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Omission and Resupply of Nitrogen Affect Physiological and Enzymatic Activities and the Gene Expression of Eucalypt Clones

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ABSTRACT: The mineral nutrient uptake of plants in the field occurs in pulses, due to variations in the substance concentrations at the root surface. The fluctuations in nutrient supply probably induce changes in the plant, which are to date unknown for *Eucalyptus*. This study evaluated these changes in plant growth, nutritional status, photosynthesis, and gene expression, which can serve as biomarkers of the nitrogen status, of four eucalypt clones exposed to N omission and resupply. A greenhouse experiment with four *Eucalyptus* clones was installed, and after initial growth exposed to N omission for 21 d, followed by N resupply in nutrient solution for 14 d. Nitrogen omission decreased the total N and photosynthetic pigments, net photosynthesis and photochemical dissipation, and increased enzyme activity especially in leaves and the gene expression in leaves and roots. Nitrogen resupply decreased these variations, indicating recovery. The total N concentration was highly and significantly correlated with net photosynthesis, enzyme activity, expression of genes GS2;1 and Gln1;3 in the leaves and AMT1;2 in the roots, contents of chlorophyll *a* and *b,* and photochemical energy dissipation. The enzymes GS and NR in the leaves and the genes AMT1;2, GS2;1 and Gln1;3 proved to be sensitive N indicators.

Keywords: nutritional efficiency of plants, fertilization and nutrition of perennial crops, plant tolerance to stress.

INTRODUCTION

Eucalyptus is the most important forest crop in Brazil, with an acreage of nearly 7 million hectares (IBGE, 2015), most of which is planted on highly weathered soils. Due to the chemical poverty, fertilization is required to ensure satisfactory yields. However, owing to variations in the nutrient transport processes and maintenance of nutrient concentrations in the rhizosphere, caused by seasonal changes in soil moisture and temperature, the nutrient uptake of plants under field conditions occurs in pulses (Veneklaas et al., 2012). This oscillation induces physiological, metabolic and molecular changes in the processes related to mineral plant nutrition, which may vary according to the genotype (Hirel et al., 2007; Liu et al., 2009; Nunes, 2010). In *Eucalyptus*, Mollica (1992) found differences in the efficiency of nutrient uptake and use of several clones in the same environment. In view of the wide genetic diversity of the genus *Eucalyptus*, these genotypic variations should be investigated in more detail, with a view to identify nutritionally more efficient varieties.

Nutritional efficiency (NE) is a complex parameter, which is best defined by an interdisciplinary approach (Hirel et al., 2007; McDonald et al., 2013). Studies that address a combined assessment of the nutritional status, growth and metabolism are very useful tools for breeding, as a guide in the selection of the most efficient varieties (Yan et al., 2006; McDonald et al., 2013). Little is known about the mechanisms involved in the control of NE of *Eucalyptus* and the plant strategies, especially in response to stress conditions, to ensure a maximal NE.

Nitrogen is absorbed as NO_3^- or NH_4^+ and, for *Eucalyptus*, growth is greater when N is supplied at a higher proportion than NH_4^+ (Locatelli et al., 1984). No toxicity has been reported caused by the dissipation of the electrochemical transmembrane gradient or the need for efflux of excess NH_4^+ (Britto and Kronzucker, 2002). However, NO_3^- can be stored in larger amounts in the vacuole and be used in periods when N supply is interrupted (Srivastava and Singh, 2006).

The regulation pathways of N metabolism begin with the uptake through protein transporters. NO_3^- is taken up by protein NRT1, and NH_4^+ by AMT1;2. Once absorbed, NO_3^- must be reduced, and the first reaction of this pathway is catalyzed by nitrate reductase (NR), regulated at the transcriptional or post-translational level (Genenger et al., 2003). After the NR action, the enzyme nitrite reductase catalyzes the reduction of nitrite to NH_4^+, which together with NH_4^+ of photorespiration or root uptake, will be assimilated by the action of the enzyme glutamine synthetase (GS), forming glutamine, the main donor of the amino group to other organic compounds (Marschner, 2012). In the regulation of this process, the genes GS2;1 and Gln1;3 (Tabuchi et al., 2002; Martin et al., 2006; Bi et al., 2007; Hirel et al., 2007) are particularly important. Part of NH_4^+ can be stored in the vacuole through aquaporin encoded by gene TIP2;1 (Rawat et al., 1999; Ludewig et al., 2007).

Once assimilated, N is part of several metabolic processes in plants, mainly for photosynthesis, which is directly related to the N status in the plant, since about 80 % of N in the leaves is contained in chlorophyll and in rubisco (Field and Mooney, 1986; Langsdorf et al, 2000). Thus, N deficiency leads to a drop of the photosynthetic potential of plants, causing reduced growth and oxidative stress (Lima et al., 2000).

Thus, it is expected that fluctuations in the supply of N will induce morphological, physiological and molecular changes at different intensities and velocities heretofore unknown to the Eucalyptus. The purpose of this study was to evaluate changes in growth, nutritional status, photosynthesis and gene expression, which can serve as biomarkers of the N status of four eucalypt clones in response to N omission and resupply.

MATERIALS AND METHODS

The experiment was conducted in a greenhouse with a mean temperature of 29 °C. Seedlings of four approximately 45-day-old *Eucalyptus* clones (I-042, I-144, VM-01 and

386) were used. Clone VM-01 is a *Eucalyptus urophylla* vs *E. camaldulensis* hybrid, and the others were hybrids of *Eucalyptus urophylla* vs *E. grandis*. The seedlings were placed in polyethylene pots with 14 L of Clark (1982) nutrient solution modified by Locatelli et al. (1984), for 60 d. Thereafter, two nutritional treatments were applied: 1) solution with continuous presence (control) of N and 2) solution with N omission (weeks 1, 2 and 3) and subsequent resupply (weeks 4 and 5). The constantly aerated nutrient solution was exchanged weekly, and the pH was adjusted daily to 5.5, using 1 mol L^{-1} HCl or NaOH. The experiment was arranged in a randomized block design with four replications per treatment, with a total of 48 experimental units, consisting of one pot with three plants.

Net photosynthesis and photochemical dissipation

Net photosynthesis (*A*) and chlorophyll *a* fluorescence (Chl *a*) were assessed five times (weeks 1 to 5) in one young fully expanded leaf (leaf diagnosis) per plant. The measurements of *A* were performed between 8:00 and 11:00 am, with an infrared gas analyzer (IRGA; LI-6400XT; LI-COR Inc., Lincoln Nebraska, USA). The photosynthetically active radiation (*PAR*) was constant at 1,400 µmol m^{-2} s^{-1}, predetermined in luminous curves for these clones, at an atmospheric CO_2 concentration of about 390 µmol mol^{-1}, environment temperature 25 to 29 °C, leaf temperature 22 °C, and air humidity 45 to 58 %. With the same device, equipped with a fluorometer, the fluorescence of Chl *a* was measured, obtaining the data of the dark and light phases of fluorescence. We decided to present only the data of the effective quantum yield of photochemical energy conversion in photosystem II - FS_{II} ($\hat{Y}_{II}$ = (Fm'-F)/Fm') (Genty et al., 1989; Hendrickson et al., 2004), where F is the fluorescence of a light-adapted sample before the saturation pulse and Fm' is the maximal fluorescence of a light-adapted sample (Fm').

Photosynthetic pigments

The diagnostic leaf was collected in the form of discs (diameter 10 mm) and the weight was determined. One disc was subjected to pigment extraction in the dark and by heat, with $CaCO_3$-saturated dimethyl sulfoxide (DMSO), for 2 h (Wellburn, 1994). The extracts were read by molecular absorption spectrometry (Thermo Scientific Evolution 60) to calculate the contents of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids.

Total nitrogen concentration and nutritional efficiency

Samples of eight diagnosis leafs were removed in the first, third, fourth, and fifth week and eight older leaves in the third and fifth week. At the end of the experiment, the plants were cut and separated in roots, stems and leaves, and separately dried, weighed and ground. These samples were subjected to sulfur digestion, followed by distillation and titration (Nelson and Sommers, 1973), to determine the total N concentration. From the N concentration and organ weight, the N concentrations in the plants were calculated, and the following nutritional efficiencies: a) nitrogen uptake efficiency (NUpE - mg g^{-1}): N content per pot (in mg)/ root dry matter weight (g) - (Swiader et al., 1994); b) N use efficiency (NUsE - g^2 g^{-1}): (dry matter weight per pot)^2 (in g^2)/N content (in g) (Siddiqui and Glass, 1981). The relative N retranslocation index (NRetI - %) was also calculated [N concentration of the old leaf in the control treatment - N concentration of the old leaf in the N treatment (g kg^{-1})]/ N concentration of the old leaf in the control treatment [(g kg^{-1}) × 100].

Other samples of diagnostic leaves and young roots were taken in the first, third, fourth and fifth week. These samples were frozen in liquid N_2 and stored at -80 °C to assess the enzyme activity and gene expression.

Enzyme activity

The extraction was carried out (Radin, 1974; Cambraia et al., 1989) with modifications for *Eucalyptus*: leaf and root samples of 0.2 g were ground in liquid N_2 in a mortar, with 0.2 g and 0.02 g PVPP (polyvinyl pyrrolidone), respectively. Then 2 mL extraction medium,

consisting of 0.1 mol L^{-1} tris-HCl buffer; pH 8.1; 4 mmol L^{-1} NiSO$_4$; 20 mmol L^{-1} reduced glutathione; and 20 µmol L^{-1} phenylmethanesulfonyl fluoride (PMSF) were added. The extracts were filtered and centrifuged at 15,000 g, for 15 min at 4 °C.

The activity of NR (NR - EC 1.0.0.1) was determined *in vitro* (Radin, 1974; Cambraia et al., 1989). Aliquots of 0.1 mL of the enzyme extract were added to 1.8 mL of a reaction medium consisting of: 200 mmol L^{-1} Tris-HCl buffer; pH 7.5; 20 mmol L^{-1} KNO$_3$; 0.1 mmol L^{-1} nicotinamide adenine dinucleotide hydride (NADH); and 1 % (v/v) triton X-100 and incubated together at 37 °C for 5 min. The reaction was interrupted by adding 2 mL of 1 % (v/v) sulfanilamide with 0.01 % (v/v) N-naphthyl ethylenediamine in 1.0 mol L^{-1} HCl. The NO$_2^-$ production was determined by molecular absorption spectrometry at 540 nm.

The GS activity (GS - EC 6.3.1.2) was assessed (Elliott, 1953) by adding 0.3 mL of the extract to 0.7 mL reaction medium, consisting of: 100 mmol L^{-1} Tris-HCl buffer; pH 7.5; 10 mmol L^{-1} 2-mercaptoethanol; 40 mmol L^{-1} MgSO$_4$.7H$_2$O; 10 mmol L^{-1} NH$_2$OHCl; 10 mmol L^{-1} ATP; and 50 mmol L^{-1} glutamate. The medium was incubated in a water bath for 5 min at 37 °C and aeration was stopped by adding 1.0 mL of the solution of 370 mmol L^{-1} FeCl$_3$; 370 mmol L^{-1} HCl and 200 mmol L^{-1} trichloroacetic acid (TCA). The sample was centrifuged for 5 min at 16,000 g and the supernatant was quantified by molecular absorption spectrometry at 540 nm.

The enzymatic activities were expressed based on the total protein content (Bradford, 1976). The control used for the enzymes was the sample to which the reaction-stopping solution was added prior to the leaf extract, followed by the other procedures.

Gene expression

The sequences of the selected genes (Table 1) were obtained from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Egrandis), the primers were established as described by the program Primer Blast, and gene expression was evaluated by the quantitative technique Real time (RT-qPCR) in a thermal cycler Bio-Rad-100.

All 256 samples were ground in liquid N$_2$, and approximately 0.5 g was removed and placed in microcentrifuge tubes (2.0 mL), to which 1.2 mL extractor (plant RNA extraction kit-Invitrogen) was added. The sample was homogenized, left to stand for 10 min at 4 °C, and centrifuged at 13,000 rpm for 5 min at 4 °C. The supernatant was collected and re-centrifuged. Thereafter, 1.0 mL of the extract was transferred to a new tube with 150 µL NaCl 5 mol L^{-1} and 450 µL chloroform P.A and centrifuged at 13,000 rpm, for 10 min at 4 °C. The supernatant was collected and an equal volume of isopropanol P.A. (~800 uL) added. The material was centrifuged at 13,000 rpm for 10 min at 4 °C, and the supernatant discarded, preserving the RNA pellet. This was centrifuged again with 1.0 mL 75 % alcohol at 13,000 rpm for 1 min at 4 °C. Thereafter, the pellet was dried in a vacuum desiccator for 10 min and resuspended in 40 µL of DEPC (diethyl pyrocarbonate) water. The RNA was quantified (QuBit), the concentration standardized at 200 µg mL^{-1} and the physical integrity assessed by 1.5 % agarose gel electrophoresis.

Table 1. Primers used for RT-PCR

Gene	Accession NCBI[1]	Fw/Rv 5′-3′ Primers
TIP2;1	AT3G16240.1	GATCCATCTGGGCTAGTGGC
NIA1	AT1G77760	TGTCCCAATTCATCACCGGG
GLn1;3	AT3G17820.1	GTGCAGACAAGGCTTTTGGG
GS2;1	AT1G66200.2	GATCCTTTCCGAGGTGGCAA
NRT1;1	AT1G12110	TGGTCCCGCAGTTCTTCTTC
AMT1;2	AT1G64780	TCATGCTCACCAACGTCCTC

[1] NCBI: National Center for Biotechnology Information.

This RNA was used for cDNA synthesis. Firstly, the treatment with Deoxyribonuclease I, Amplification Grade (Invitrogen) was applied, and Superscript II (Invitrogen) was used for cDNA synthesis, according to the manufacturer's instructions.

The primers were validated according to the manufacturer of the thermal cycler (Bio-Rad-100) and the constitutive genes of histone H2B and Hist1 chosen as reference. The PCR reactions were composed of 1 µL cDNA; 0.8 µL of 2.5 mmol L^{-1} primer; 3.2 µL water and 5 µL Fast SYBR Green Master Mix. The device Bio-Rad 100 was used, with triplicate readings. The calculations were based on the relative expression ($2^{-\Delta\Delta C_T}$), comparing the Ct (threshold cycle) of each treatment with the respective control and both with the constitutive genes.

Statistical analysis

For evaluations of plant growth, N content, NUpE and NUsE, analysis of variance and the F test were performed, comparing the treatments of N omission/resupply with the control, for each clone, at the end of the experiment. The same procedure was applied for N concentrations and photosynthetic pigments, A, $\hat{Y}_{II}$, and enzyme activity at each evaluation. For NRetI and gene expression analysis of variance was performed and the means between clones compared by the Tukey test.

RESULTS

Nitrogen omission followed by resupply reduced plant growth of clones 386, I-042 and I-144, with the most remarkable drop (50.3 %) for clone 386, compared to its control (Table 2). This was the fastest-growing clone in the control treatment, but also the most affected by N omission, while the mass of clone VM-01 was not affected by the nutritional restriction.

There was a reduction in total N concentration in the diagnosis leaves, in the first week, in the mean 39.2 % and in the third week 55.4 %. After N resupply, in the fourth and fifth week, there was an increase in N concentration of all clones, and the N concentration of plants in the omission treatment was higher than in the control treatment. The N concentrations of old leaves in the omission treatments decreased by 42.4 %.

Table 2. Dry matter weight, concentration total nitrogen in diagnostic and old leaf, and nitrogen content in eucalypt clones (386, I-144, I-042 and VM-01) subjected to nitrogen omission (weeks 1 and 3) and N resupply (weeks 4 and 5) and control treatment

Week	386		I-144		I-042		VM-01	
	Control	N	Control	N	Control	N	Control	N
Dry matter mass (g per pot)								
5	112	56[**]	85	62[**]	83	67[**]	75	73[ns]
Total N concentration - Diagnostic leaf (g kg^{-1})								
1	32.94	18.97[**]	34.72	20.61[**]	33.36	21.63[**]	28.90	17.76[**]
3	33.70	12.87[**]	30.46	15.22[**]	33.94	14.32[**]	33.77	16.20[**]
4	30.94	24.56[**]	30.82	23.28[**]	29.44	22.82[**]	32.01	24.29[**]
5	34.26	35.72[*]	33.34	36.62[**]	33.68	37.46[**]	34.42	36.50[*]
Total N concentration - Old leaf (g kg^{-1})								
3	27.09	15.45[**]	27.78	15.90[**]	26.85	14.62[**]	26.62	16.42[**]
5	25.77	20.96[**]	23.82	20.02[**]	26.91	22.33[**]	23.68	21.22[**]
N content (g per pot)								
5	2.27	1.05[**]	1.82	1.12[**]	1.93	1.31[**]	1.69	1.38[**]

[ns] and [**]: not significant and significant at 1 % probability, respectively, by the F test compared to the control of each clone.

The NUpE decreased under N omission/resupply in all clones, although for clone 386 the difference was minor and insignificant (Table 3). The greatest relative reduction in NUpE (31.4 %) was observed for clone I-042. Nitrogen use efficiency (NUsE) in the control treatment was highest in clone 386, but this clone also had the lowest NUsE in the N omission/resupply treatment. For clone VM-01 on the other hand, NUsE increased by 13.1 %. The values of NRetI differed little between the clones and the lowest value was observed for VM-01.

Nitrogen omission decreased the net photosynthetic rate (*A*) in all clones and clone VM-01 was least affected by N deficiency. For clone 386, *A* decreased most (Table 4), in agreement with the recorded growth data. There was also a decrease in $\hat{Y}_{II}$ values, indicating the amount of energy used in photosynthesis (Table 4). The excess energy of the photochemical processes was dissipated, avoiding photo-oxidative damage, which resulted in drops in electron transport rate (data not shown). The same pattern was observed for the photosynthetic pigments (Table 5).

Table 3. Nitrogen uptake efficiency (NUpE), nitrogen use efficiency (NUsE) and translocation index (NRetI) of nitrogen in four Eucalyptus clones (386, I-144, I-042 and VM-01) in response to nitrogen omission and resupply, and control treatments

	386	I-144	I-042	VM-01
	NUpE (mg g^{-1})			
Control	78.3	81.4	104.0	125.6
N	76.1^{ns}	67.6**	71.3**	93.5**
	NUsE (g^2 mg^{-1})			
Control	5,579	3,982	3,573	3,364
N	2,967**	3,414**	3,436^{ns}	3,805**
	NRetI (%)			
	43.0 a	42.7 a	45.5 a	38.3 b

^{ns} and **: not significant and significant at 1 % probability by the F test, compared to the control treatments; Means followed by the same letter do not differ by the Tukey test at 1 %.

Table 4. The net photosynthesis (A) and effective quantum efficiency of the linear electron flux in photosystem II ($\hat{Y}_{II}$) in Eucalyptus clones subjected to nitrogen omission (weeks 1 and 3) and nitrogen resupply (weeks 4 and 5) and control treatments

Clone	Week	A		$\hat{Y}_{II}$	
		Control	N	Control	N
		μmol m^{-2} s^{-1}			
386	1	20.2	14.7**	0.27	0.17**
I-144		24.4	12.9**	0.26	0.24**
I-042		20.6	14.2**	0.31	0.15**
VM-01		19.2	14.5**	0.25	0.17**
386	2	18.0	7.9**	0.22	0.09**
I-144		16.0	8.4**	0.25	0.11**
I-042		16.7	7.9**	0.22	0.09**
VM-01		17.8	10.1**	0.33	0.17**
386	3	17.0	7.2**	0.19	0.11**
I-144		17.3	7.6**	0.22	0.11**
I-042		17.5	9.1**	0.24	0.11**
VM-01		15.6	9.0**	0.21	0.09**
386	4	17.5	12.4**	0.18	0.18^{ns}
I-144		18.3	13.6*	0.19	0.17^{ns}
I-042		17.8	15.8**	0.19	0.15**
VM-01		14.6	15.0^{ns}	0.17	0.16^{ns}
386	5	20.6	21.1^{ns}	0.22	0.22^{ns}
I-144		19.9	23.2^{ns}	0.21	0.21^{ns}
I-042		20.6	19.6**	0.2	0.26**
VM-01		15.8	20.3**	0.19	0.21**

^{ns} and **: not significant and significant at 1 % probability by the F test, compared to the control treatments.

The activity of enzymes NR and GS was stimulated by N deficiency, with different responses of the clones (Table 6). After N resupply, the activity of these enzymes decreased, similar to their activity in the control treatment, with exception of clone I-144, for which the relative enzyme activity was highest in the fifth week. There was an increase in NR activity, although approximately 62 times lower than that of GS. The sensitivity to N omission of the analyzed genes differed. The greatest change compared to the control was observed for gene Gln1;3 in clone I-042, followed by gene GS2;1 in clone I-144 (Table 7).

Table 5. Chlorophyll a, b and carotenoids, and chlorophyll a/b in leaves of four Eucalyptus clones (386, I-144, I-042, and VM-01) subjected to nitrogen omission (weeks 1 and 3) and nitrogen resupply (weeks 4 and 5) and control treatments, grown in nutrient solution

Clone	Week	Chlorophyll a		Chlorophyll b		Carotenoids		Chlorophyll a/b	
		Control	N	Control	N	Control	N	Control	N
					mg g^{-1}				
386	1	1.83	1.01**	0.78	0.45**	0.38	0.33^{ns}	2.36	2.23^{ns}
I-144		2.57	1.44**	0.74	0.56**	0.57	0.43**	3.47	2.55**
I-042		2.35	1.85**	0.71	0.66*	0.57	0.52*	3.29	2.82**
VM-01		2.02	1.29**	0.67	0.48**	0.50	0.36**	3.00	2.69**
386	3	2.18	1.19**	0.53	0.20**	0.55	0.35**	4.13	5.90**
I-144		2.72	0.85**	0.66	0.12**	0.68	0.33**	4.14	6.95**
I-042		2.50	1.29**	0.70	0.18**	0.61	0.33**	3.60	7.29**
VM-01		2.18	1.06**	0.53	0.17**	0.55	0.33**	4.08	6.25**
386	4	2.25	1.47**	0.77	0.45**	0.57	0.39**	2.93	3.27*
I-144		2.39	1.39**	0.74	0.37**	0.63	0.36**	3.21	3.73*
I-042		2.22	1.09**	0.70	0.33**	0.52	0.32**	3.17	3.32^{ns}
VM-01		2.29	1.17**	0.54	0.31**	0.52	0.33**	4.26	3.78**
386	5	2.27	2.47*	0.76	0.83**	0.55	0.55^{ns}	3.00	2.96^{ns}
I-144		2.25	2.45*	0.77	0.80*	0.57	0.54^{ns}	2.95	3.08^{ns}
I-042		2.34	2.26^{ns}	0.78	0.75^{ns}	0.48	0.49^{ns}	3.02	3.02^{ns}
VM-01		1.99	1.84*	0.66	0.56*	0.44	0.43^{ns}	3.00	3.26*

^{ns}, * and **: not significant and significant effect at 5 or 1 % probability, respectively, by the F test.

Table 6. Activity of the enzymes nitrate reductase (NR) and glutamine synthetase (GS) in diagnostic roots and leaves of Eucalyptus clones (386, I-144, I-042 and VM-01) subjected to nitrogen omission (weeks 1 and 3) and nitrogen resupply (weeks 4 and 5) and control treatments

Clone	Week	Leaf				Root			
		NR		GS		NR		GS	
		Control	N	Control	N	Control	N	Control	N
			μmol mg^{-1} min^{-1}				nmol mg^{-1} min^{-1}		
386	1	1.06	6.96**	43.0	341.2**	0.28	1.06**	374.7	485.0**
I-144		1.01	14.15**	88.2	1186.3**	0.36	0.82**	259.9	355.2**
I-042		1.62	21.43**	141.6	1486.2**	0.82	1.55**	217.6	285.3**
VM-01		1.08	3.79**	78.7	242.5**	1.47	3.48**	272.4	336.3**
386	3	1.11	35.39**	43.7	1997.2**	0.59	1.63**	389.5	693.4**
I-144		1.07	45.86**	46.4	3442.6**	0.42	1.52**	433.2	611.6**
I-042		1.09	49.93**	96.3	4262.2**	0.58	2.42**	357.9	630.4**
VM-01		1.18	38.89**	104.4	3069.1**	1.08	3.42**	319.7	447.9**
386	4	1.23	48.51**	54.0	2397.5**	0.48	4.00**	302.9	418.9**
I-144		3.66	22.62**	260.4	1675.4**	1.14	3.33**	126.0	129.6
I-042		4.67	54.52**	461.6	4565.0**	0.89	2.21**	357.4	159.9**
VM-01		3.52	28.67**	159.8	1719.8**	0.96	1.09^{ns}	158.4	332.8**
386	5	1.39	4.67**	56.4	182.3**	1.18	6.56**	318.6	362.1**
I-144		4.65	16.07**	270.6	789.2**	0.48	4.30**	363.9	332.9*
I-042		4.15	4.52^{ns}	238.4	268.3^{ns}	1.10	1.15^{ns}	309.4	286.5*
VM-01		3.43	3.30^{ns}	159.9	170.5^{ns}	1.03	1.63**	327.0	467.5**

^{ns} and **: not significant and significant at 1% probability, respectively, by the F test in the control of each clone.

Table 7. Relative gene expression related to nitrogen metabolism in roots and leaves of Eucalyptus clones (386, I-144, I-042 and VM-01) in response to nitrogen omission (weeks 1 and 3) and nitrogen resupply (weeks 4 and 5)

Clone	Week	Leaf				Root			
		NIA1	TIP2;1	GS2;1	Gln1;3	NRT1;1	TIP2;1	AMT2;1	Gln1;3
386	1	1.83 a	1.21 a	1.06 a	2.39 b	1.62 a	0.83 a	0.45 c	4.08 a
	3	1.96 a	1.24 a	1.67 a	7.72 a	1.00 b	1.26 a	1.69 a	2.16 b
	4	0.96 b	1.03 a	1.14 a	1.46 b	0.53 c	0.94 ab	1.15 b	1.34 c
	5	1.11 b	0.81 a	1.03 a	1.15 b	0.87 a	0.47 c	0.60 c	0.72 d
I-144	1	1.73 b	0.50 b	1.44 c	2.28 a	1.13 b	1.82 b	1.96 b	1.71 a
	3	3.71 a	2.51 a	8.13 a	0.95 b	1.92 a	2.43 a	3.29 a	0.84 b
	4	1.32 b	1.04 b	2.64 b	3.61 a	1.17 b	1.39 a	0.89 c	1.99 a
	5	1.30 b	0.34 c	0.98 d	1.14 b	1.22 b	0.37 a	0.41 c	1.13 b
I-042	1	2.37 a	0.73 b	2.65 a	2.32 b	2.52 a	1.19 b	2.46 a	6.54 a
	3	1.01 b	1.93 a	1.19 b	15.00 a	1.07 b	1.87 a	1.20 b	3.20 b
	4	0.97 b	0.91 b	1.40 b	1.62 c	0.69 a	1.44 b	1.10 b	1.56 c
	5	1.35 b	0.58 b	1.04 b	1.22 a	1.28 b	0.55 c	0.71 c	1.2 c
VM-01	1	3.00 a	2.37 a	4.05 a	5.00 b	2.49 a	1.71 b	4.17 b	2.65 b
	3	2.86 a	1.33 b	3.53 a	6.30 a	2.86 a	2.81 a	6.08 a	5.12 a
	4	0.92 c	1.02 b	2.72 a	2.67 c	0.90 b	1.60 b	2.10 c	1.60 c
	5	1.15 b	0.51 c	1.15 b	1.17 d	1.23 b	0.74 c	0.55 d	1.35 c

Means with different letters indicate differences by the Tukey test at 1 % probability for each clone.

DISCUSSION

Nitrogen omission induced different plant responses, which differed in the variable, duration of treatment and clone. After N resupply, the variations tended to decrease, approaching the control treatment, demonstrating the resilience of the evaluated clones.

In the plant, the N cycle begins with the plant uptake, mediated by transporter proteins. There was induction in the expression of double-affinity transporter NRT1;1, which operates in NO_3^- transport and AMT1;2, a high-affinity NH_4^+ transporter, when under N deficiency (Loqué and von Wirén, 2004). In clone 386 the induction response was low or absent, while VM-01 was the most responsive. The activity of transporter gene TIP2;1, which operates in tonoplast controlling the NH_4^+ movement, was highest in leaves and roots of clone VM-01, in the first and third week of N omission, respectively. A similar action of this gene was reported in roots and shoots of *Arabdopsis* by Rawat et al. (1999), who concluded that this gene is essential for species that tolerate high NH_4^+ levels, e.g., *Eucalyptus*.

This study allows the conclusion that NO_3^- reduction and NH_4^+ assimilation in *Eucalyptus* occur primarily in the leaves and that the activity of GS was much higher than that of NR, regardless of the evaluated tissue. This suggests that high NH_4^+-N levels are taken up, assimilated and tolerated by *Eucalyptus*, resulting in energy savings (Campbell, 1999). This information contributed to the understanding of the high energy efficiency of *Eucalyptus*, since these processes in the leaves optimize ATP, which is generated by photosynthesis and re-translocation to the roots is not necessary, aside from the lower energy demand of the NH_4^+-N than of the NO_3^--N cycle

Generally speaking, major activities of NR are related to a greater absorption of NO_3^- and consequently, to higher active N concentrations in leaves (Beevers and Hageman, 1969;

Hirel at al., 2005, 2007). But this NO_3^- may be contained in the vacuoles, without toxicity symptoms and, therefore, lower NR activities are related to the tolerance to N deficiency (North et al., 2009). Thus, the increase in NR activity under N omission observed in this study can be explained by the use of NO_3^- by the cell vacuoles, which occurred mainly in clone 386, which had the highest N concentration in the control treatment. Gene NIA1 proved responsive to the N status and NR activity, but had a weak correlation with enzyme NR, indicating different turnover rates of NR (North et al., 2009; Cai et al., 2013).

Higher GS activities were related to the increased use of NH_4^+-N and N remobilization (Tabuchi et al., 2002; Unno et al., 2006), which is the main function in the N stress period. The GS participates in N reassimilation from degraded organic compounds, since glutamine is the most efficient way to translocate N in the plant (Nakasathien et al., 2000). In the leaves, the GS activity was highest in clone I-144 in the third week, at the same time of highest GS2;1 expression, encoding the plastidic GS and demonstrating the regulation of this pathway (Lima et al., 2006; Bernard and Habash, 2009). In the other clones, gene Gln1;3 was most sensitive and had the highest expression in leaves and roots, indicating differences between the varieties under study. Similar results relating the expression of these genes with the GS activity were reported in other crops (Lian et al., 2005; Bi et al., 2007). The study and manipulation of GS can elucidate processes involved in the highest NUsE (Bernard and Habash, 2009), since increases in their activity are responsible for variations in N concentration, at the same level of availability (Hirel et al., 2007).

The decreases in *A* observed here can be attributed to the damages in the photosynthetic apparatus: reductions in the chlorophyll *a* and *b* contents and lower $\hat{Y}_{II}$ (Damour et al., 2010; Warren, 2011). The increase in the chl *a/b* ratio in the third week indicates the dominance of the reaction centers (PS_{II}) and lower action of the solar radiation collection system (Anderson, 1986; Terashima and Hikosaka, 1995). Furthermore, the increase in carotenoid content strongly indicates an anti-stress mechanism (Hendry and Price, 1993). These are strategies that adjust the radiation absorption capacity to the current energy demand for the photosynthesis processes (Warren, 2011). Nitrogen omission decreased the photochemical dissipation ($\hat{Y}_{II}$) of the absorbed radiation, and this change is associated with abiotic stress (Golding and Johnson, 2003; Joliot and Joliot, 2006) and used in the selection of new genotypes (Damatta et al., 2002; Murchie and Lawson, 2013). Clones VM-01 and I-042 fit best to this model, since their avoidance mechanisms of oxidative stress were most effective (Ort and Baker, 2002; Smirnoff, 2005).

With regard to growth, which involves the entire plant metabolism, clone 386 performed best in the control treatment, corroborating the findings of Pinto et al. (2011). However, under N omission, the growth of this clone was drastically reduced, indicating its higher nutritional demand and the lack of effective resistance and resilience mechanisms to nutritional stress. Opposite to this performance, no growth reduction was observed for clone VM-01, indicating a greater resistance and quick recovery to nutritional stress. VM-01 also had the highest expressions of N transporters in the roots, AMT1,2 and NRT1;2, although the induction of the GS and NR activity and NRetI were lower than for the other clones. This indicates the use of other forms of N accumulation in plant roots and stems in forms that can be retranslocated when N availability is low (Lima, 1996). The N movement in *Eucalyptus* is very high, as mentioned earlier by Silva et al. (2013), who found N retranslocation of 70 % when no mineral fertilization was applied, which explains the frequently observed irresponsiveness to N fertilization under field conditions (Miller, 1995; Santos, 2001). This high internal cycling observed in all clones is one of the plant strategies that ensures greater nutritional efficiency and is essential for the sustainability of the plantation.

CONCLUSIONS

The responses observed between clones indicate different resistance and resilience strategies to nitrogen-deficiency stress.

The total N concentration in the diagnostic leaves was high and significantly correlated with the measured variables: A, NR and GS activity, and expression of genes GS2;1 and Gln1;3 in the leaves and AMT1;2 in the roots, chlorophyll-a and chlorophyll-b contents and $\hat{Y}_{II}$.

The enzymes GS and NR in the leaves and the genes AMT1;2, GS2;1 and Gln1;3 proved most sensitive as N indicators.

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