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Nitrate addition improves photosynthesis and flooding tolerance of rubber tree plants

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Abstract

The role of nitrogen in plants under oxygen deficiency is not well understood. Some studies indicate that nitrate reduction can act as an alternative electron sink (on proton consumption and $NAD(P)^+$ regeneration) reducing the cytoplasmic acidosis induced by anaerobiosis. In this study, we evaluate the role of nitrogen (applied in nutrient solution as either 8mM NO_3^-or 8mM NH_4^+) in the metabolism and physiology of rubber trees (*Hevea Brasiliensis*) subjected to oxygen deficiency. The experiment lasted 21 days, with measurements of gas exchange, biochemical and anatomical analyses taken every 7 days (day 7, 14 and 21). Net photosynthesis, stomatal conductance and transpiration rate in stressed plants significantly decreased (around 50%) at 14 days of the treatment compared to control plants, regardless the nitrogen source. However, in plants treated with nitrate even under stress photosynthesis remained relatively high until the end of the experiment. When exposed to stress, plants treated with nitrate showed a higher efficiency of the antioxidant system enzymes (superoxide dismutase and ascorbate peroxidase), so the H_2O_2 content was higher in leaves of plants treated with ammonium and exposed to hypoxia. Moreover, plants grown in nitrate in waterlogged conditions were characterized by earlier aerenchyma formation than plants grown with ammonium. Therefore, our results indicate that NO_3^- is more beneficial than NH_4^+ for rubber tree plants metabolism under oxygen deficiency conditions.

Keywords: *Hevea brasiliensis*, Hypoxia, Nitrogen metabolism, Antioxidant system, Anatomy.

Abbreviations: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), nitrate reductase (NR), reactive oxygen species (ROS), net CO_2 assimilation rate (P_N), stomatal conductance (g_S), (E) transpiration, photosynthetic photon flux density (PPFD).

Introduction

Plants are frequently exposed to conditions of excessive soil moisture (waterlogging) in which the root system is hampered by the low diffusion rate of oxygen (O_2) in water (Armstrong, 1980). Given the fundamental importance of O_2 in the aerobic metabolism of plants, waterlogging stress can strongly affect growth and survival in both agricultural and natural ecosystems. Indeed, O_2 depletion can negatively influence the productivity of many economically important species (Irfan et al., 2010). From a metabolic perspective, O_2 deficiency decreases oxidative phosphorylation and, consequently, causes a relative increase in ATP production by cytosolic glycolysis. O_2 limitation also affects the terminal acceptor of mitochondrial respiratory chain, resulting in the inhibition of energy-demanding processes and the stimulation of fermentative pathways for anaerobic ATP production (Bailey-Serres and Voesenek, 2008). At the physiological level, O_2 deficiency in roots affects key functions such as nutrient and water uptake. Furthermore, whole-plant processes are also affected, including liquid-CO_2 assimilation, stomatal conductance/ transpiration and carbohydrate translocation (Kreuzwieser et al., 2004). There is also an uncontrolled increase of free radicals in cells. These diverse responses to O_2 depletion vary according to the species and to the severity and duration of stress (Shao 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 (Manivannan et al., 2008; Jaleel et al., 2009). Many plants also undergo morphological changes in response to low oxygen availability such as the production of adventitious roots, lenticels and aerenchyma. These adaptations help minimize the oxygen deficit, increasing the level of tolerance of the plant to water stress (Kawase, 1981). The formation of aerenchyma in stress tolerant species can occur in nodes, rhizomes, stems, and leaves, although they are mainly observed in submerged roots. (Drew, 1997; Jackson and Armstrong, 1999; Drew et al., 2000; Gibberd et al., 2001).

The application of nitrate (NO_3^-) - but not ammonium (NH_4^+) - 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). Indeed, comparisons of NO_3^- and NH_4^+ indicate that these nitrogen forms may induce distinct metabolic responses under water stress (Escobar et al., 2006; Patterson et al., 2010). NO_3^- appears to be reduce to nitrite (NO_2^-) through nitrate reductase (NR) under O_2 deficiency (Botrel et al. 1996). 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 $NAD(P)^+$ regeneration by NR

(Stoimenova et al., 2003; Libourel et al., 2006). This observation 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 a compound derived from it (Libourel et al. 2006). Nevertheless, the effect of hypoxia on plant metabolism is still not completely understood (Sousa and Sodek 2002a; Bailey-Serres and Voesenek, 2008). The objective of this study was to identify and quantify the physiological (gas exchanges), metabolic (enzymatic activity) and anatomical changes (aerenchyma development) in the rubber tree plants (*Hevea Brasiliensis Müll.Arg.*) subjected to waterlogging and treated with two forms of exogenous nitrogen (NO_3^- or NH_4^+). Specifically, we studied the mechanisms of tolerance to hypoxia by examining changes in gas exchange, the antioxidant system of leaves and anatomy of roots.

Results

Gas exchange

Net CO_2 assimilation rate in plants cultivated in solution with no nitrogen exhibited maximum values of 2.5 µmol CO_2 $m^{-2}.s^{-1}$ in normal levels of oxygen or hypoxia. Plants treated with nitrate exhibited higher net assimilation rate under both normoxia and hypoxia. Under normoxia, photosynthesis reached 8.2 µmol CO_2 $m^{-2}.s^{-1}$ at 21 days, whereas the assimilation peak occurred at 7 days reaching 9.2 µmol CO_2 $m^{-2}.s^{-1}$ under hypoxia. There was a small reduction in net assimilation rate to 5.5 µmol CO_2 $m^{-2}.s^{-1}$ after 14 and 21 days under hypoxia. In plants treated with ammonium, the results were much lower, waterlogging caused an increase in net CO_2 assimilation rate, reaching 4.5 µmol CO_2 $m^{-2}.s^{-1}$ at 7 days. However, a large reduction in this parameter was observed at 14 days (1.5 µmol CO_2 $m^{-2}.s^{-1}$) and too at 21 days (1.3 µmol CO_2 $m^{-2}.s^{-1}$). (Fig. 1). Stomatal conductance and transpiration were similar throughout experiment period. Flooded plants cultivated under hypoxia had higher values than the control for these two parameters at 7 days. However, at 14 and 21 days, plants under stress were affected negatively by the deficiency of oxygen (Fig. 2). In the presence of ammonium under normal oxygen conditions, stomatal conductance reached a maximum value of 0.1 mol H_2O $m^{-2}.s^{-1}$. The same value was observed at 7 days for the waterlogged root systems - this parameter decreasing to 0.01 (14 days) and 0.006 (21 days). In both hypoxic and normal oxygen conditions, plants treated with nitrate had significantly higher stomatal conductance in comparison to plants treated with ammonium or without addition of exogenous nitrogen (Fig. 2a). At 14 and 21 days under hypoxia, there was a decrease in transpiration regardless the nitrogen source. However, for this parameter results found in plants treated with nitrate were higher than other treatments even under stress conditions (Fig. 2b).

Antioxidant metabolism

Plants without the addition of exogenous nitrogen exhibited a lower activity of superoxide dismutase enzyme (SOD) when compared to those ones treated with nitrate or ammonium (Fig. 3a). This difference was maintained throughout the experimental period regardless of oxygen availability. Intermediate values for SOD activity were found in plants treated with ammonium, with waterlogging causing an average increase of 4.5 % in enzyme activity. The highest SOD activity was observed in flooded plants cultivated with nitrate, with SOD activity reaching the maximum value (422.0) 21 days after waterlogging. A significant increase in ascorbate peroxidase (APX) activity was observed in all waterlogged treatments in comparison to their respective controls. In the ammonium treatment under hypoxia, it was observed a moderate increase in relation to the control. Within the nitrate treatment, the low oxygen availability led to an intense increase in APX activity reaching 143 at 7 days, 92 at 14 days and 90 at 21 days (Fig. 3b). Consistent with results previously described, the leaf H_2O_2 content was higher in plants exposed to hypoxia and in plants treated with ammonium (Fig. 3c).

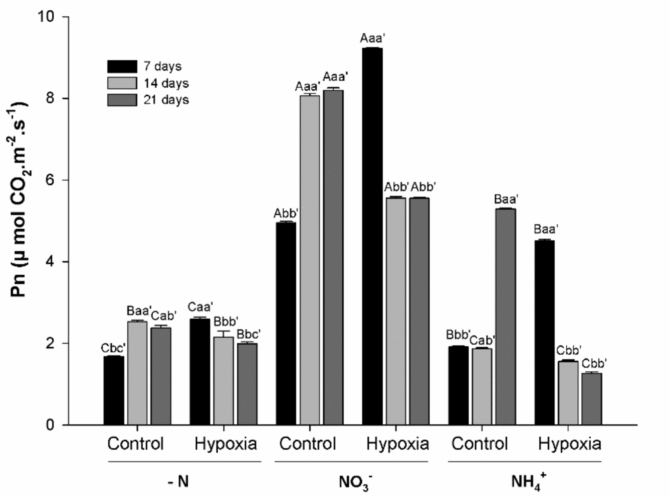

Fig 1. Effect of oxygen availability and nitrogen source on the net photosynthesis (Pn) of rubber tree seedlings. Capital letters compare the nitrogen sources at each sampling time within each oxygen condition (control and hypoxia); lowercase compare oxygen availability in each sampling time within each nitrogen source, and lowercase followed by an apostrophe compare the sampling times within each oxygen condition (control and hypoxia) for each nitrogen source. Different letters indicate significant differences with 0.05 probability.

On average, catalase activity (Fig. 3d) was 100-fold lower in comparison to SOD and APX. Interactions between ammonium and hypoxia (as well as between nitrate and hypoxia) resulted in a significant increase in CAT activity only at 21 days.

Aerenchyma formation

Control plants (not subjected to waterlogging) did not form aerenchyma. The roots of hypoxic plants treated with ammonium (Fig. 4) showed aerenchyma only by 21days. The most interesting result was that, hypoxic plants cultivated with nitrate showed signs of aerenchyma formation by day 14 and, by 21 days, a more pronounced development of this morphological adaptation was observed (Fig. 5).

Discussion

Our investigation of gas exchange, antioxidant system enzymes and root anatomy in rubber tree seedlings subjected to O_2 deficiency clearly demonstrated that addition of both nitrate and ammonium considerably modified the metabolic effects of root hypoxia. As an energy-consuming process, nitrogen assimilation has important implications for the energy state of vegetal cells (Bloom and Sukrapanna, 1992).

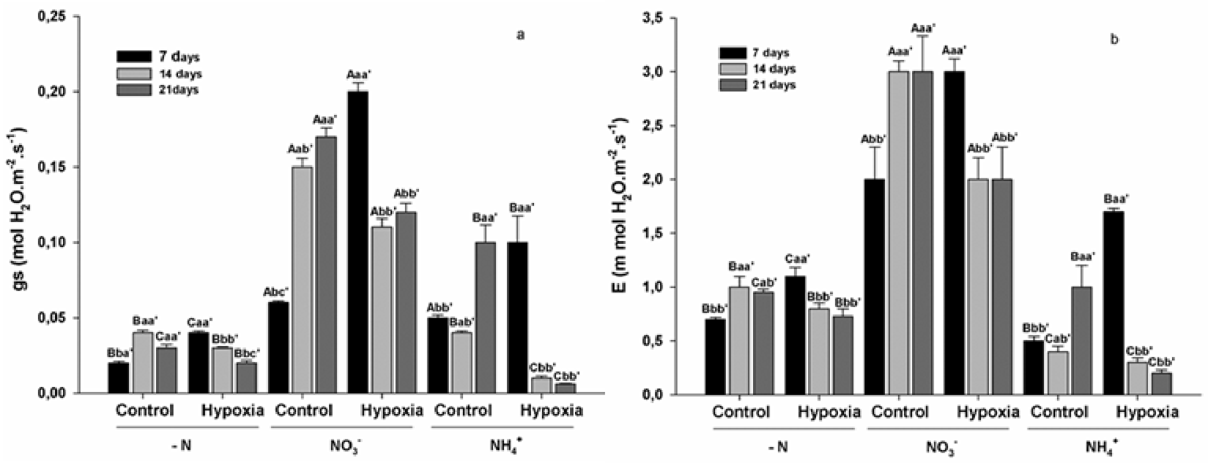

Fig 2. Effect of oxygen availability and nitrogen source in the (gs) stomatal conductance (a) and (E) transpiration (b) of rubber tree seedlings. Capital letters compare the nitrogen sources at each sampling time within each oxygen condition (control and hypoxia); lowercase compare oxygen availability in each sampling time within each nitrogen source, and lowercase followed by an apostrophe compare the sampling times within each oxygen condition (control and hypoxia) for each nitrogen source. Different letters indicate significant differences with 0.05 probability.

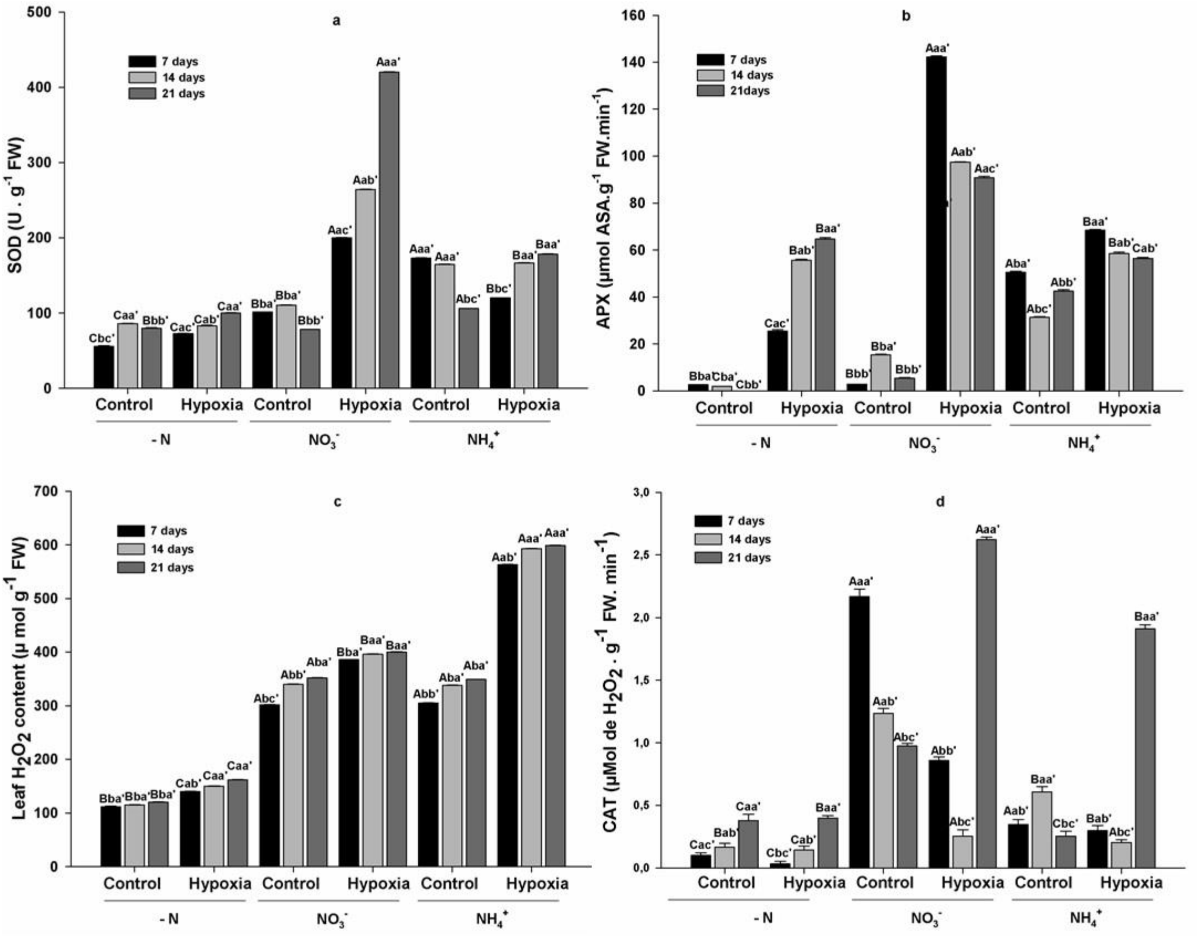

Fig 3. Effect of oxygen availability and of the nitrogen source in the activity of the enzymes superoxide dismutase a, peroxidase ascorbate b, H_2O_2 content c and catalase d, of rubber tree seedlings. Capital letters compare the nitrogen sources at each sampling time within each oxygen condition (control and hypoxia); lowercase compare oxygen availability in each sampling time within each nitrogen source, and lowercase followed by an apostrophe compare the sampling times within each oxygen condition (control and hypoxia) for each nitrogen source. Different letters indicate significant differences with 0.05 probability.

Moreover, the moderating influence of nitrogen may become more important in conditions of O_2 deficiency such as waterlogging, when there is a decrease in the ATP/ADP relation due to the low yield of fermentative processes (Bailey-Serres and Voesenek, 2008). It has been suggested that the conversion of NO_3^- in NO_2^- by nitrate reductase in plants subjected to NO_3^- could regenerate NAD $(P)^+$ and consume protons, providing an alternative to fermentation during hypoxia (Roberts et al., 1985). However, some authors have raised questions about this hypothesis (Stoimenova et al., 2003; Libourel et al., 2006). Specifically, Stoimenova et al. (2007) demonstrated that NO_2^- may play a role as an alternative receptor of electrons, replacing O_2 in the respiratory chain and oxidizing NAD(P)H for ATP synthesis. Thus, NO_2^- synthesis from NO_3^- might play an important role in the maintenance of mitochondrial functionality during O_2 deficiency (Gupta & Igamberdiev, 2011). Plants cultivated with ammonium do not reduce nitrate to nitrite or reduce nitrite to ammonium. The lack of these processes is detrimental because equivalent reducers are not recycled, especially in plants under hypoxia. Furthermore, the first reaction that takes place when ammonium is absorbed is catalyzed by glutamine synthetase, which depends on ATP. Since this enzyme has decreased activity in roots under O_2 deficiency, the ratio of ATP/ADP is reduced (Limami et al., 2008). Lower glutamine synthetase activity may lead to NH_4^+ accumulation, high levels of which have deleterious effects on vegetal cell (Givan, 1979). In the present study, we observed in plants treated with ammonium a large reduction in stomatal conductance and transpiration. When this occurs, there is a decrease in absorption of water by the roots and a lower rate of CO_2 diffusion into the plant. Consequently, photosynthesis drops because Rubisco is affected by the reduction of leaf hydric potential or lack of substrate (CO_2). However, plants subjected to nitrate exhibited assimilation rate over 5 µmol CO_2 $m^{-2}.s^{-1}$ even in hypoxic conditions (see Fig. 1). Unlike ammonium, nitrate is transported through the xylem and, therefore, nitrate assimilation can occur in roots and leaves. Thus, the reactions, which convert nitrate into amino acids, function as an electron sink, preventing cell super reduction that occurs due to accumulation of ATP and NADPH under hypoxic conditions. 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 (Giannakoula et al., 2010). Stress caused by low oxygen availability leads to a higher SOD activity, which determines O_2^- and H_2O_2 concentration - key defense mechanisms necessary to prevent the formation of OH^- radicals (Jaleel et al., 2007). 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). APX as well as other peroxidases, unlike CAT, have high affinity for H_2O_2, metabolizing it even in low concentrations (Gechev et al., 2006). Several APX isoforms are widely distributed in nearly all cell organelles, and abiotic stresses frequently induce increases in both gene expression and APX activity to compensate for deficiencies in CAT activity. 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 plant seedlings under hypoxia exhibited higher activity of anti-oxidant system enzymes, especially in plants treated with nitrate. The maintenance of photosynthetic rate and the lowest content of peroxide due to the efficiency of the antioxidant system proves the positive influence of nitrate when we subject these plants to flooding. This strongly suggests that this nitrogen source positively affects the removal of reactive-oxygen species (ROS) preventing H_2O_2 accumulation. In our study, higher SOD and APX activity and lower content of H_2O_2 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. In low oxygen availability conditions 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 and APX controlling the action of free radicals (H_2O_2) and oxidative stress. Therefore, it is believes that less

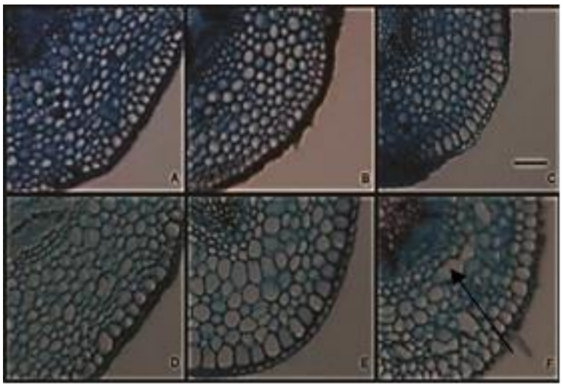

Fig 4. Effect of oxygen availability and nitrogen source in the aerenchyma formation in cross-sectional lateral roots of rubber tree seedlings grown with 8 mM Ammonium and subjected to two oxygen availability conditions: without waterlogging (A, B and C) and waterlogging (D, E and F), D, E and F corresponds 7, 14 and 21 days after the induction of waterlogging. The bar (——) corresponds to 100 micrometers. The arrow to point aerenchyma formation.

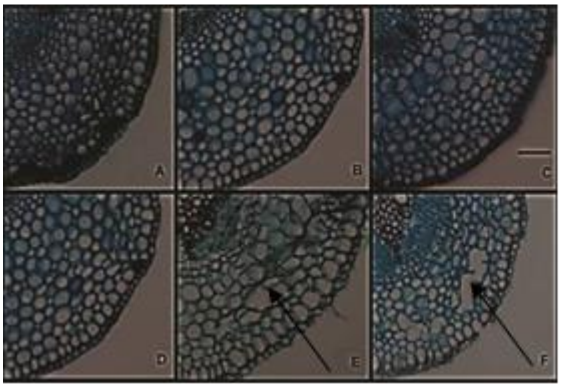

Fig 5. Effect of oxygen availability and nitrogen source in the aerenchyma formation in cross-sectional lateral roots of rubber tree seedlings grown with 8 mM Nitrate and subjected to two oxygen availability conditions: without waterlogging (A, B and C) and, waterlogging (D, E and F), D, E and F corresponds 7, 14 and 21 days after the induction of waterlogging. The bar (——) corresponds to 100 micrometers. The arrow to point aerenchyma formation.

damage occurs in the membranes of organelles and cells leads to the maintenance of metabolic processes, as demonstrated by photosynthesis, culminating in cell homeostasis and a normal plant development. Another important point is that under low O_2 concentrations leads to the formation of aerenchyma in tolerant plant species. The large number of studies have shown the beneficial effects of aerenchyma by providing a pathway for the diffusion of gases to the submerged organs (Loureiro et al., 1995), and rubber tree seedlings growing in solution with nitrate, developed aerenchyma, an adaptation associated with low availability of O_2, earlier than plants growing in ammonium. Second Thomas and Sodek, (2005), the addition of nitrate in soybean plants apparently form a more compact aerenchyma, with more cells and intercellular spaces. Therefore, nitrate positively affects the natural plasticity of plant rubber trees to survive in areas where periodic flooding can happen - a common occurrence in native habitats of this species.

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; average air relative humidity around 75%; maximum photosynthetic photon flux density (PPFD) of 1500 mmol m^{-2}. s^{-1} and 12h photoperiod. Seeds (cultivar RRIµ600) previously selected for size and weight were germinated in pots (5L) filled with sand. Eight days after germination, seedlings of similar height and morphological characteristics were transplanted to pots (2L) filled with nutrient solution (Bolle-Jones 1957). 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 12 months-old they were divided into six treatments consisting of three nitrogen concentrations in nutrient solution (0 mM nitrogen, 8 mM nitrate (KNO_3) and 8 mM ammonium ($(NH_4)_2SO_4$) and two conditions of oxygen availability (control plants kept under normoxia and flooded plants kept under root hypoxia). Vegetal material (leaf and root) were sampled seven (7), fourteen (14) and twenty-one (21) days after the induction of treatments. Sampling was carried out simultaneously to allow accurate comparisons of the antioxidant metabolism enzymes activity (SOD, CAT, APX) and anatomical changes of the root system over time.

Gas exchange measurements

Gas exchange measurements were performed in the third fully expanded leaf was monitored throughout the experiment. Every seven days for three weeks, gas exchange was evaluated at 09:00 or 10:00 in the central leaflet. Net CO_2 assimilation rate (P_N) and stomatal conductance (g_S) were regularly evaluated using a portable gas exchange system (IRGA LI-6400XT, LI-COR, Lincoln, NE, USA).

Enzyme extraction, activity assays and H_2O_2 content

Enzyme extract was obtained with liquid nitrogen mortification of 0.3 g leaf blades, to which was added 1.5 mL of extraction buffer containing 375 µL potassium phosphate buffer 400 mM (pH 7.8), 15 µL EDTA 10 mM, ascorbic acid 200 mM, 22 mg PVPP and 1035 µL water. The extract was centrifuged at 13000g per 10 min at 4 ºC. 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, H_2O_2 content was determined according Velikova et al. (2000).

Anatomical measurements

Fragments of secondary roots were fixed with FAA 50 (Johansen, 1940) and stored in 70% ethanol. Material was sectioned on microtome; cross-sections were made on the third centimeter of pilifera zone. Cross-sections were cleared, rinsed and subsequently stained with safranin 7.5: Astra blue 2.5 (Bukatsch, 1972). Semi-permanent slides were mounted with glycerin and photomicrographs obtained in optical microscope coupled with a digital camera.

Statistical analysis and experimental design

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

Conclusion

Lastly based on our results, NO_3^- is clearly more beneficial than NH_4^+ for the metabolism of rubber trees under hypoxia. However, a definitive explanation for the positive effect of NO_3^- on plant growth during O_2 deficiency is not known yet.

Acknowledgements

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