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

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Abstract

The plants may be exposed to excessive soil moisture condition, in which oxygen supply (O2) to the root system becomes difficult due to the low diffusion rate gas in water. Given the fundamental importance of O2 in plants aerobic metabolism, the flooding can affect the plants development in ecosystems. O2 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 (NO3−) has been observed to increase the plant tolerance subjected O2 deficiency. The nitrogen role in plants under O2 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 O2 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 (KNO3) or with 8 mM nitrogen (NH4)2SO4 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 (O2) 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 O2 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 O2 supply to submerged tissues due to the low diffusion rate of this gas in aqueous medium (Licausi et al., 2011). Plants submitted to O2 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 Voeseaenek 2008). In addition to primary carbon metabolism, nitrogen assimilation is also affected in hypoxic roots (Rocha et al. 2010a). Under normal conditions, nitrate (NO3−) absorbed by roots is reduced by nitrate reductase (NR) to nitrite (NO2−), in turn reduced to ammonium (NH4+) by nitrite reductase (NiR). NH4+ 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 NH4+ assimilation under stress conditions, such as low oxygen availability, has also been suggested (Skopelitis et al., 2006). The application of NO3−, has been observed to increase the survival of plant species subjected O2 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$_3^-$ or by nitric oxide (NO). Other studies have reported that addition of NO$_3^-$ under hypoxic conditions leads to an increased production of NO$_3^-$ and/or NO by plant tissues of various species (Brandão and Sodek, 2009; Oliveira et al., 2013). Stoimenova et al. (2007) have demonstrated that NO$_3^-$ 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 Igambersiev, 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 1b).

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 2b) 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 (Table 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$_4^+$ treatment.
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 21 days after treatment induction.

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<th>(-)N 7 days</th>
<th>(-)N 14 days</th>
<th>(-)N average</th>
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<th>NO$_3$(^-) 14 days</th>
<th>NO$_3$(^-) average</th>
<th>NH$_4$(^+) 7 days</th>
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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 21 days after stress induction.

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<th>14days</th>
<th>21days</th>
<th>Average</th>
<th>NO$_3^-$</th>
<th>7days</th>
<th>14days</th>
<th>21days</th>
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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 cytoplasmatic 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$_3^-$ 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$_3^+$ reoxidation 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 (Vanlerberghhe 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). Vanlerberghhe et al. (1991) suggested that alanine accumulation is directly dependent on anaerobic assimilation of NH$_3^+$ and relay fermentation metabolism to the N "status" of the plant. Thus, the synthesis of alanine would allow detoxification of NH$_3^+$, 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 (Souza 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 (Serra 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 (Kimersley and Turano, 2000). Nevertheless, a role of GABA turnover in the tolerance to O$_2$ deficiency has been proposed, since its catabolism to $\gamma$-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$_4$)$_2$SO$_4$) and two oxygen availability conditions (normoxia-control plants: roots kept under nutrient solution and continuous aeration and hypoxic-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 Ratajczak 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-oxoglutarate, 4 mM CaCl$_2$ and 100 mM (NH$_4$)$_2$SO$_4$ (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-phthalaldehyde) 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.

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