

Effect of nitrogen source and oxygen deficiency on carbon metabolism and antioxidant system of rubber tree plants (*Hevea* spp.)**Pollyanna Aparecida de Carvalho¹, Luiz Edson Mota de Oliveira¹, Débora Domiciano¹, Jullyanna Nair de Carvalho², Débora de Oliveira Prudente¹, Rubens José Guimarães³**¹Fisiologia Vegetal, Departamento de Biologia, Universidade Federal de Lavras, Lavras, Minas Gerais, Brasil²Universidade Federal do Vale do São Francisco, Petrolina, Pernambuco, Brasil³Departamento de Agricultura, Universidade Federal de Lavras, Lavras, Minas Gerais, Brasil***Corresponding author: pollycarvalho@lavraviva.com.br****Abstract**

Oxygen deficiency in roots affects key functions such as nutrient and water uptake; furthermore, liquid-CO₂ assimilation, stomatal conductance/ transpiration and carbohydrate translocation are also affected. Under oxygen depletion, there is also an uncontrolled increase of free radicals in cells. The nitrogen application has been observed to increase the plant tolerance to oxygen deficiency. Indeed, NO₃⁻ and NH₄⁺ (nitrogen forms) may induce distinct metabolic responses under hypoxia. Nevertheless, the beneficial effects of N during hypoxia it is not fully explained. In this study, the nitrogen (N) role in the physiology and metabolism of rubber plants (*Hevea brasiliensis*) subjected to oxygen deficiency was evaluated. The experiment was conducted with plants supplied with KNO₃ (8 mM N) or (NH₄)₂SO₄ (8 mM N) in nutrient solution for 3 days. The experiments were arranged in a completely randomized design (CRD) in a factorial arrangement (2x2x4). The experiment had four treatments and four time points (12, 24, 48 and 72 hours) for stress measurements with four replicates, totaling 64 plants. Data were analyzed using analysis of variance (ANOVA), and the means were compared using the Scott-Knott test ($p \leq 0.05$). Gas exchange measurements and biochemical analyzes were performed at 12, 24, 48 and 72 hours after treatment induction. The results showed that, net photosynthesis, stomatal conductance and transpiration rate in stressed plants decreased significantly when compared to the control plants, regardless of nitrogen source. However, under hypoxia, plants treated with NO₃⁻ showed significantly higher CO₂ assimilation, overcoming the treatment with NH₄⁺. Therefore, when comparing plants treated with NO₃⁻ and those treated with NH₄⁺ under flooding conditions (for 3 days), the first presented higher sucrose production, consequently higher invertase isoform activity. Under hypoxia, NO₃⁻ treated plants also showed higher antioxidant system efficiency. Therefore, H₂O₂ content was higher in plants treated with NH₄⁺. Thus, we concluded that carbon metabolism such as carbohydrate anabolism, catabolism and transport were negatively affected by hypoxia. However, NO₃⁻ addition in contrast to NH₄⁺ treatment reduced damage caused by oxygen deficiency increasing the level of tolerance of the plant to stress.

Keywords: *Hevea brasiliensis*, Nitrogen, Oxygen deficiency, Carbohydrates metabolism, Invertase isoforms, Antioxidant enzymes.**Abbreviation:** SOD_Superoxide dismutase, CAT_catalase, APX_ ascorbate peroxidase, NR_nitrate reductase, ROS_reactive oxygen species, P_N_net CO₂ assimilation rate, g_s_stomatal conductance, E_transpiration, PPFD_photosynthetic photon flux density, VIN_vacuolar invertase, NIN_neutral invertase, CWIN_cell wall invertase.**Introduction**

Oxygen deficiency and low soil redox potential, induced by flooding, negatively affect several aspects of plant physiology, such as changes in carbon assimilation, macronutrient and water absorption, carbohydrate translocation, suppression of the respiratory metabolism of the roots, causing decline in rate ATP / ADP (Kreuzwieser et al., 2004). In addition, hypoxic conditions affect the terminal receptor of the mitochondrial respiratory chain, resulting in the inhibition of demanding energy processes (Bailey-Serres and Voeseenek, 2008). From the moment that the oxygen concentration present becomes insufficient to maintain the normal rate of root respiration, by definition, the system enters the state of hypoxia. Given the fundamental importance of O₂ in aerobic plants metabolism, waterlogging stress can strongly affect growth and survival in both

agricultural and natural ecosystems. Indeed, O₂ depletion can negatively influence the productivity of many economically important species (Irfan et al., 2010).

Throughout the evolutionary process, tolerant species developed a variety of strategies that enabled them to occupy areas subject to flooding. Among these strategies, morpho-anatomical changes occur that aid the internal plant aeration, and metabolic alterations, such as decrease in energy consumption and less investment in growth (Medri et al. 2007). In most species, the success strategy combines morphological, anatomical, and physiological responses. In general these responses may include reduced growth and development of plants (Kozłowski 1997), the stomatal closure and the decrease in photosynthesis and transpiration rate, as well as changes in the transport and partitioning of photoassimilates (Kreuzwieser et al. 2004) ,

and changes in the action of the plants antioxidant system (Jaleel et al. 2009). Currently, the positive effect of nitrogen addition on the roots of flooded plants is known, however, the effect of interaction between hypoxia and carbon and nitrogen metabolism is still not completely understood (Carvalho et al., 2015a). It is known that the application of nitrate (NO_3^-) has been observed to increase the tolerance of plant species subjected O_2 deficiency (Allegre et al. 2004; Thomas and Sodek 2005; Horchani et al. 2010; de Carvalho et al. 2015b). Indeed, comparisons of NO_3^- and NH_4^+ indicate that these nitrogen forms may induce distinct metabolic responses under water stress and hypoxia (Escobar et al., 2006; Patterson et al., 2010). At the whole plant level, however, maintaining the metabolism of sugars at appropriate levels, followed by correct partitioning of these molecules associated with energy, are prerequisites for survival to adverse conditions (Goggin, 2007). Invertase (EC 3.2.1.26) hydrolyzes sucrose into glucose and fructose and plays a major role in plant development and in response to biotic and abiotic stresses (Essmann et al. 2008). The resultant hexoses are both important signaling molecules for regulating gene expression and essential substrates for energy (ATP) generation and various metabolic and biosynthetic processes, including starch and cellulose synthesis (Rolland et al. 2006). Since sucrose catabolism interferes in the cellular redox state, influencing the coordination of carbon and nitrogen metabolism (Nunes-Nesi et al. 2010).

Stress caused by low oxygen availability also leads to an increase of free radicals in cells, vary according to the species and to the severity and duration of stress (Hong-Bo et al. 2008). Tolerates plants increased SOD activity, which determines O_2^- and H_2O_2 concentration - key defense mechanisms necessary to prevent the OH^- radical formation (Jaleel et al. 2009). Among enzymes involved in ROS deletion, SOD is considered a key enzyme because it is the first line of defense against oxidative stress (Pompeu et al. 2008). Plants have developed a complex enzymatic defense system as a protective strategy against oxidative damage. Key constituents of this system include superoxide dismutase (SOD), that catalyzes the conversion of superoxide anion into H_2O_2 and O_2 , catalase (CAT) and ascorbate peroxidase (APX), that can break down H_2O_2 to H_2O and O_2 (Jaleel et al., 2009). The removal of ROS has a direct influence on the metabolism of nitrogen, as well as photosynthesis and carbohydrate metabolism as a whole is affected by the poll of free radicals in the cell (Carvalho et al. 2015a). Therefore, the objective of this study was to identify physiological (gas exchanges) and metabolic (enzymatic activity) rubber tree plants (*Hevea brasiliensis* Müll. Arg.) responses under hypoxia and nitrogen (sources: NO_3^- or NH_4^+). The mechanisms of tolerance to hypoxia, were studied, by gas exchange evaluation and carbohydrates metabolism and antioxidant system analysis in the leaves and roots.

Results

Dry weight and chlorophyll content

After 72 hours of the experiment, seedlings did not show significant differences in shoot or root dry weight; this characteristic remained at the same level throughout the

experiment Fig. 1. The dry weight of shoot was, on average, 30 g per plant Fig. 1A, while dry weight of root was 12 g per plant Fig. 1B. Root dry weight did not increase in the control treatment and did not increase after 72 hours in the hypoxic treatment. Rubber tree seedlings subjected to hypoxia showed significant decreases in the levels of chlorophyll, whose values then remained constant until the end of the experiment Fig. 1C. In contrast, the levels of this pigment remained unchanged in control seedlings. The controls maintained their chlorophyll concentration (0.035 mg.cm^{-2}). Throughout the experiment, the values of chlorophyll were 15 % lower in oxygen deficient seedlings when compared to control regardless of nitrogen source.

Gas exchange

Variance analysis for net photosynthetic rate (A), ratio between internal carbon and external carbon (Ci/Ca), transpiration rate (E) and stomatal conductance (gs) indicates significance for interaction nitrogen source versus O_2 availability, to these four evaluated variables (Fig 2). In both nitrogen treatments, plants under normoxia had higher photosynthesis. Treatment with NO_3^- , photosynthesis decreased from $4.0 \mu\text{mol CO}_2.\text{m}^{-2}.\text{s}^{-1}$ under normoxia to 2.0 under hypoxia, down of 50%. In the presence of NH_4^+ , the decrease in this variable is even greater than $5.0 \mu\text{mol CO}_2.\text{m}^{-2}.\text{s}^{-1}$ to 1.2 it is drop of 76% (Fig. 2A). Similarly, the ratio Ci/Ca (Fig. 2B) was significantly reduced when the root system was subjected to hypoxia. The decreases were 0.6 to 0.4 in NH_4^+ treatment and 0.8 to 0.5 in NO_3^- treatment. Stomatal conductance as well as A and ratio Ci/Ca , was adversely affected by O_2 deficiency it was found (Fig. 2C). A reduction of approximately 50% in plants under flooding, regardless nitrogen source applied in solution was found. Plants treated with NO_3^- , there was a reduction of $0.04 \text{ mol H}_2\text{O}.\text{m}^{-2}.\text{s}^{-1}$ to 0.02 , while in the treatment with NH_4^+ thr gs decreased from $0.07 \text{ mol H}_2\text{O}.\text{m}^{-2}.\text{s}^{-1}$ to $0,035$. Under normoxia, transpiration rate showed values of $2.2 \text{ mmol H}_2\text{O}.\text{m}^{-2}.\text{s}^{-1}$ and 3.6 , while under hypoxia, the results reduced (approximately 50%) to $1.0 \text{ mmol H}_2\text{O}.\text{m}^{-2}.\text{s}^{-1}$ and 1.7 in treatment with NO_3^- and NH_4^+ respectively (Fig. 2D).

Carbohydrate metabolism

Invertase isoforms activity

The activity of all isoforms of invertase on leaves and in the roots and regardless of the source, was reduced under flooding. In addition, under O_2 deficit, in all evaluations, and in both tissues, the results obtained with NO_3^- were greater than to the results with NH_4^+ (Figs. 3 and 4). In leaf tissues, the activities of the three isoforms of invertase were higher than in root tissue. The vacuole acid invertase (VIN) (Figs. 3A and 4A) and the neutral cytosol invertase (NIN) (Figs. 3B and 4B) showed lower activities compared to acid invertase cell wall (CWIN) (Figs. 3C and 4C). As shown in Figs. 3 and 4, the VIN activity, in plants with NO_3^- reduced on average by 44% in the leaf, and 40% at the root. Already in plants with NH_4^+ these reductions reached 57 and 67% respectively. As mentioned above, the NIN was higher than in plants under normoxia. Under hypoxic, differences were observed between treatments with NO_3^- and NH_4^+ . Under hypoxia, in leaves and roots, NO_3^- treatment was higher than to

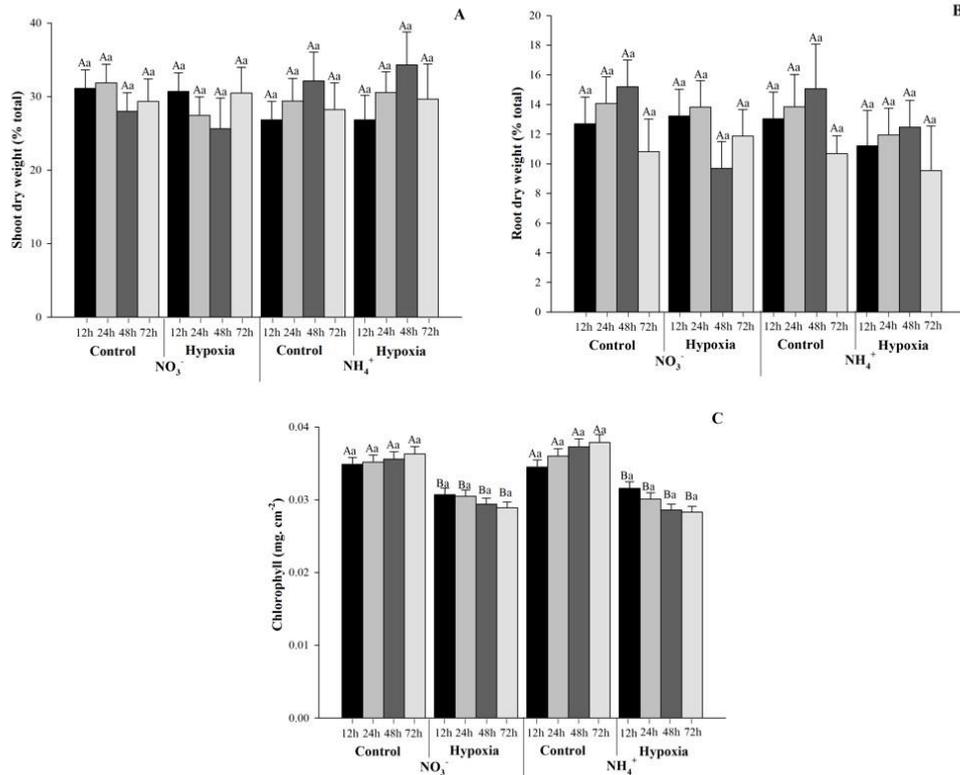


Fig 1. Effect of oxygen availability and of the nitrogen source on shoot (A) and root (B) dry weight and chlorophyll content (C) of rubber tree seedlings. Capital letters compare oxygen availability (control and hypoxia) at each sampling time (12, 24, 48 and 72 hours) within each nitrogen source (NO_3^- 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.

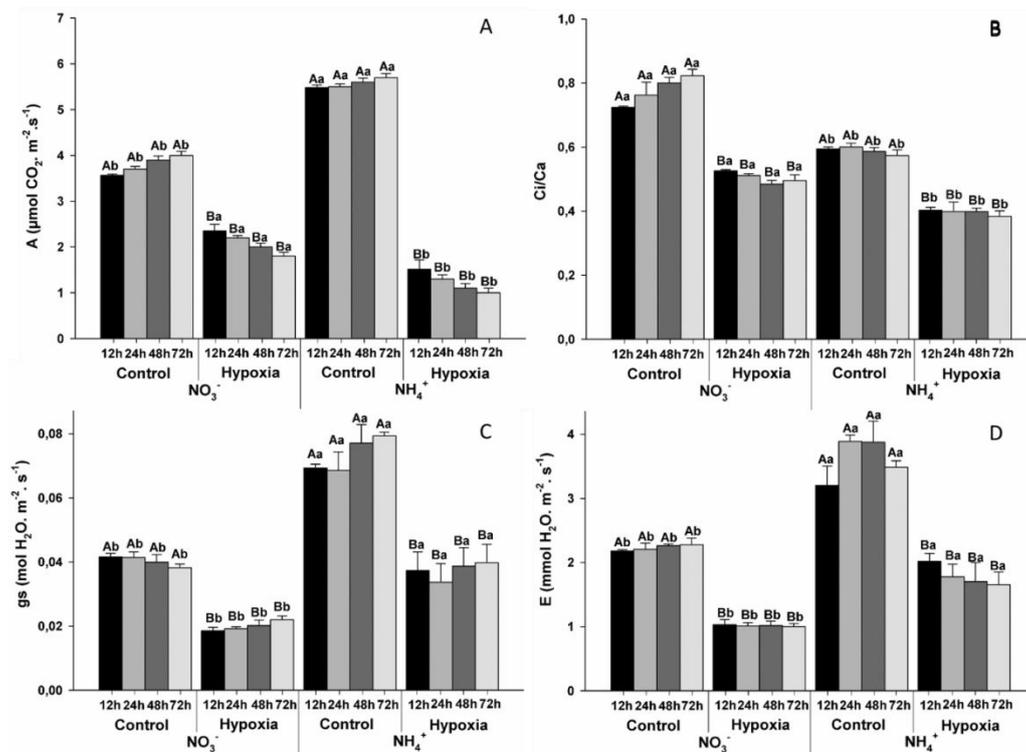


Fig 2. Effect of oxygen availability and of the nitrogen source on net assimilation rate (A), ratio Ci/Ca (B), stomatal conductance (C) and transpiration (D) of rubber tree seedlings. Capital letters compare oxygen availability (control and hypoxia) at each sampling time (12, 24, 48 and 72 hours) within each nitrogen source (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.

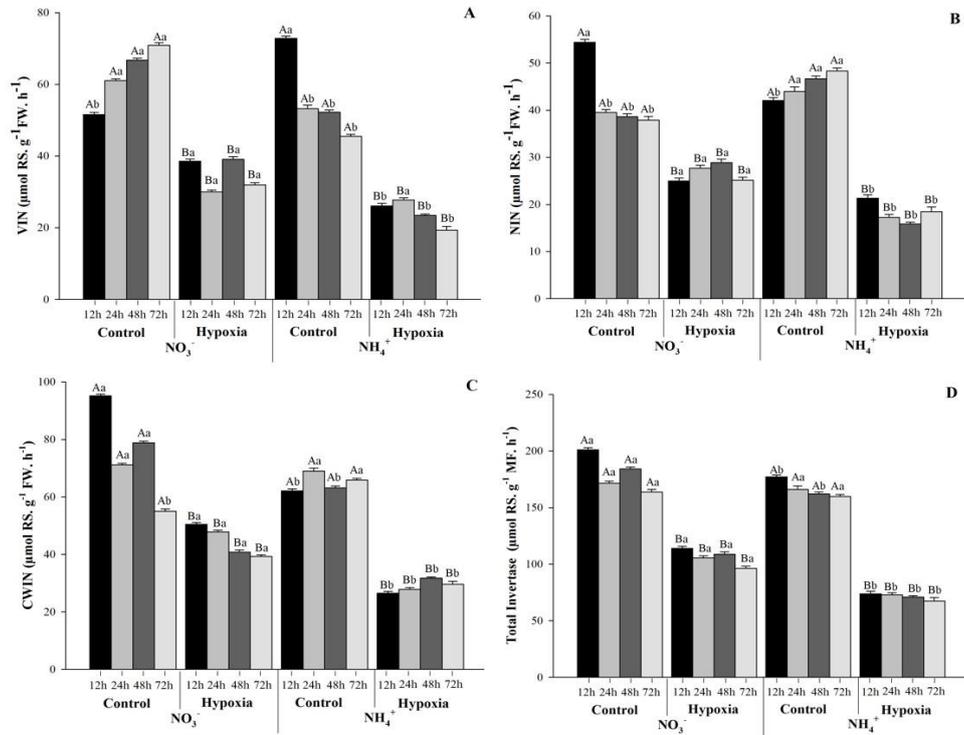


Fig 3. Effect of oxygen availability and of the nitrogen source in the activity of the enzymes vacuolar invertase (A), neutral invertase (B), cell wall invertase (C) and total invertase (D), in leaf of rubber tree seedlings. Capital letters compare oxygen availability (control and hypoxia) at each sampling time (12, 24, 48 and 72 hours) within each nitrogen source (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.

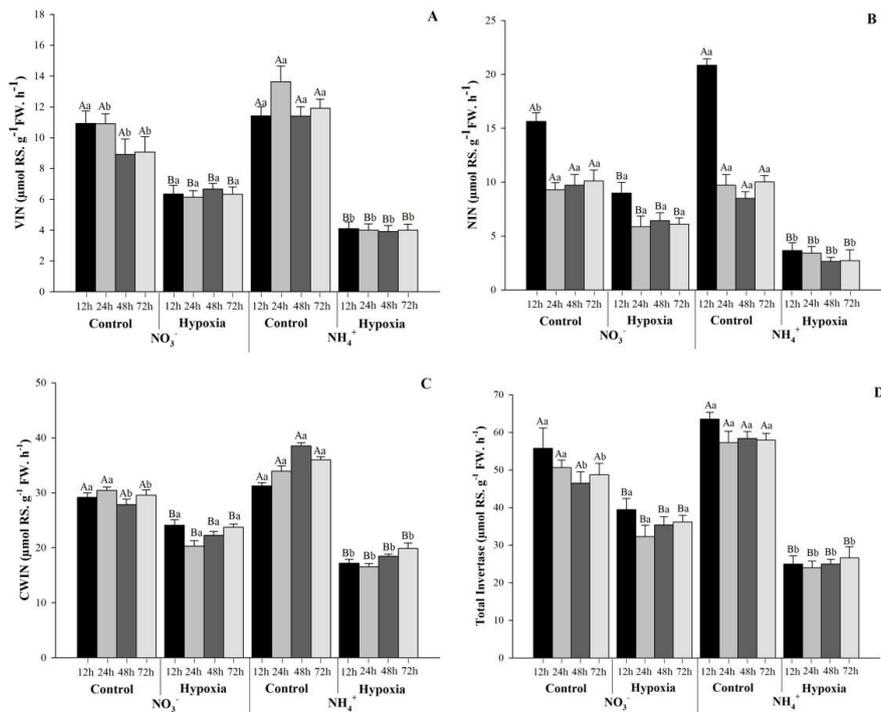


Fig 4. Effect of oxygen availability and of the nitrogen source in the activity of the enzymes vacuolar invertase (A), neutral invertase (B), cell wall invertase (C) and total invertase (D), in roots of rubber tree seedlings. Capital letters compare oxygen availability (control and hypoxia) at each sampling time (12, 24, 48 and 72 hours) within each nitrogen source (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.

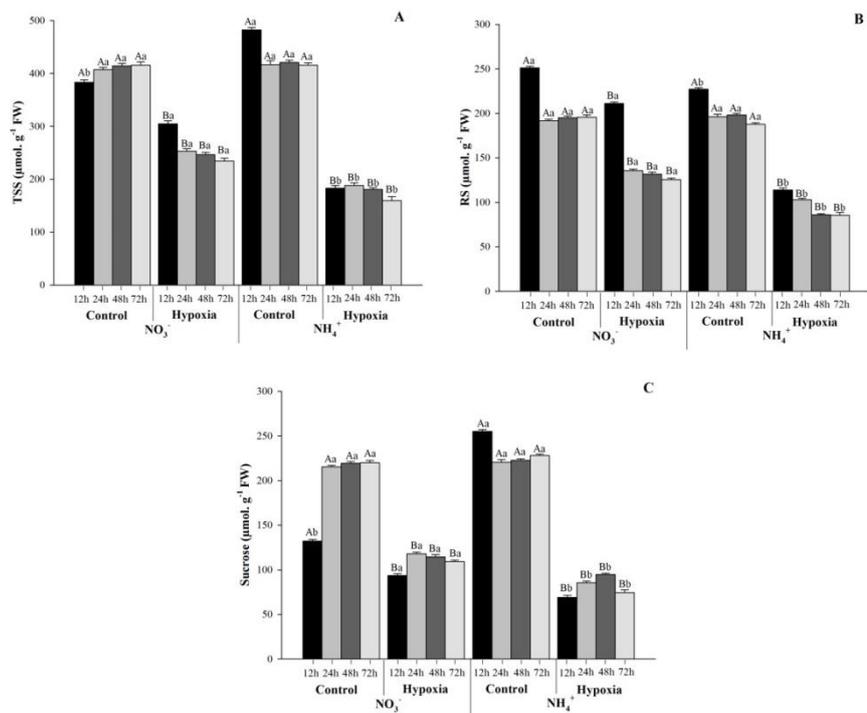


Fig 5. Effect of oxygen availability and of the nitrogen source in leaf content of total sugar soluble (A), reducing sugar (B) and sucrose (C) of rubber tree seedlings. Capital letters compare oxygen availability (control and hypoxia) at each sampling time (12, 24, 48 and 72 hours) within each nitrogen source (NO₃⁻ and NH₄⁺), whereas lowercase letters compare the nitrogen sources at each sampling time within each oxygen condition. Different letters indicate significant differences with 0.05 probability.

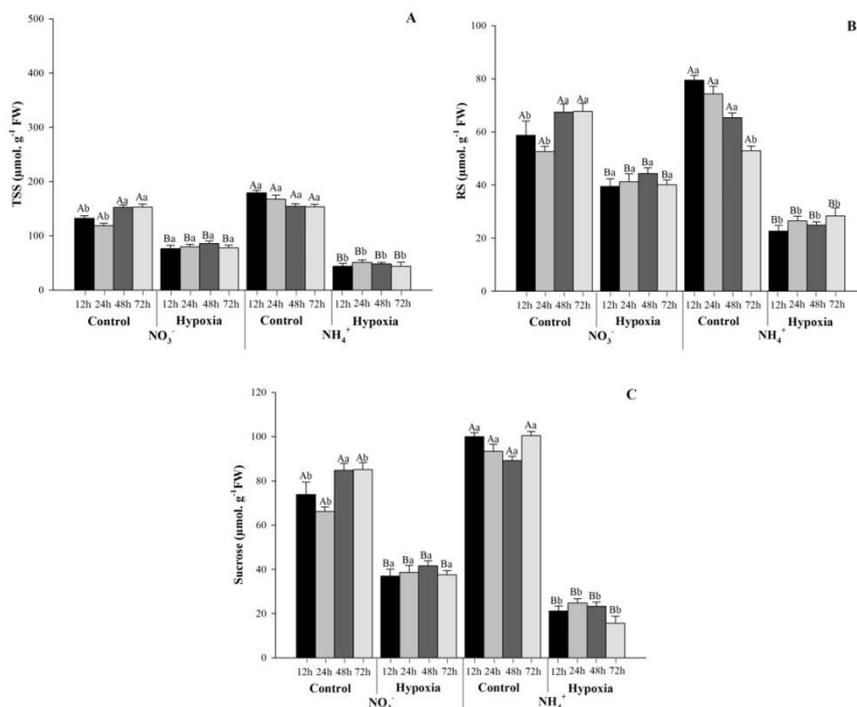


Fig 6. Effect of oxygen availability and of the nitrogen source in root content of total sugar soluble (A), reducing sugar (B) and sucrose (C) of rubber tree seedlings. Capital letters compare oxygen availability (control and hypoxia) at each sampling time (12, 24, 48 and 72 hours) within each nitrogen source (NO₃⁻ and NH₄⁺), whereas lowercase letters compare the nitrogen sources at each sampling time within each oxygen condition. Different letters indicate significant differences with 0.05 probability.

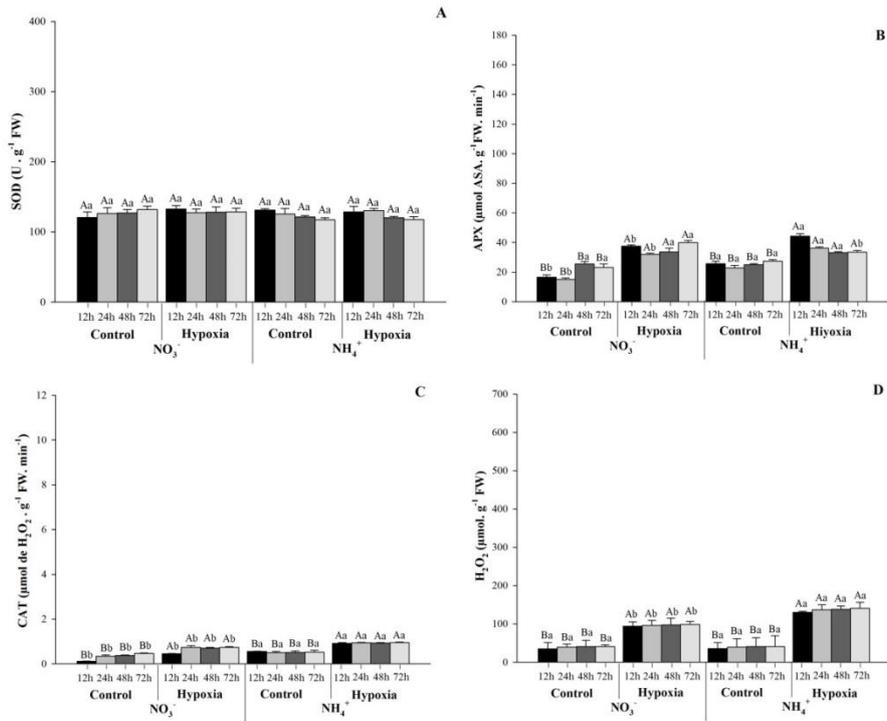


Fig 7. Effect of oxygen availability and of the nitrogen source in the activity of the enzymes superoxide dismutase (A), peroxidase ascorbate (B), catalase (C) and H_2O_2 content (D), in leaf of rubber tree seedlings. Capital letters compare oxygen availability (control and hypoxia) at each sampling time (12, 24, 48 and 72 hours) within each nitrogen source (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.

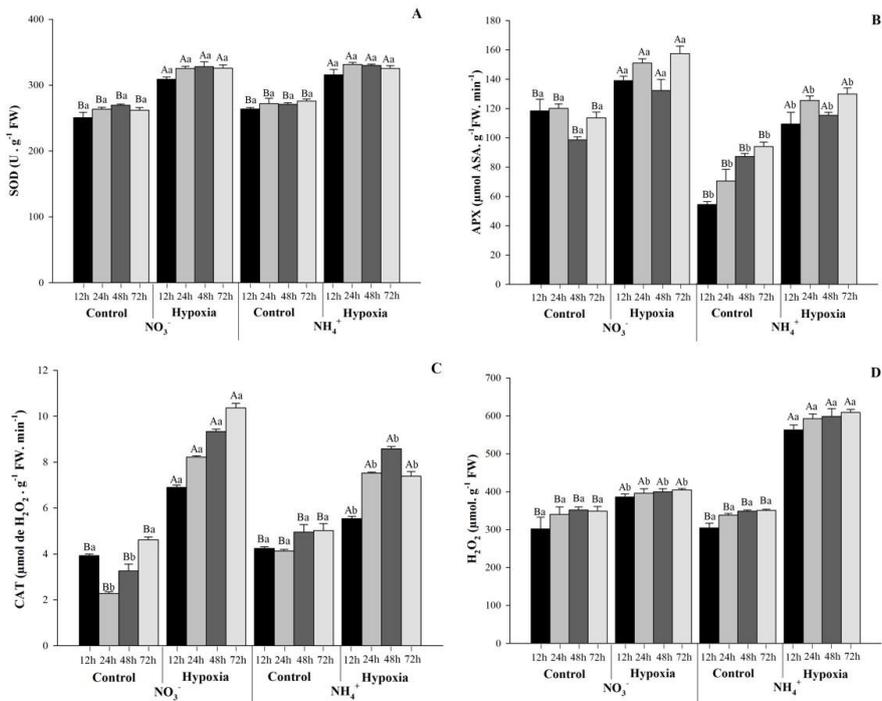


Fig 8. Effect of oxygen availability and of the nitrogen source in the activity of the enzymes superoxide dismutase (A), peroxidase ascorbate (B), catalase (C) and H_2O_2 content (D), in roots of rubber tree seedlings. Capital letters compare oxygen availability (control and hypoxia) at each sampling time (12, 24, 48 and 72 hours) within each nitrogen source (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.

treatment with NH_4^+ . In the leaves, value of $26 \mu\text{mol RS.g}^{-1} \text{FW. h}^{-1}$ in the treatment NO_3^- and $18 \mu\text{mol RS.g}^{-1} \text{FW. h}^{-1}$ in the treatment with NH_4^+ (70%) were observed (Fig. 3B). In roots, $6.0 \mu\text{mol RS.g}^{-1} \text{FW. h}^{-1}$ in the treatment with NO_3^- and $3.0 \mu\text{mol RS.g}^{-1} \text{FW. h}^{-1}$ with NH_4^+ (50%) were the results (Fig. 4B). Low O_2 availability, also, provided reductions in CWIN activity. In the leaves, , when compared control and hypoxia, the reduction was on average, 40% in the plants treated with NO_3^- and 50% with NH_4^+ (Fig. 3C). In the roots, once again, NO_3^- had lower reduction than NH_4^+ , the decrease was $29.0 \mu\text{mol RS.g}^{-1} \text{FW. h}^{-1}$ to 22.0 (25%) and 35.0 to 18.0 (49%) respectively (Fig. 4C).

Total soluble sugars, reducing sugars and sucrose contents

The carbohydrates as well as the invertase activities were significantly reduced after flooding induction, this was observed in leaves and roots for both nitrogen sources. When comparing control and hypoxia, in the NO_3^- treatment, in the leaves, total soluble sugars decreased of approximately $400 \mu\text{mol.g}^{-1} \text{FW}$ to 250 (37.5%) and of $400 \mu\text{mol.g}^{-1} \text{FW}$ to 190 (52.5%) with NH_4^+ . In the roots, the decrease was approximately of $140 \mu\text{mol.g}^{-1} \text{FW}$ to 80 (42.8%) with NO_3^- and of $170 \mu\text{mol.g}^{-1} \text{FW}$ to 50 (70%) with NH_4^+ (Figs 5A and 6A).

Reducing sugars as part of total sugars also reduced sharply. Under normoxia, the results for both NO_3^- and for NH_4^+ were around $200 \mu\text{mol.g}^{-1} \text{FW}$ in the leaves. In addition, about $65 \mu\text{mol.g}^{-1} \text{FW}$ in the roots. However, in hypoxia, NO_3^- showed values of $150 \mu\text{mol.g}^{-1} \text{FW}$ in leaves and roots was of $40.0 \mu\text{mol.g}^{-1} \text{FW}$. With NH_4^+ , the mean values were of $100 \mu\text{mol.g}^{-1} \text{FW}$ and of 25 respectively (Figs 5B and 6B). Sucrose content was higher in control plants, in both tissues evaluated, hypoxic treatments leads to a decrease of approximately 50% in the treatment with NO_3^- and of 70% with NH_4^+ . (Figs 5C and 6C). Practically, no differences in carbohydrate content between experimental times were observed.

Antioxidant metabolism

The SOD activity, in leaves, there was no difference between treatments (Fig. 7A). The result was approximately $126 \text{ U g}^{-1} \text{FW}$, which remained constant throughout the experimental period. In the roots (Fig. 8A), when analyzing the effect of the O_2 availability, there was a significant increase in SOD activity in plants under hypoxia, the values increased 22% ($265 \text{ U g}^{-1} \text{FW}$ to 325) in both NO_3^- and NH_4^+ treatments. In the treatments under hypoxia, there was significant increase in the APX activity compared to their respective controls, regardless of nitrogen source applied and evaluated tissue. The activity of this enzyme in the leaves under flooding, increased on average 75% in plants treated with NO_3^- and 40% in plants treated with NH_4^+ (Fig. 7B). In roots, higher APX activities were observed in flooded plants treated with NO_3^- . The results from this anion were significantly higher whether the O_2 availability, and 24 hours after induction flooding, the activity reached $155 \mu\text{mol ASA. min}^{-1} \text{g}^{-1} \text{FW}$. While, in plants under hypoxia, subjected to NH_4^+ , the maximum value was $130 \mu\text{mol ASA. min}^{-1} \text{g}^{-1} \text{FW}$ only after 72 hours (Fig. 8B). CAT activity, in both, in the leaves and in root tissue was enhanced under hypoxia. In the leaves of plants treated with NO_3^- the CAT activity doubled, and

increased 80% in the plants treated with NH_4^+ (Fig. 7C). In the roots, under hypoxia, the increase in CAT activity, compared to the control was on average of 150% in plants treated with NO_3^- and 60% in plants treated with NH_4^+ (Fig. 8C). In agreement with results previously described, the H_2O_2 content was higher in plants subjected to hypoxia, regardless of tissue evaluated and nitrogen source. In the leaves, under normoxia, no differences between treatments with NO_3^- and NH_4^+ were observed. However, under hypoxia, plants treated with NH_4^+ showed higher H_2O_2 production, overcoming treatment with NO_3^- in 40%. In the roots, this variable has behaved similarly the leaves, although at much larger scale. In this plant tissue, H_2O_2 accumulation in the NH_4^+ presence and O_2 absence, was even more significant, that is 65% higher when compared to treatment with NO_3^- (Figs. 7D and 8D).

It is worth mentioning also that for the three enzymes of the antioxidant system and H_2O_2 content, the data found in root tissue, overcame the results in leaf tissues in all evaluations regardless of the O_2 availability and nitrogen source.

Discussion

Rubber tree plants to survive in areas where periodic flooding can happen - a common occurrence in native habitats of this species. This investigation about gas exchange, carbohydrate metabolism and antioxidant system enzymes in rubber tree seedlings subjected to O_2 deficiency, showed that NO_3^- addition reduced effects of root hypoxia. Therefore, nitrate positively affects the natural plasticity of rubber tree plants. 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).

In the present study was aimed to evaluate similar plants and as consequence of the experimental times (hours) no significant differences in the dry weight were detected. However, large metabolic changes in gas exchange, chlorophyll and sugar content and carbohydrate and antioxidant metabolism were found. In addition, was verified the plants response to the interaction nitrogen source *versus* low O_2 availability.

Photosynthesis is the primary carbon source for higher plants when impaired under low O_2 availability, it affects the entire dynamics of the carbon metabolism. At leaf level, stomatal conductance decline to prevent loss water along with the decrease of Rubisco activity constitutes a first response against O_2 deprivation. (Carvalho et al. 2015a), also observed reduction of 33% and 71% in photosynthesis of rubber tree seedlings treated with NO_3^- and NH_4^+ respectively, after 21 days under flooding. Plants treated with ammonium showed large reduction in stomatal conductance and transpiration resulting in decrease of water absorption by roots and a lower CO_2 diffusion rate into the leave. Consequently, Rubisco is affected by the reduction of leaf hydric potential or lack of substrate. In fact, although variable, the preservation of the photosynthetic activity is associated with plants tolerance to flooding (Arbona et al. 2009). In this work, the CO_2 assimilation was higher in plants treated with NO_3^- which showed greater conservation of the photosynthetic process, under O_2 deficit. Carbohydrates are the main carbon stocks

of plants and their quantities, maintain your metabolism at appropriate levels and the correct partitioning of these molecules associated with energy are prerequisites for the stresses survival (Goggin 2007). The results showed that when under hypoxia, a significant decrease in soluble sugars concentration, particularly sucrose, is accompanied by a reduction in activity of all isoforms invertase and therefore the amount of reducing sugars. Some explanations are possible for the decline of activity of invertase in wetland environments, including pre-existing enzymes degradation and substrate limitation to induce enzyme. Thus, the significant activity decrease of soluble and insoluble invertase treatment with NH_4^+ probably due to substrate limitation in these plants. Metabolic adaptations to hypoxia include the down-regulation of storage metabolism (van Dongen et al. 2004), the energy conserving shift from the invertase to the sucrose synthase route of sucrose degradation (Huang et al. 2008) thereby using pyrophosphate rather than ATP, and a decrease in mitochondrial respiratory activity at low oxygen (Zabalza et al. 2009). Decreases in vacuolar invertase activity are associated with responses to low oxygen and drought-induced early seed abortion (Andersen et al. 2002). In contrast to the CWIN, VIN and the NIN is not glycosylated invertase so the NIN activity is inhibited by breaking sucrose. Enzymes involved in sucrose breakdown are; soluble invertase, neutral invertase and sucrose synthase (Komor 2000). The futile cycle of sucrose, and constant cleavage and re-synthesis of the molecule have been proposed as the mechanism that allows greater flexibility and control over the carbohydrate metabolism and its partition. This cycling promotes the unloading phloem through a sucrose concentration gradient (Rontein et al. 2002).

The changes in demand under adverse conditions alter carbohydrate carbon partitioning in accordance with the relation source-drain, and the charge potential and are dependent on biochemical signaling pathways and molecular complex. Among the enzymatic pathways affected, those involved in sucrose degradation have been extensive study object. Thus, along with vascular changes in physical properties, as well as the loading and transport in the phloem, contribute to different responses observed. Therefore, catabolic pathways regulation contributes to better responses to stress (Bailey-Serres and Voesenek 2008). In our study, higher SOD and APX activity and lower H_2O_2 content was observed in hypoxic plants treated with nitrate. The efficient action of these enzymes is an extremely important component of physiological tolerance, since their actions prevent free radicals from damaging cells and membranes organelles such as mitochondria and chloroplasts. Second Allen et al. (1997), increases in anti-oxidant enzyme expression may increase tolerance to oxidative stress, which increases in low O_2 availability conditions. Under low O_2 availability excessive reducing power and surfeit of ATP leads to cell super reduction, triggering the production of reactive oxygen species (ROS) which are highly detrimental to cell structures. Increased enzyme activity (such as SOD and APX) associated with ROS deletion is thus related with increased stress tolerance in plants exposed to adverse environmental conditions (Giannakoula et al. 2010). Rubber tree plants under hypoxia presented higher antioxidant enzymes activity, especially in plants treated with nitrate. Nitrate application reduces the

formation of reactive oxygen species because NR consumes NADH reducing power, preventing that accumulates and causes over cellular reduction. Furthermore, nitrate improves the efficiency of enzymes SOD, CAT and APX controlling the action of free radicals (H_2O_2) and oxidative stress.

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 18 °C (minimum) and 40 °C (maximum) with an average temperature of 29 °C; average air relative humidity around 56%; maximum photosynthetic photon flux density (PPFD) of 1500 $\mu\text{mol m}^{-2} \cdot \text{s}^{-1}$ and 12h photoperiod. Seeds (cultivar GT1) previously selected for size and weight were germinated in sand. Fifteen days after germination, seedlings of similar height and morphological characteristics were transplanted to pots (5L) filled with nutrient solution of Bolle-Jones (1957)1/2 strength. The solution volume was refilled 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 6 months-old they were divided into four treatments consisting of two nitrogen source in nutrient solution (8 mM nitrate (KNO_3) and 8 mM ammonium ($(\text{NH}_4)_2\text{SO}_4$) and two conditions of oxygen availability (control plants, kept under normoxia and flooded plants, kept under root hypoxia). The experiment was conducted over a period of 3 days, measurements of gas exchange and biochemical analyzes (sample plant material: leaf and root) performed at twelve (12), twenty four (24), forty-eight (48) and seventy-two (72) hours after treatment induction. Sampling was carried out simultaneously to allow accurate comparisons among gas exchanges, carbohydrate metabolism enzymes activity (invertase isoforms and carbohydrate content) and antioxidant metabolism enzymes activity (SOD, CAT, APX).

Shoot and root dry weight

The seedlings were divided into roots and shoots. The plant material was dried at 70 °C to constant weight and the dry weight measured.

Gas exchange and chlorophyll content measurements

Gas exchange measurements were performed in the third fully expanded leaf was monitored throughout the experiment. For three days (12, 24, 48 and 72 hours after treatment induction.) gas exchange was evaluated at 09:00 or 10:00 in the central leaflet. Net CO_2 assimilation rate (P_N), stomatal conductance (g_s), intracellular and environmental concentrations of CO_2 (Ci and Ca) and the transpiration rate (E) were regularly evaluated using a portable gas exchange system (IRGA LI-6400XT, LI-COR, Lincoln, NE, USA). The

chlorophyll content was determined using the chlorophyll FT GREEN LLC atLEAF⁺ model.

Carbohydrate metabolism enzyme assay (CWIN, VIN and NIN) and sugar measurement

Enzyme extract was obtained with liquid nitrogen mortification of 0.5 g leaf blades or roots, to which was added 1.5 mL of extraction buffer containing potassium phosphate buffer 100 mM (pH 7.5), PMSF 1 mM, MgCl₂ 5 mM and DTT 1 mM. The extract was centrifuged at 18000g per 20 min at 4 °C (Cairo et al. 2009). Supernatant fraction was used to analyze the soluble activity (VIN and NIN). Extract for insoluble activity (CWIN) was obtained from pellet fraction according to Fahrendorf; Beck (1990). Activities of vacuolar (VIN) and cytoplasmic (NIN) invertases were determined with MgCl₂ 5 mM e sucrose 200 mM, phosphate buffer (0,4M) at pH 7.5 for neutral invertase or pH 4.5, for acidic invertase. Invertase cellular wall activity (CWIN) was determined with sodium acetate buffer 0,1 M e pH 3,5, MgCl₂ 5 mM and sucrose 200 mM. The reactions proceeded for 1 h at 37°C when the formation of reducing sugars was analyzed (Miller 1959). Carbohydrates extraction was performed from 1000 mg of dry weight were homogenized in 5 mL of potassium phosphate buffer 100 mM (pH 7,0) and then placed in a water bath for 30 min at 40 °C. Homogenate was centrifuged at 5,000 g for 10 min and the supernatant was collected. The process was repeated twice and supernatants were combined. Sucrose and total soluble sugars were quantified as described by Dische (1962), and level of reducing sugars was quantified according to Miller (1959).

Antioxidant metabolism enzymes activity (SOD, CAT, APX) and H₂O₂ content

Enzyme extract was obtained with liquid nitrogen mortification of 0.2 g leaf blades or roots, to which was added 1.5 mL of extraction buffer containing potassium phosphate buffer 400 mM (pH 7.8), EDTA 10 mM, ascorbic acid 200 mM and 22 mg PVPP. The extract was centrifuged at 13000g per 10 min at 4 °C. Supernatant was used to analyze the superoxide dismutase (SOD), catalases (CAT) and ascorbate peroxidases (APX) enzymes (Biemelt et al. 1998). SOD activity (EC 1.15.1.1) was evaluated by the ability of the enzyme to inhibit photo-reduction of nitroblue tetrazolium (NBT), as proposed by Giannopolitis and Ries (1977). CAT (EC 1.11.1.6) was evaluated according to Havir and McHale (1987) and APX activity (EC 1.11.1.11) was determined according Nakano and Asada (1981). Lastly, H₂O₂ content was determined according Velikova et al. (2000).

Statistical analysis and experimental design

The experiments were arranged in a completely randomized design (CRD) in a factorial arrangement (2x2x4). The experiment had four treatments and four time points (12, 24, 48 and 72 hours) for stress measurements with four replicates, totaling 64 plants. Data were analyzed using analysis of variance (ANOVA), and the means were compared using the Scott-Knott test ($p \leq 0.05$).

Conclusion

Carbon assimilation, carbohydrate metabolism, sugar distribution and antioxidant system action are strongly affected by hypoxia in rubber tree plants. Plants treated with NH₄⁺ are more sensitive to hypoxia than plants treated with NO₃⁻. NH₄⁺ treatment presented greater reduction of the photosynthesis and carbohydrate content, lower efficient antioxidant activity for the removal of oxygen reactive species. Therefore, NO₃⁻ addition reduces damage caused by oxygen deficiency promoting response to hypoxia was less intense than with NH₄⁺ thus NO₃⁻ increase the tolerance of rubber tree plants to stress.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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