Nitrogen metabolism in the roots of rubber tree (*Hevea brasiliensis*) plants supplied with nitrate or ammonium as nitrogen source during hypoxia

Pollyanna Aparecida de Carvalho[1*], Luiz Edson Mota de Oliveira[1], Ladaslav Sodek[2], Jullyanna Nair de Carvalho[3]

[1]Setor de Fisiologia Vegetal, Departamento de Biologia, Universidade Federal de Lavras, CP 3037, 37200-000 Lavras, Minas Gerais, Brasil
[2]Departamento de Fisiologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, CP 6109, 13083-970 Campinas, SP, Brasil
[3]Colegiado de Engenharia Agronômica, Universidade Federal do Vale do São Francisco, Campus de Ciências Agrárias, CP 252, 56300-990Petrolina, PE, Brasil

*Corresponding author: pollycarvalhoufla@yahoo.com.br

Abstract

The plants may be exposed to excessive soil moisture condition, in which oxygen supply (O_2) to the root system becomes difficult due to the low diffusion rate gas in water. Given the fundamental importance of O_2 in plants aerobic metabolism, the flooding can to affect the plants development in ecosystems. O_2 lack in the cultivation substrates negatively influences the productivity of many economically important species such as rubber tree (*Hevea brasiliensis* Müll.Arg.). Amazonian species undergoes flooding periods at different lifecycle stages. The application of nitrate (NO_3^-) has been observed to increase the plant tolerance subjected O_2 deficiency. The nitrogen role in plants under O_2 deficiency is very important, but is not well understood. In this study we evaluate nitrogen metabolism changes (roots enzymatic assimilation) and the nitrogen compounds transport to the shoot in rubber trees under O_2 deficiency treated with nitrate or ammonium. Rubber tree plants were grown in nutrient solutions of Bolle-Jones (1957) 1/2 strength. When plants were 12 months-old they were divided into six treatments consisting of the complete nutrient solution of Bolle-Jones (1957) free nitrogen, with 8 mM nitrogen (KNO_3) or with 8 mM nitrogen (($NH_4)_2SO_4$) and two oxygen availability conditions (normoxia-control plants: roots kept under nutrient solution and continuous aeration and hypoxia- flooded plants: roots kept under nutrient solution without aeration). Root and sap xylem material was harvested for biochemical analysis every 7 days (days 7, 14 and 21). The results showed that under hypoxia, nitrate treated plants showed higher nitrate reductase (NR) activity compared to the control. Glutamine synthetase (GS), glutamine-2-oxoglutarate aminotransferase (GOGAT) and glutamate dehydrogenase (GDH) activities were significantly decreased in stressed plants regardless of the nitrogen source. However, under normoxia or hypoxia, nitrate treated plants showed higher (GS, GOGAT and GDH) activity, in comparison with the ammonium treatment. Moreover, under hypoxia nitrate treated plants, presented higher alanine and Gaba concentrations in the xylem than plants treated with ammonium. Therefore nitrogen assimilation, amino acid biosynthesis and transport are in general less affected by hypoxia, however, nitrate addition attenuates stress effects.

Keywords: *Hevea brasiliensis*, Hypoxia, Nitrogen metabolism, HPLC, nitrate, ammonium.
Abbreviations: NR_nitrate reductase, NiR_nitrite reductase, GS_glutamine synthetase, GOGAT_glutamine-2-oxoglutarate aminotransferase, GDH_glutamate dehydrogenase, Gaba_γ-aminobutyric acid.

Introduction

Molecular oxygen (O_2) acts as the final electron acceptor of the mitochondrial respiratory chain and is essential for the activity of diverse enzymes, having an indispensable role in plant growth and metabolism (Licausi et al., 2011). However, during their life cycle, plants and particularly their root systems can often be submitted to conditions of O_2 deficiency (Irfan et al. 2010). This stress occurs, for instance, after heavy rainfalls or excessive irrigation, situations in which the soil, depending on its drainage capacity, may become waterlogged, affecting O_2 supply to submerged tissues due to the low diffusion rate of this gas in aqueous medium (Licausi et al., 2011). Plants submitted to O_2 deficiency respond through several biochemical modifications, including the decrease of ATP synthesis by oxidative phosphorylation and a consequent stimulus of fermentation processes (Bailey-Serres and Voesenek 2008). In addition to primary carbon

metabolism, nitrogen assimilation is also affected in hypoxic roots (Rocha et al. 2010a). Under normal conditions, nitrate (NO_3^-) absorbed by roots is reduced by nitrate reductase (NR) to nitrite (NO_2^-), in turn reduced to ammonium (NH_4^+) by nitrite reductase (NiR). NH_4^+ is then incorporated into amino acids by the system glutamine synthetase-glutamine-2-oxoglutarate aminotransferase (GS/GOGAT). Glutamine (GLN) and glutamate (GLU) are the first products and then other amino acids are produced by aminotransferases action. The amino acids are used in protein synthesis or transported to other plant parts (Lea 1993). The involvement of glutamate dehydrogenase (GDH) in NH_4^+ assimilation under stress conditions, such as low oxygen availability, has also been suggested (Skopelitis et al., 2006). The application of NO_3^-, has been observed to increase the survival of plant species subjected O_2 deficiency (Allegre et al., 2004; Thomas and

Sodek, 2005; Horchani et al., 2010). It is well established that NO_3^- but not NH_4^+, exerts a beneficial effect on growth and metabolism of plants submitted to hypoxia (Horchani et al., 2010). Nevertheless, the mechanism how this happens is still not completely understood (Allegre et al., 2004). Indeed, comparative studies with NO_3^- and NH_4^+ indicate that these nitrogen forms may induce distinct metabolic responses under stress (Escobar et al., 2006; Patterson et al., 2010). Nevertheless, it is difficult to provide a convincing explanation for the beneficial effect of NO_3^- during hypoxia based solely on its direct effects: NO_3^- reduction by NR and NAD^+ regeneration by NR (Libourel et al., 2006). Libourel et al., 2006 also suggests that NO_3^- effects the regulation of cytoplasmic pH during O_2 deficiency, and by extrapolation, tolerance to this stress might be mediated by NO_2^- or by nitric oxide (NO). Other studies have reported that addition of NO_3^- under hypoxic conditions leads to an increased production of NO_2^- and/or NO by plant tissues of various species (Brandão and Sodek, 2009; Oliveira et al., 2013). Stoimenova et al. (2007) have demonstrated that NO2- can act as an alternative electron acceptor for the respiratory chain, being reduced to NO in a process coupled to NAD^+ regeneration and ATP synthesis. Therefore, NO synthesis from N O_2^- would have a relevant role in maintaining mitochondrial functionality during O_2 deficiency (Gupta and Igamberdiev, 2011). NO_3^- utilization and reduction can occur under hypoxia (Botrel et al., 1996). Under anaerobiosis, the GS/GOGAT system appears to play an important role in the accumulation of free amino acids, especially alanine and γ-amino butyric acid (Gaba), through its participation in the synthesis of glutamate (Reggiani et al., 2000). Plants whose root system is flooded can undergo biochemical and metabolic changes in order to tolerate hypoxic stress. Thus, the objective of this study was to analyze the changes in nitrogen metabolism of 1-year-old rubber tree plants subjected to anoxia (waterlogging) and treated with two forms of exogenous nitrogen (NO_3^- or NH_4^+). Specifically, we focused on the more important changes relating to the enzyme activities of N assimilation (NR, GS, GOGAT and GDH) together with emphasis on NO_3^-, NH_4^+ and amino acid transport in the xylem sap.

Results

Assimilation of NO_3^- and NH_4^+ by roots

NR activity was detected only in the NO_3^- treatment (Fig 1a). The NR activity was lower under normoxia in comparison with hypoxia. The NR activity of the roots increased sharply during the 21-day period of hypoxia of the root system, that is, it doubled, rising from 0.1 μmol NO_2^-.g^{-1} FW.h^{-1} at 7 days to 0.2 at 21 days. Under normoxia, the initial value was 0.06 and the activity rose reaching 0.13 μmol NO_2^-.g^{-1} FW.h^{-1} at 21 days. A significant reduction in GS, GOGAT and GDH activity was observed in the hypoxia treatments when compared with respective controls. However, NO_3^- treatment suffered the lowest influence of hypoxia compared to the NH_4^+ treatment. The N-free treatment exhibited lower enzyme activities when compared with both the NO_3^- and NH_4^+ treatments. This difference remained throughout the experimental period regardless of oxygen availability. At 21 days GS activity under hypoxia was lower than for normoxia both in the presence of NO_3^- (3.3 vs. 2.4 μmol GHA.g^{-1}FW.h^{-1}) and NH_4^+ (1.6 vs. 0.7 μmol GHA.g^{-1}FW.h^{-1}) indicating that O_2 deficiency strongly affects GS activity in the roots (Fig 1 b).

Consistent with this result, GOGAT activity in roots submitted to hypoxia presented an approximately 50% reduction in activity regardless of the nitrogen source and at all times studied (Fig 1c). However, treatment with NO_3^- resulted in activities that were higher (about twice) than those with NH_4^+, under the same O_2 availability condition. Over the experimental period hypoxia in the presence of NO_3^- resulted in a lower decrease in root GDH activity (on average around 36 to 26 μmol Glu.g^{-1}FW.min^{-1}) a decrease of 27% in comparison with the control (Fig 1d). On the other hand, with the NH_4^+ treatment, when normoxia and hypoxia are compared, a greater decrease of 76% (on average around 13 to 3 μmol Glu.g^{-1}FW.min^{-1}) was observed.

Nitrogen compounds translocation in the xylem sap
NO_3^- transport

Data for NO_3^- content of the xylem bleeding sap are shown in Fig 2a. NO_3^- transport was observed only in the NO_3^- treatment. The concentration of NO_3^- in the xylem sap was significantly lower under hypoxia than normoxia for any time period studied. Under normoxia the values ranged from 3500 to 2800 nmol.mL^{-1} compared with 2000 to 1400 nmol.mL^{-1} under hypoxia. Under hypoxia in plants supplied with NH_4^+ (Fig 2a), there was a decline in NO_3^- content of the xylem sap over the experimental period, where the NO_3^- concentration decreased from 2.0 nmol.mL^{-1} at 7 days to 1.5 at 21 days. These data are consistent with the increased NR activity in the roots during hypoxia, since greater rates of NO_3^- reduction would decrease the concentration of NO_3^- available for transport.

NH_4^+ transport

The concentration of NH_4^+ transported in the xylem to the shoot (Fig 2 b) were very low but certainly higher in the treatment with NH_4^+. Quantities of NH_4^+ increased significantly after the root system had been subjected to hypoxia, most clearly observed in the treatment with NH_4^+. Under hypoxia the concentration of NH_4^+ ranged from 1.0 to 3.5 nmol.mL^{-1} in the NO_3^- treatment and from 18.0 to 30.0 nmol.mL^{-1} with NH_4^+. These data are, therefore, consistent with reduced GS activity in the roots during hypoxia, where lower assimilation rates could increase the availability of NH_4^+ for transport. On the other hand, under normoxia, concentrations remained similar at all time points of the experimental period, that is, approximately 1.0 nmol.mL^{-1} for the NO_3^- treatment and 14.0 nmol.mL^{-1} for the NH_4^+ treatment.

Amino acids transport

Under the normoxia condition, glutamate and glutamine were the amino acids that predominate in the xylem sap independent of the nitrogen source. These two together represent approximately 70% of the total amino acids for the NO_3^- treatment and 60% for the NH_4^+ treatment (Tab. 1). Following these, in order of abundance, we find for the NO_3^- treatment, alanine, Gaba, aspartate, arginine, valine, glycine, threonine and serine which together add up to 27% of the total while "others" represent 3.0%. In the NH_4^+ treatment, after glutamine and glutamate, the order found was: Gaba, arginine, alanine, aspartate, valine, serine, threonine and glycine (together, 38% of the total) while "others" represent 2.0%. Among the amino acids that accumulate under anaerobiosis, alanine and Gaba stand out. They together represent 92% in the NO_3^- treatment and 50% in the NH_4^+

Table 1 Effect of nitrogen treatments on the amino acid composition (mol %) of xylem sap of plants under normoxia. NO_3^- (8 mM), NH_4^+ (8 mM) or solution without nitrogen (–N) was added to the nutrient solution in which the roots were maintained under normoxia for 21 days. Amino acid concentrations were determined by HPLC at 7, 14 and 21days after treatment induction.

	Normoxia											
	–N				NO_3^-				NH_4^+			
	7days	14days	21days	average	7days	14days	21days	average	7days	14days	21days	average
Glu	45.6	31.5	15.3	30.8 B	12.5	39.7	62.3	38.2 A	19.4	27.5	31.1	26.0 C
Gln	1.4	17.6	1.3	6.8 B	47.5	38.9	0.6	29.0 A	27.3	30.0	33.7	30.3 A
Gaba	1.5	7.1	5.2	4.6 B	3.4	5.4	8.8	5.9 B	10.3	16.2	14.2	13.5 A
Ala	2.6	3.9	8.1	4.9 A	3.2	7.6	10.1	7.0 A	5.9	6.8	7.5	6.7 A
Asp	9.0	14.6	0.8	8.1 A	10.6	0.9	2.3	4.6 B	9.5	1.2	0.3	3.7 B
Gly	0.3	1.7	6.4	2.8 A	0.8	0.1	2.9	1.3 A	0.9	0.7	0.6	0.7 A
Ser	0.5	3.1	0.2	1.2 A	2.3	0.2	0.4	0.9 A	3.2	0.2	0.2	1.2 A
Arg	0.1	1.6	1.6	1.1 C	7.7	1.1	2.1	3.6 B	11.4	7.3	4.4	7.7 A
Val	0.7	1.9	0.3	0.9 A	2.2	2.4	1.6	2.1 A	1.9	2.7	3.7	2.7 A
Thr	0.6	2.0	8.4	3.7 A	1.0	0.1	2.8	1.3 B	1.1	0.8	0.5	0.8 B
Others	1.3	3.6	0.5	1.8 B	6.2	0.7	3.8	3.6 A	3.3	1.5	0.8	1.9 B
Total	63.6	88.6	48.1	66.7 B	97.4	97.1	97.7	97.5 A	94.2	94.9	97.0	95.2 A

Letters compare nitrogen source (-N, NO_3^- and NH_4^+) to each amino acids. Different letters indicate significant differences with 0.05 probability. ''Others'' includes: asparagine, histidine, leucine, lysine, isoleucine, phenylalanine, methionine and tyrosine.
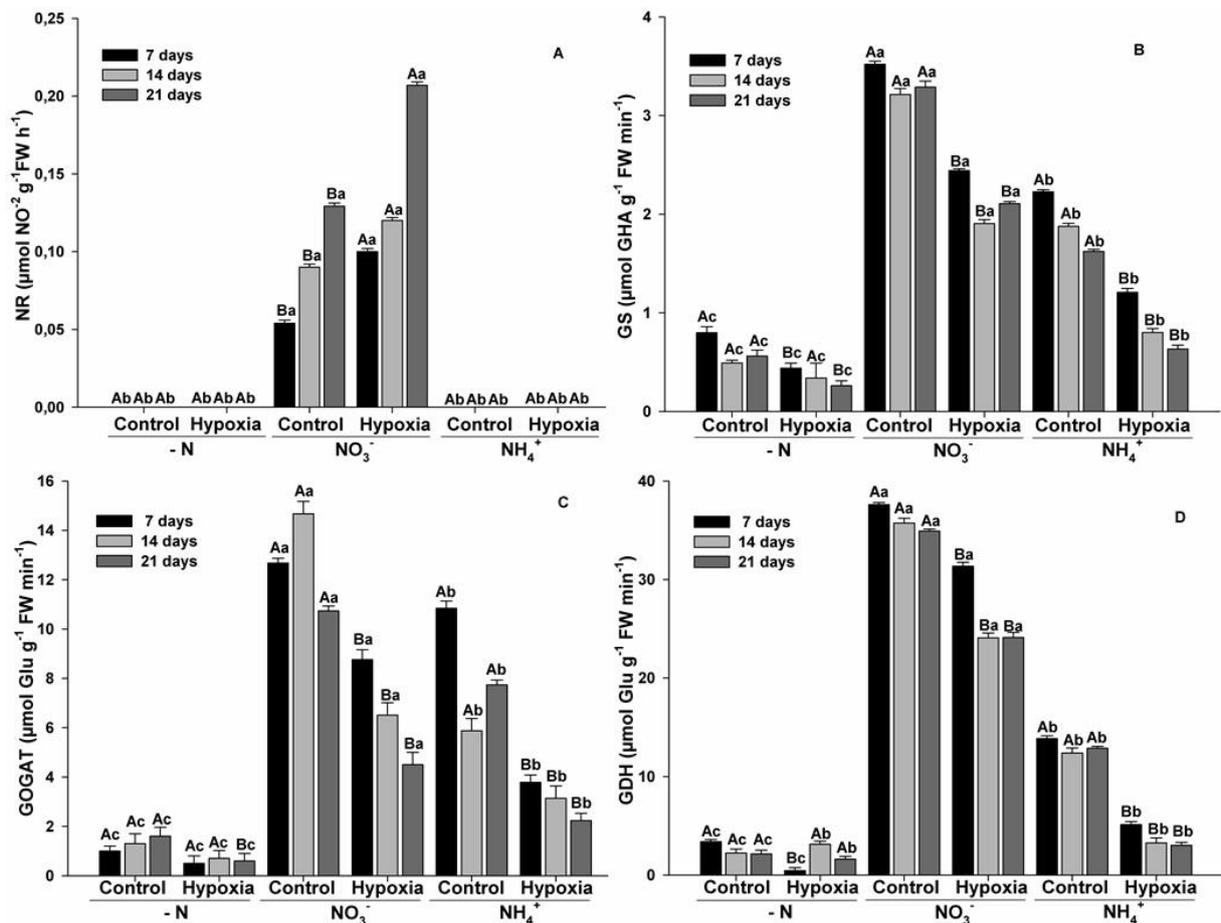
Fig 1. Effect of oxygen availability and of the nitrogen source in the activity of the enzymes nitrate reductase (A), glutamine synthetase (B), glutamine-2-oxoglutarate aminotransferase (C) and glutamate dehydrogenase (D), in roots of rubber tree seedlings. Capital letters compare oxygen availability (control and hypoxia) at each sampling time (7, 14 and 21 days) within each nitrogen source (- N, NO_3^- and NH_4^+), whereas lowercase letters compare the nitrogen sources at each sampling time within each oxygen condition. Different letters indicate significant differences with 0.05 probability.

Table 2 Effect of nitrogen treatments on the amino acid composition (mol %) of xylem sap of plants under hypoxia. NO_3^- (8 mM), NH_4^+ (8 mM) or solution without nitrogen (–N) was added to the nutrient solution in which the roots were maintained under hypoxia for 21 days. Amino acid concentrations were determined by HPLC at 7, 14 and 21days after stress induction.

	Hypoxia											
	– N				NO_3^-				NH_4^+			
	7days	14days	21days	average	7days	14days	21days	average	7days	14days	21days	average
Glu	26.8	38.0	42.4	35.7 A	26.8	4.6	9.0	13.5 C	8.6	11.8	30.4	16.9 B
Gln	4.9	12.5	0.1	5.8 A	1.1	4.3	0.7	2.0 B	8.4	9.0	5.4	7.6 A
Gaba	10.5	4.7	11.5	8.9 C	41.2	76.4	63.5	60.4 A	28.8	32.9	35.4	32.4 B
Ala	3.7	5.1	0.4	3.1 C	24.2	32.4	15.8	24.1 A	20.1	0.7	2.1	7.6 B
Asp	16.1	12.1	7.4	11.9 A	0.6	0.6	1.2	0.8 B	2.4	0.5	0.1	1.0 B
Gly	0.2	1.7	2.7	1.5 B	1.2	4.0	7.4	4.2 A	1.1	0.7	0.1	0.6 A
Ser	4.0	0.8	0.1	1.6 A	0.2	0.1	0.4	0.2 B	4.3	0.3	0.1	1.6 A
Arg	0.4	0.8	0.1	0.4 B	1.0*	0.4	1.1	0.8 B	7.4	6.4	9.7	7.8 A
Val	1.4	1.6	0.0	1.0 A	1.4	0.1	0.2	0.5 A	1.9	2.3	1.5*	1.9 A
Thr	0.2	0.9	3.0	1.3 B	1.4	4.3	9.3	5.0 A	1.1	0.8	0.3	0.7 B
Others	4.3	3.6	0.2	2.7 A	1.7	0.3	1.3	1.1 B	5.8	2.3	0.3	2.8 A
Total	72.5	81.8	67.9	73.9 B	100.8	127.5	109.9	112.6 A	89.9	67.7	85.4	80.9 B

Letters compare nitrogen source (-N, NO_3^- and NH_4^+) to each amino acids. Different letters indicate significant differences with 0.05 probability. ''Others'' includes: asparagine, histidine, leucine, lysine, isoleucine, phenylalanine, methionine and tyrosine.
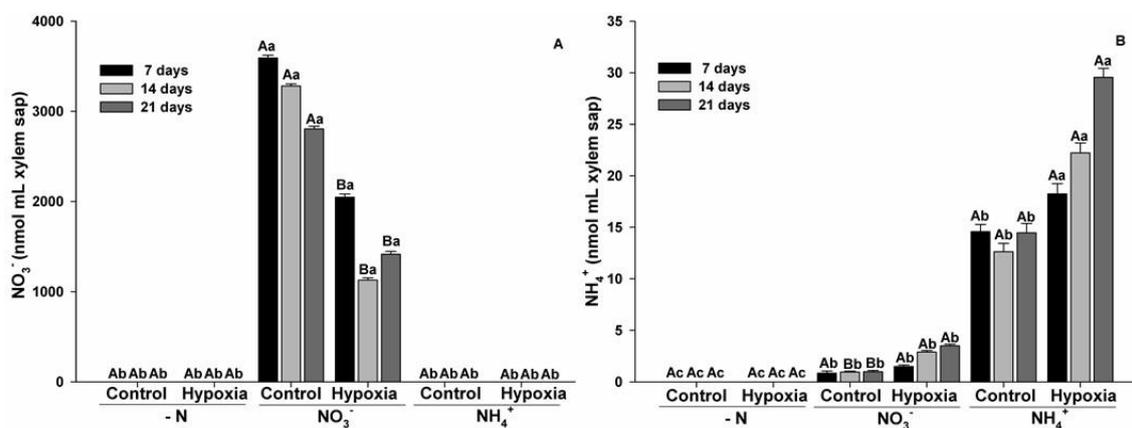
Fig 2. Effect of oxygen availability and of the nitrogen source on nitrate (A) and ammonium (B) transport in the xylem sap of rubber tree seedlings. Capital letters compare oxygen availability (control and hypoxia) at each sampling time (7, 14 and 21 days) within each nitrogen source (- N, NO_3^- and NH_4^+), whereas lowercase letters compare the nitrogen sources at each sampling time within each oxygen condition. Different letters indicate significant differences with 0.05 probability.
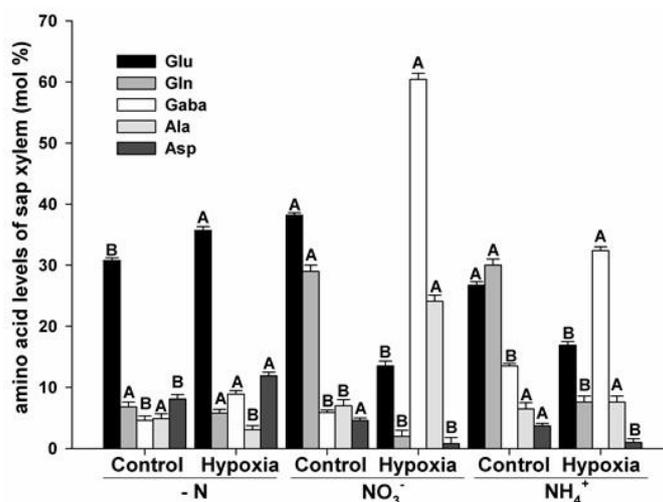
Fig 3. Effect of oxygen availability and of the nitrogen source on amino acid transport in the xylem sap of rubber tree seedlings. Letters compare oxygen availability (control and hypoxia) within each nitrogen source (-N, NO_3^- and NH_4^+). Different letters indicate significant differences with 0.05 probability.

treatment. Furthermore, the total amino acid quantities in the xylem sap of the, NO_3^- treatment increased sharply under hypoxia (Tab.2), in comparison with normoxia (Tab.1). Independent of the nitrogen source, the analysis of amino acid content in the xylem sap indicates that hypoxia led to the redirection of amino acid metabolism, particularly towards alanine and Gaba synthesis, amino acids that presented the highest concentrations in this situation (Fig.3). Moreover, under the same condition a proportional decrease in aspartate, glutamate and glutamine concentrations was detected, for both NO_3^- and NH_4^+ treatments.

Discussion

The present study provides an analysis of the effect of O_2 deficiency on nitrogen assimilation in roots and nitrogen transport in the xylem sap of rubber tree plants. In addition, was verified the plants response to the interaction nitrogen source *versus* low O_2 availability.Currently, it is believed that NO_3^- has effect on anaerobic metabolism. Its presence in the surrounding medium increases tolerance to flooding (Kaiser and Huber, 2001). A hypothesis that NO_3^- respiration may explain the beneficial effect on flooding tolerance was proposed, since the recycling of NAD^+ by NO_3^- reduction would substitute for ethanol formation in this role (Roberts et al., 1985). In the present study, we observed NR activity was detected only in the NO_3^- treatment. NR activity was higher under hypoxia in comparison with normoxia, possibly due to high reducing power availability in the cytosol (*in vivo* assay). Moreover, the NR activity increased sharply during the 21 days period of hypoxia of the root system. Consistent with this hypothesis, Garcia Novo and Crawford (1973) found increased NR activities (*in vivo* assays) in roots of many species under flooding. The reductive assimilation of NO_3^- to NH_4^+ may have two functions in plants under O_2 deficiency. Both contributing to alleviate cytoplasmic acidity. Firstly, the process consumes 4 moles of NADH (8 electrons) and 8 protons per reaction cycle (Salsac et al., 1987), being more efficient in NAD^+ generation and consumption of protons than any of the fermentation reactions (Fan et al., 1997). Moreover, the process generates hydroxyl ions (Smirnoff and Stewart, 1985) that can counteract the protons generated by hypoxia. Therefore, NO_3^- reduction should provide an alternative sink for electrons and in doing so reduce the formation of fermentation products such as ethanol and lactate. Recent evidence suggests that the beneficial effect of NO_3^-, in plant metabolism under hypoxia, may involve the metabolic product NO_2^- (Stoimenova et al., 2007). Overall, the data of Oliveira et al. (2013) indicate a negative correlation between the process of NO production from NO_2^- and the intensity of root fermentation, nevertheless, a direct effect of NO_2^- in modulating hypoxic metabolism could not be excluded. Moreover, these authors have demonstrated that NO_2^- can act as an alternative electron acceptor for the respiratory chain, being reduced to NO in a process coupled to NAD^+ regeneration and ATP synthesis. Plants cultivated with NH_4^+ will not carry out the reduction of NO_3^- to NO_2^- nor that of NO_2^- to NH_4^+ in view of the absence of NO_3^-. The lack of these processes is detrimental because NAD^+ are not recycled, especially in plants under hypoxia. Furthermore, the first reaction that takes place when NH_4^+ is absorbed is catalyzed by GS, which depends on ATP. This enzyme has decreased activity in roots under O_2 deficiency, where the ratio of ATP/ADP is reduced (Limami et al., 2008). Lower GS activity may lead to NH_4^+ accumulation, high quantities of which have deleterious effects on the plant cell (Givan, 1979). In this study, NH_4^+

quantities transported in the xylem to the shoot were significantly increased. On the other hand the nitrate amount in xylem sap was significantly reduced after the root system had been subjected to hypoxia, regardless nitrogen source, although only trace concentrations were observed in the NO_3^- treatment. These data are consistent with increased NR activity and reduced GS activity under hypoxia. It is known that GS activity is decreased under conditions of O_2 privation, which may result from its dependence on ATP. The possible involvement of GDH in NH_4^+ assimilation under stress conditions has been suggested (Skopelitis et al., 2006). In our study, GDH activity was affected by low O_2 availability, however, under hypoxia, in the presence of NH_4^+ the decreased was more intense than with NO_3^-. GDH catalyzes a reversible reaction and in the direction of glutamate formation it consumes NADH. Under stress, possibly the reaction in the direction that produces glutamate is favored due to high NADH availability. Greater GOGAT activity was found in the NO_3^- treatment. Under hypoxia GOGAT activity was twice as high as that in the NH_4^+ treatment. The GS/GOGAT cycle contributes to NH_4^+ reassimilation and primary assimilation of NO_3^-, constantly producing glutamate for the synthesis of other compounds. Under anaerobiosis, the GS/GOGAT system appears to play an important role in the accumulation of free amino acids (Reggiani et al., 2000). Studies carried out with plants where the roots or shoots are subjected to O_2 deficiency revealed increases in quantities and interconversion of free amino acids (Fan et al., 1997; Reggiani et al., 2000). According to our results, the total amino acid level of the xylem sap of the NO_3^- treatment was statistically higher than that of the NH_4^+ treatment. Oliveira et al. (2013b) demonstrated that O_2 deficiency enhanced, for both root and xylem, the endogenous levels of alanine and Gaba resulting in an increment of total amino acid content. In our study, among the amino acids that accumulate under anaerobiosis, alanine and Gaba stand out. Independent of the nitrogen source, the overall analysis of amino acid content in the xylem sap indicate that hypoxia led to the redirection of amino acid metabolism, particularly towards alanine and Gaba synthesis. Moreover, a decrease was detected in aspartate and glutamate levels, consistent with the role of these amino acids as precursors to alanine and Gaba synthesis (Reggiani et al., 2000; Oliveira and Sodek, 2013). It is believed that transamination, indirectly with aspartate, via aspartate aminotransferase (Vanlerberghe et al., 1991) and directly with glutamate (Reggiani et al., 1988) through alanine aminotransferase, is the mechanism responsible for supplying the N of alanine (Good and Crosby, 1989b). Vanlerberghe et al. (1991) suggested that alanine accumulation is directly dependent on anaerobic assimilation of NH_4^+ and relate fermentation metabolism to the N "status" of the plant. Thus, the synthesis of alanine would allow detoxification of NH_4^+, without affecting the energy state or redox potential of the cell and, form a pool of N that could be used in the synthesis of amino acids. As our data show, NO_3^- also favored alanine biosynthesis. Alanine formation does not cause toxicity to the cell, an important property for hypoxia tolerance. Indeed, it has been suggested that alanine acts as a non-toxic form of carbon and nitrogen storage during hypoxia, since it is able to form pyruvate and to participate in the synthesis of other amino acids by transamination during the recovery period (Sousa and Sodek, 2003). Furthermore, alanine may acts in regulating cytosolic pH under hypoxia (Rocha et al., 2010b; Shingaki-Wells et al., 2011). Another amino acid that accumulates under several stress conditions is Gaba (Serraj et al., 1998), including O_2 deficiency (Reggiani et al., 2000). Its

synthesis occurs principally by the decarboxylation of glutamate, a reaction catalysed by glutamate decarboxylase (Crawford et al., 1994). In view of the sharp acid pH optimum of glutamate decarboxylase (Snedden et al., 1992), increased synthesis of Gaba can be explained by the effect of reduced cytoplasmic pH that follows O_2 deficiency, together with reduced Gaba shunt activity (Kinnersley and Turano, 2000). Nevertheless, a role of Gaba turnover in the tolerance to O_2 deficiency has been proposed, since its catabolism to γ-hydroxybutyrate contributes to NAD^+ regeneration under hypoxia (Breitkreuz et al., 2003) while synthesis of Gaba via glutamate decarboxylase consumes H^+, thereby counterbalancing the detrimental effects of cytosolic acidification during hypoxia (Crawford et al., 1994). The majority of authors agree that interconversion of amino acids aids cellular pH regulation in plant organs under O_2 deficiency (Streeter and Thompson, 1972a; Crawford et al., 1994; Fan et al., 1997; Reggiani, 1999), a characteristic considered fundamental for the survival of species under hypoxia (Ricard et al., 1994).

Materials and Methods

Plant cultivation and experimental conditions

Rubber trees were grown from seed in a greenhouse (21°14'S, 45°00'W, altitude 918 m). Environmental conditions inside the greenhouse were: mean air temperature between 14 °C (minimum) and 36 °C (maximum) with an average temperature of 25 °C; 75% average air relative humidity; maximum photosynthetic photon flux density (PPFD) of 1500 μmol $m^{-2}.s^{-1}$ and around 12h photoperiod. Seeds (cultivar RRIM600) previously selected for size and weight were germinated in pots (5L) containing sand. Eight days after germination, seedlings of similar height and morphological characteristics were transplanted to pots (2L) containing nutrient solution of Bolle-Jones (1957) 1/2 strength with NO_3^- (2mM) and NH_4^+ (2mM). The solution was topped up daily. The pH of the solution was adjusted daily to 5.5 ± 0.5 and solutions were completely replaced at weekly intervals.

Treatments and harvesting

When plants were 12 months-old they were divided into six treatments consisting of the complete nutrient solution of Bolle-Jones (1957) free nitrogen, or with 8 mM nitrogen (KNO_3) or with 8 mM nitrogen (($NH_4)_2SO_4$) and two oxygen availability conditions (normoxia-control plants: roots kept under nutrient solution and continuous aeration and hypoxia-flooded plants: roots kept under nutrient solution without aeration). Plant material (roots and xylem sap) were sampled seven, fourteen and twenty-one days after the treatments initiation. Sampling was carried out simultaneously to allow accurate comparisons of the behavior of nitrogen assimilation enzymes (NR, GS, GOGAT and GDH) and transport of nitrogen compounds (amino acids, NO_3^- and NH_4^+) in xylem sap, over time.

Enzyme extraction, activity assays

NR activity (E.C.1. 6. 6. 1) was measured, *in vivo*, as proposed by Meguro and Magalhães (1983). For the other enzymes the extract was obtained by grinding 1 g of root tissue in a mortar with liquid nitrogen followed by the addition of 5 mL of extraction medium containing 100 mM potassium phosphate buffer (pH 7.5), 1 mM PMSF, 100 mM

EDTA, 10% PVPP and 2 mM DTT. The extract was centrifuged at 13000 x *g* for 20 min at 4 °C. The supernatant was used to assay GS, GOGAT and GDH enzymes. GS activity (EC 6. 3. 1. 2) was evaluated as proposed by Ratajczack et al. (1981). NADH-GOGAT (EC 1. 4. 1. 7) activity was assayed in 50 mM potassium phosphate buffer, pH 7.5, containing 20 mM 2-mercaptoethanol, 0.2 mM NADH, 15 mM 2-oxoglutarate, 15 mM L-glutamine and 10 mM KCl (Groat and Vance, 1981). NADH-GDH (EC 1. 4. 1. 3) activity was determined with 100 mM Tris-HCl, pH 7.80, 0.2 mM NADH, 10 mM 2-oxtoglutarate, 4 mM $CaCl_2$ and 100 mM ($NH_4)_2SO_4$ (Groat and Vance, 1981). Both GOGAT and GDH were measured spectrophotometrically, monitoring the oxidation of NADH at 340 nm.

Amino acids, NO_3^- and NH_4^+ determinations

After cutting the stem 20 cm above the cotyledonary node, xylem sap was collected using a Scholander pump. Samples of the xylem exudates were used directly for biochemical analysis. The separation and analysis of amino acids in the xylem sap was carried out by reverse-phase HPLC (High-performance liquid chromatography) using OPA (ortho-phthaldialdehyde) amino acid derivatives, as described previously (Puiatti and Sodek, 1999). NO_3^- and NH_4^+ content were determined according Cataldo et al., 1975 and McCullough, 1967 respectively.

Statistical analysis and experimental design

The experiments were arranged in a completely randomized (CDR) design in a factorial arrangement (3x2x3). The experiment had six treatments and three harvesting times (7, 14 and 21 days) for stress measurements with four independent replicates, totaling 72 plants. Data were analyzed using analysis of variance (ANOVA), and the means were compared using the Scott-Knott test ($p < 0.05$).

Conclusion

Nitrogen assimilation, amino acid biosynthesis and transport are strongly affected by hypoxia of the root system of 1-year-old rubber tree plants. However, in the presence of NO_3^- the response to hypoxia was less intense than with NH_4^+. Increased xylem transport of amino acids associated with tolerance to hypoxia (alanine and Gaba) reflects the main change in amino acid metabolism under hypoxia of the root system.

References

Allegre A, Silvestre J, Morard P, Kallerhoff J, Pinelli, E (2004) Nitrate reductase regulation in tomato roots by exogenous nitrate: a possible role in tolerance to long-term root anoxia. J Exp Bot. 55: 2625-2634.

Bailey-Serres J, Voesenek LACJ (2008) Flooding stress: acclimations and genetic diversity. Annu Rev Plant Biol. 59:313-339.

Bolle-Jones EW (1957) Copper: its effects on the growth of the rubber plant (*Hevea brasiliensis*). Plant and Soil. 10:168-178.

Botrel A, Magné C, Kaiser WM (1996) Nitrate reduction, nitrite reduction and ammonium assimilation in barley roots in response to anoxia. Plant Physiol Biochem. 34: 645-652.

Brandão, AD, Sodek, L (2009) Nitrate uptake and metabolism by roots of soybean under oxygen deficiency. Braz J Plant Physiol. 21:13-23.

Breitkreuz KE, Allan WL, Van Cauwenberghe OR, Jakobs C et al (2003) A novel γ-hydroxybutyrate dehydrogenase: identification and expression of an Arabidopsis cDNA and potential role under oxygen deficiency. J Biol Chem. 17:41552-41556.

Cataldo DA, Haroon M, Schrader LE, Youngs VL (1975) Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun. Soil Sci Anal. 6:71-80.

Crawford LA, Bown AW, Breitkreuz KE, Guinel FC (1994) The synthesis of γ-aminobutyric acid in response to treatments reducing cytosolic pH. Plant Physiol. 104:865-871.

Fan TW-M, Higashi RM, Frenkiel TA, Lane AN (1997) Anaerobic nitrate and ammonium in flood-tolerant rice coleoptiles. J Exp Bot. 48:1655-1666.

Garcia-Novo F, Crawford RMM (1973) Soil aeration, nitrate reduction and flooding tolerance in higher plants. New Phytol. 72:1031-1039.

Givan CV (1979) Metabolic detoxification of ammonia in tissues of higher plants, Phytochem. 18:375-382.

Good AG, Crosby W (1989b) Anaerobic induction of alanine aminotransferase in barley root tissue. Plant Physiol. 90:1305-1309.

Groat RG, Vance CP (1981) Root nodule enzymes of ammonia assimilation in alfalfa (*Medicago sativa* L.). Developmental patterns and response to applied nitrogen. Plant Physiol. 67:1198-1203.

Gupta KJ, Igamberdiev AU (2011) The anoxic plant mitochondrion as a nitrite: NO reductase. Mitochondrion 11:537–543.

Horchani F, Aschi-Smiti S, Brouquisse R (2010) Involvement of nitrate reductase in the tolerance of tomato (*Solanum lycopersicum* L.) plants to prolonged root hypoxia. Acta Physiol Plant. 32: 1113-1123.

Irfan M, Hayat S, Hayat Q, Afroz S, Ahmad A (2010) Physiological and biochemical changes in plants under waterlogging. Protop. 241:3-17.

Kaiser WM, Huber SC (2001) Post-translational regulation of nitrate reductase: mechanism, physiological relevance and environmental triggers. J Exp Bot. 52:1981-1989.

Kinnersley AM, Turano FJ (2000) Gamma aminobutyric acid (GABA) and plant responses to stress. Critical Rev. Plant Sci. 19:479-509.

Lea PJ, Ireland RJ (1999) Nitrogen metabolism in higher plants. In: Singh BK (ed.), Plant Amino Acids. Biochem Biotechnol. 1-47.

Libourel IG, Van Bodegom PM, Fricker MD, Ratcliᵉe RG (2006) Nitrite reduces cytoplasmic acidosis under anoxia. Plant Physiol. 142:1710-1717.

Licausi F, Kosmacz M, Weits DA, Giuntoli B, Giorgi FM, et al. 2011. Oxygen sensing in plants is mediated by an N-end rule pathway for protein destabilization. Nature 479:419–22

Limami AM, Ricoult C, Cliquet J, Planchet E (2008) Concerted modulation of alanine and glutamate metabolism in young *Medicago truncatula* seedlings under hypoxic stress, J Exp Bot. 59:2325-2335.

McCullough H (1967) Determination of ammonia in whole blood by a direct colorimetric method. Clin Chim Acta. 17:297-304.

Meguro NE, Magalhães AC (1983) Water stress affecting nitrate reduction and leaf diffusive resistance in *Coffea arabica* L. cultivars. J Horticultural Sci. 58:147-152.

Oliveira HC, Sodek L (2013) Effect of oxygen deficiency on nitrogen assimilation and amino acid metabolism of soybean root segments. Amino Acids 44:743–755.

Oliveira HC, Salgado I, Sodek L (2013a). Involvement of nitrite in the nitrate-mediated modulation of fermentative metabolism and nitric oxide production of soybean roots during hypoxia. Planta, 237:255-264.

Oliveira HC, Freschi L, Sodek L (2013b) Nitrogen metabolism and translocation in soybean plants subjected to root oxygen deficiency. Plant Physiol Biochem. 66:141–149.

Prioul JL, Guyot C (1985) Role of oxygen transport and nitrate metabolism in the adaptation of wheat plants to root anaerobiosis. Physiol Veg. 23:175-185.

Puiatti M, Sodek L (1999) Waterlogging affects nitrogen transport in the xylem of soybean. Plant Physiol Bioch. 37:767-773.

Ratajczak L, Ratajczak W, Mazurowa H (1981) The effect of different carbon and nitrogen sources on the activity of glutamine synthetase and glutamate dehydrogenase in lupine embryonic axes. Physiol Plant. 51:277-280.

Reggiani R (1999) Amino acid metabolism under oxygen deficiency. Phytochem. 2:171-174.

Reggiani R, Cantú CA, Brambilla I, Bertani A (1988) Accumulation and interconversion of amino acids in rice roots under anoxia. Plant Cell Physiol. 29:982-987.

Reggiani R, Nebuloni M, Mattana M, Brambilla I (2000) Anaerobic accumulation of amino acids in rice roots: role of the glutamine synthetase/glutamate synthase cycle. Amino Acids. 18:207-217.

Ricard B, Couée I, Raymond P, Saglio PH, Saint-Ges V, Pradet A (1994) Plant metabolism under hypoxia and anoxia. Plant Physiol Biochem. 32:1-10

Roberts JKM, Andrade F, Anderson IC (1985) Further evidence that cytoplasmic acidosis is a determinant of flooding intolerance in plants. Plant Physiol. 77:492-494.

Rocha M, Licausi L, Araújo WL, Nunes-Nesi A, Sodek L, Fernie AR, Dongen JT (2010a) Glycolysis and the TCA-cycle are linked by alanine aminotransferase during hypoxia induced by waterlogging of *Lotus japonicus.* Plant Physiol. 152:1501-1513.

Rocha M, Sodek L, Licausi F, Hameed MW et al (2010b) Analysis of alanine aminotransferase in various organs of soybean (Glycine max) and in dependence of different nitrogen fertilizers during hypoxic stress. Amino Acids 39:1043-1053.

Salsac L, Sylvain C, Morot-Gaudry J-F, Lesaint C, Jolivet E (1987) Nitrate and ammonium in plants. Plant Physiol Biochem. 25:805-812.

Serraj R, Barry JS, Sinclair TR (1998) Accumulation of γ-aminobutyric acid in nodulated soybean in response to drought stress. Physiol Plant. 102:79-86.

Shingaki-Wells RN, Huang S, Taylor NL, Carroll AJ et al (2011) Differential molecular responses of rice and wheat coleoptiles to anoxia reveal novel metabolic adaptations in amino acid metabolism for tissue tolerance. Plant Physiol. 156:1706-1724.

Skopelitis DS, Paranychianakis NV, Paschalidis KA, Pliakonis ED et al (2006) Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis in tobacco and grapevine. Plant Cell. 18:2767-2781.

Smirnoff N, Stewart GR (1985) Nitrate assimilation and translocation by higher plants: comparative physiology and ecological consequences. Physiol Plant. 64:133-140

Snedden WA, Chung I, Pauls RH, Bown AW (1992) Proton/L-glutamate symport and the regulation of intracellular pH isolated mesophyll cells. Plant Physiol. 99:665-671.

Sousa CAF, Sodek L (2003) Alanine metabolism and alanine aminotransferase activity in soybean (Glycine max) during hypoxia of the root system and subsequent return to normoxia. Environ Exp Bot. 50:1-8.

Stoimenova M, Igamberdiev AU, Gupta KJ, Hill RD (2007) Nitrite-driven anaerobic ATP synthesis in barley and rice root mitochondria. Planta. 226:465-474.

Thomas AL, Sodek L (2005) Development of nodulated soybean plant after flooding of the root system with different sources of nitrogen. Braz J Plant Physiol. 17:291-297.

Vanlerberghe GC, Turpin DH (1990) Anaerobic metabolism in the N-limited green alga *Selenastrum minutum*. II. Assimilation of ammonium by anaerobic cells. Plant Physiol. 94:1124-1130.

Vanlerberghe GC, Joy KW, Turpin DH (1991) Anaerobic metabolism in the N-limited green alga *Selenastrum minutum*. III. Alanine is the product of anaerobic ammonium assimilation. Plant Physiol. 95:655-658.