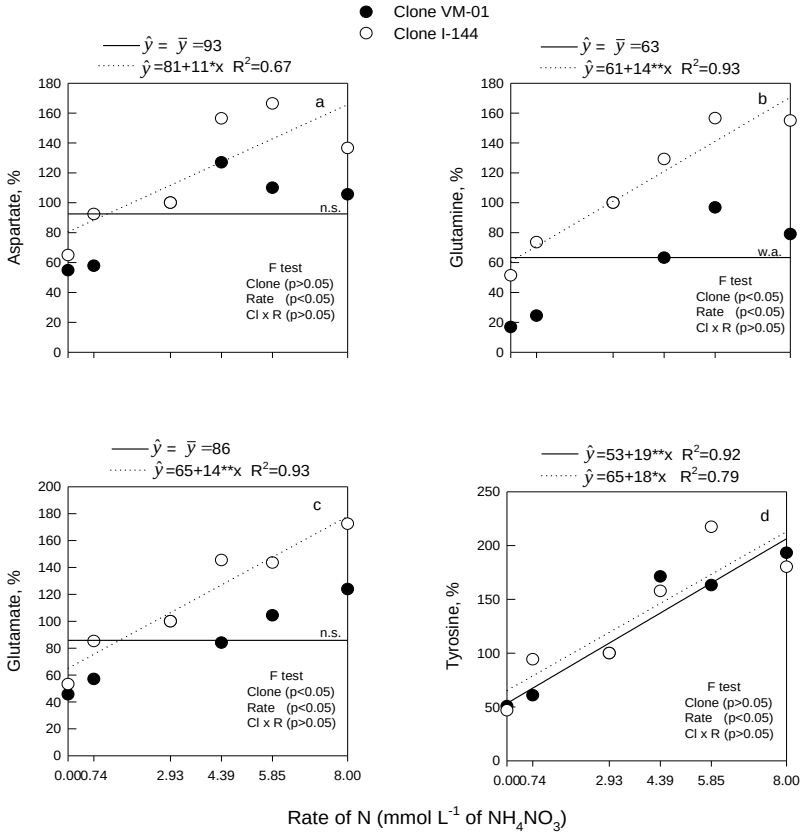

Fig 4. Amino acids aspartate (a), glutamine (b), glutamate (c), and tyrosine (d) in leaves of eucalyptus clones under N rates in a nutrient solution. $^{n.s.}$, * and ** indicate, respectively, not significant, significant at 5 and 1 % by the F test ($p<0.05$). Cl (clone), R (rate) and w.a. (without adjustment). Values (%) are in relation to the control rate (2.93 mmol L^{-1} of NH_4NO_3).

Study location

The experiment was carried out in a greenhouse at the Department of Soils of the Universidade Federal de Viçosa, Viçosa, MG, Brazil (20º45'14"S, 42º52'53" W), over three months (from July to September 2011). The first month was for acclimatization of the plants in the nutrient solution and the two following months for application of the treatments.

Plant acclimatization phase

During acclimatization, the concentration of the nutrient solution (Clark, 1975) was increased weekly (to 25, 50, 75, and 100 % of the original concentration) in collective trays (11 L). After that, two homogenous plants were selected and transferred to individual pots (6 L) and the treatments were applied, as describe below.

Treatments and experimental procedure

A randomized block experimental design in a 2 × 6 factorial arrangement with five replications was used, the treatments being two eucalyptus clones (VM-01 and I-144) and six N application rates (0, 0.74, 2.93, 4.39, 5.85, and 8 mmol L^{-1} of NH_4NO_3).We used Clark's solution (Clark, 1975) adapted to contain NH_4^+/NO_3^- ratio equal 1 (Locatelli et al., 1984) and twice P concentration (Caldeira et al., 1994). More details can be found in Ferreira (2013). The nutrient solution had permanent aeration; it was changed weekly with deionized water and its pH was monitored daily and maintained at 5.5 (±0.05) by adding NaOH or H_2SO_4 (0.1 mol L^{-1}). Plants were harvested sixty days after application of the treatments. The organs (roots, stems, branches, and leaves) were separated and washed in deionized water and then placed in a laboratory oven (60 ºC) for 72 h to obtain dry matter.

Laboratory analyses

Leaves and roots were ground, and total N was determined by the Kjeldahl method (Bremner, 1996). We also collected leaves (without the midrib) from middle branches, and root tips of the plants for amino acid analysis, put them in Al foil, and kept them in liquid N until placing them in a freezer at -80 ºC. These samples were ground in liquid N and stored once more in a -80 ºC freezer. They were subsequently lyophilized to be taken to the chemistry laboratory of Duke University (USA). These samples were weighed (~100 mg) into Eppendorf tubes and 1 mL of ultrapure water was added to each sample and then they were placed in a vortex mixer for 2 min. The following day, the samples were centrifuged at 5,000 rpm for 10 min, 0.5 mL of ultrapure water was added, and then the supernatant was filtered through a 0.45 µm nylon syringe filter with the aid of a vacuum pump. After that, 150 µL of the supernatant was transferred into a vial for analysis. When out of the -80 ºC freezer, the samples were always kept on ice. All possible underivatized free amino acids were determined by high performance liquid chromatography with detection by mass spectrometry (HPLC/MS method) (Özcan and Senyuva, 2006). This method provided useful results for most of the amino acids but not all; the method was not able to detect glycine and cysteine in the samples. There were no readings for some amino acids, such as hydroxy-proline, histidine, and cystine (roots and leaves) and asparagine, lysine, and arginine

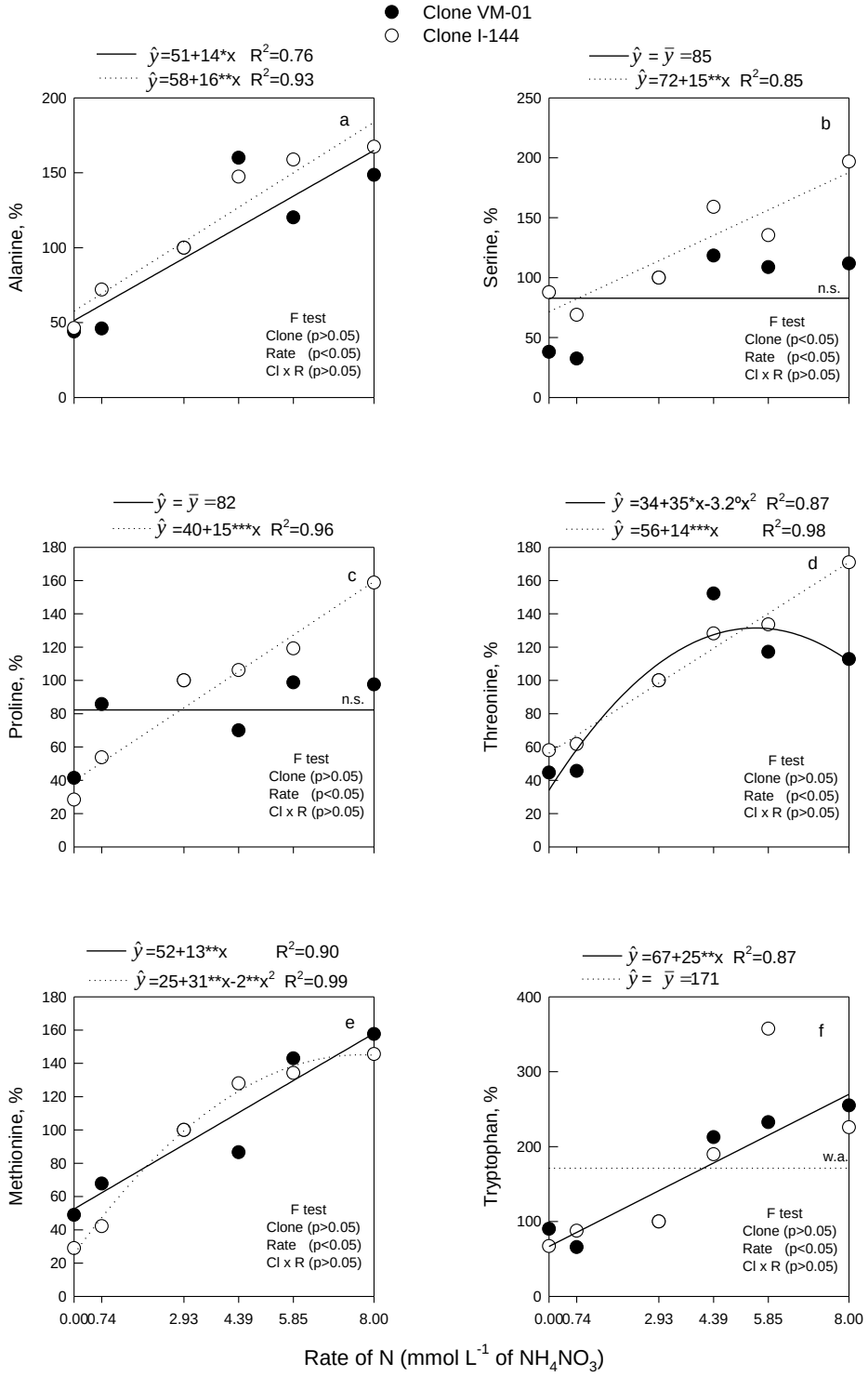

Fig 5. Amino acids alanine (a), serine (b), proline (c), threonine (d), methionine (e), and tryptophan (f) in leaves of eucalyptus clones under N rates in the nutrient solution. $^{n.s.}$, *, **, and *** indicate, respectively, not significant, significant at 5, 1, and 0.1 % by the F test ($p<0.05$). Cl (clone), R (rate) and w.a. (without adjustment). Values (%) are in relation to the control rate (2.93 mmol L^{-1} of NH_4NO_3).

(leaves) in most of the samples. So, we assumed that they were below the level of detection, which means that they were absent or present only at very low concentrations. Thus, we assessed the amino acids alanine, serine, proline, valine, threonine, leucine, isoleucine, asparagine, aspartate, glutamine, lysine, glutamate, methionine, phenylalanine, arginine, tyrosine, and tryptophan. All samples were analyzed in duplicate and each sample was examined individually for each compound to access whether the component was present or not, and at what level. We used two criteria to decide if an amino acid was present: first, did it have the correct exact mass within 50 mg L^{-1}, and second, did the compound elute from the HPLC column with the same retention time as the standard compound within 0.03 min. Single injections of one blank and one standard were run after every five samples to test whether the analytical procedure was working. The

relative concentration for a particular amino acid was based on the intensity of the peak area. The peak area for each amino acid was divided by the mass of its sample to provide a relative amount. Since it was not possible to make a calibration curve, all the amounts are relative to each amino acid; thus we divided the prior relative amount of each amino acid of all treatments (N rates) by the relative amount of the same amino acid found in the reference treatment. We chose the rate of 2.93 mmol L^{-1} of NH_4NO_3 as a reference treatment because it is the rate used in Clark's solution, and it was also at this rate that we found the peak values for most of the growth variables (Ferreira et al., 2015c). Thus, the values for all amino acids were presented as percentage in relation to the reference treatment, which was standardized as 100 %.

Statistical analyses

We performed analysis of variance (F test) for all the data with SAS software (SAS, 2004) to evaluate the effects of the treatments on relative concentrations of each amino acid in relation to the reference treatment. For the amino acids that were not influenced ($p>0.05$) by interaction between N rates and clones, we only showed the mean values in a table, and the other amino acids that were statistically influenced ($p\leq0.05$) by N rates or by interaction between N rates and clones we showed them on graphs with regression equations. For the latter, we fitted linear, quadratic, and quadratic root equations; and we chose the model ($p\leq0.05$) that showed the highest determination coefficient (R^2).

Conclusion

Our results demonstrated that there is great variation in amino acid concentration as a function of the N supply, and that this variation depends on the organ (root or leaf) and the clone evaluated. We also observed greater number of amino acids in roots than in leaves (17 and 14, respectively), most likely due to higher amino acid synthesis or translocation to the roots. For both clones, N deficiency induces a significant decrease in the concentrations of proline, arginine, and methionine in roots and tyrosine, alanine, threonine, and methionine in leaves. It is noteworthy that this decrease is also likely associated with lower total N concentration and total dry matter of the eucalyptus clones.

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