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Water relations, nitrogen compounds and enzyme activities in leaf and root of young Yellow Lapacho (*Tabebuia serratifolia*) plants subjected to flooding

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Abstract

The aim of this study was to investigate the (i) behavior of young *Tabebuia serratifolia* (Yellow Lapacho) plants exposed to flooding condition, (ii) measuring the physiological and biochemical parameters in leaf and root, and (iii) to discover if this species can be used in areas where the flooding occurs frequently. A completely randomized block design with two water conditions (control and flooding) was used. The parameters measured were the leaf water potential, stomatal conductance, transpiration rate, nitrate reductase activity, nitrate, free ammonium, glutamine synthetase activity, total soluble amino acids, total soluble proteins, alcohol dehydrogenase activity, and lactate dehydrogenase activity. The data showed significant differences in all parameters. The reductions were observed in water relations and nitrogen compounds. However, increases were determined in alcohol dehydrogenase and lactate dehydrogenase activities after flooding. Regarding with organs evaluated, modifications more intense into root for many parameters such as glutamine synthetase and lactate dehydrogenase enzymes, and leaf was more responsive in nitrate, free ammonium, and alcohol dehydrogenase activity. Therefore, *Tabebuia serratifolia* plants are sensitive to flooding, and are not suitable for areas prone to flooding.

Keywords: *Tabebuia serratifolia*, flooding, anoxia, nitrogen, lactate, alcohol.
Abbreviations: O_2 – oxygen, CO_2 - carbon dioxide, N - nitrogen, NO_3^- - nitrate, NO_2^- - nitrite, NH_4^+ - ammonium, photosynthetic active radiation - PAR, GS - glutamine synthetase, GOGAT - glutamine oxoglutarate aminotransferase, GDH - glutamate dehydrogenase, ADH - alcohol dehydrogenase, LDH - lactate dehydrogenase, ATP - adenosine-5'-triphosphate, TCA cycle - tricarboxylic acid cycle.

Introduction

Abiotic stress caused by water saturation in substrate is called flooding, and is responsible for eliminating soil spaces previously filled by air, limiting gas exchange in plants. Under flooding situations, oxygen (O_2) dissolved in water can be consumed by root and microorganisms, resulting in a hypoxic or anoxic environment (Bailey-Serres and Voesenek, 2008). Substrates with low or no O_2 supply induce changes in anatomical (Pereira et al., 2008), morphological (Medri et al., 2007), physiological (Islam et al., 2010), and biochemical parameters of plants (Ferreira et al., 2009). A plant with specific response to stresses such flooding as can be classified as tolerant or sensitive (Armstrong et al., 1994). Injury level and survival capacity in higher plants to flooding are influenced by water depth, oxygen (O_2) supply and carbon dioxide (CO_2) concentration, pH, and water turbidity (Ito et al., 1999). In other words, these factors will negatively affect many essential processes such as photosynthesis and water relations (Chen et al., 2005), nutrient absorption (Lizaso et al., 2001; Conaty et al., 2008), and partitioning of photoassimilates (Pezeshki, 2001).

Growth and distribution of plants in ecosystems is normally controlled by adequate water supply and favorable edaphoclimatic condition. Flooding can be caused by insufficient drainage, large pluviometric index, and tidal volume (Haddad et al., 2000). Some plants are sensitive to inundation during early growth stages resulting in a low rate of survival. On the other hand, tolerant plants have a capacity to temporarily remain under anoxic conditions and their survival is higher, compared with sensitive plants (Bailey-Serres and Voesenek, 2008). *Tabebuia serratifolia* (Yellow Lapacho) is a native tree species in Brazil with forestry potential, with economical, ornamental, and ecological functions. This tree is caducifolium during flowering period, and flowers are intense yellow in color (Biondi and Reissmann, 2002). This tree is frequently used in revegetation of degraded areas, mainly in areas previously explored for petroleum in Amazon region. However, several problems have occurred in its utilization, mainly plant loss during periods of higher pluviometric index and consequent flooding.

The aim of this study was to investigate the (i) behavior of young *Tabebuia serratifolia* plants exposed to flooding condition, (ii) to measure physiological and biochemical parameters in leaf and root, and (iii) discovering if this species can be used in areas prone to flooding.

Results

Consequences of flooding on water potential, stomatal conductance, and transpiration

Water potential in leaf was affected by flooding, compared with control plants (Fig 1 A). Water potential in the control plants and under flooding was -0.2 and -2.3 MPa, respectively, demonstrating a decrease of 105%. Stomatal conductance was significantly influenced (Fig 1 B), with control and flooded plants at 0.38 and 0.02 mol m^2 s^{-1}, respectively. The flooding promoted a decrease of 94.7% in stomatal conductance, in relation to control plants. Similarly, transpiration rates of control and flooding plants were 1.80 and 0.02 mol m^2 s^{-1}, respectively (Fig 1 C). Transpiration rate in plants under flooding was reduced by 89%, compared with control plants.

Effect of flooding on nitrate reductase, nitrate and free ammonium in leaf and root

Nitrate reductase activity was influenced by flooding in leaf and root of plant (Fig 2A). In leaf of plants, nitrate reductase activity was 0.01 µmol NO_2^- g^{-1} FM h^{-1} under flooding condition, with a reduction of 96.8 %, compared with control plants (0.32 µmol NO_2^- g^{-1} FM h^{-1}). In roots, the values were 0.35 and 0.01 µmol NO_2^- g^{-1} FM h^{-1} in control and flooding treatments, respectively, with 97% reduction in flooding plants. Leaf nitrate concentrations of control and flooding treatments were 0.68 and 0.01 µmol NO_3^- g^{-1} DM, respectively. The root nitrate levels were 0.58 and 0.02 µmol NO_3^- g^{-1} DM, respectively (Fig 2B). Hence, leaf and root nitrate concentrations of plants under flooding showed a decrease of 98.5% and 96.5%, respectively, compared to control plants. The free ammonium amount in leaves was 18.6 and 10.3 µmol GGH g^{-1} DM in control and flooded plants, respectively (Fig 2C). In addition, these data revealed a fall of 44.6% in flooded plants, compared with the control. In the same way, levels of root free ammonium of control and flooding were 14.1 and 6.2 µmol GGH g^{-1} DM, respectively, indicating a reduction of 56% in plants subjected to flooding.

Changes produced by flooding on glutamine synthetase, total soluble amino acids, and total soluble proteins in leaf and root

Glutamine synthetase activity in leaf was significantly reduced in flooded (2,4 µmol GGH g^{-1} DM), compared with control plants (27 µmol GGH g^{-1} DM) (Fig 3A). Enzyme activity in leaf indicated a reduction of 91%, compared with the control. Activity of this enzyme in root was 96.2% lower in flooded (1.4 µmol GGH g^{-1} DM), compared with control plants (37 µmol GGH g^{-1} DM). Concentrations of total soluble amino acids in leaf were negatively influenced by flooding, with control and flooded plants at 59.53 and 14.1 µmol g^{-1} DM (Fig 3B), respectively, characterizing a decrease of 76.2% in plants under flooding. Regarding with roots, the total soluble amino acids were 51.74 and 6.43 µmol g^{-1} DM, for control and flooded plants, respectively. These data

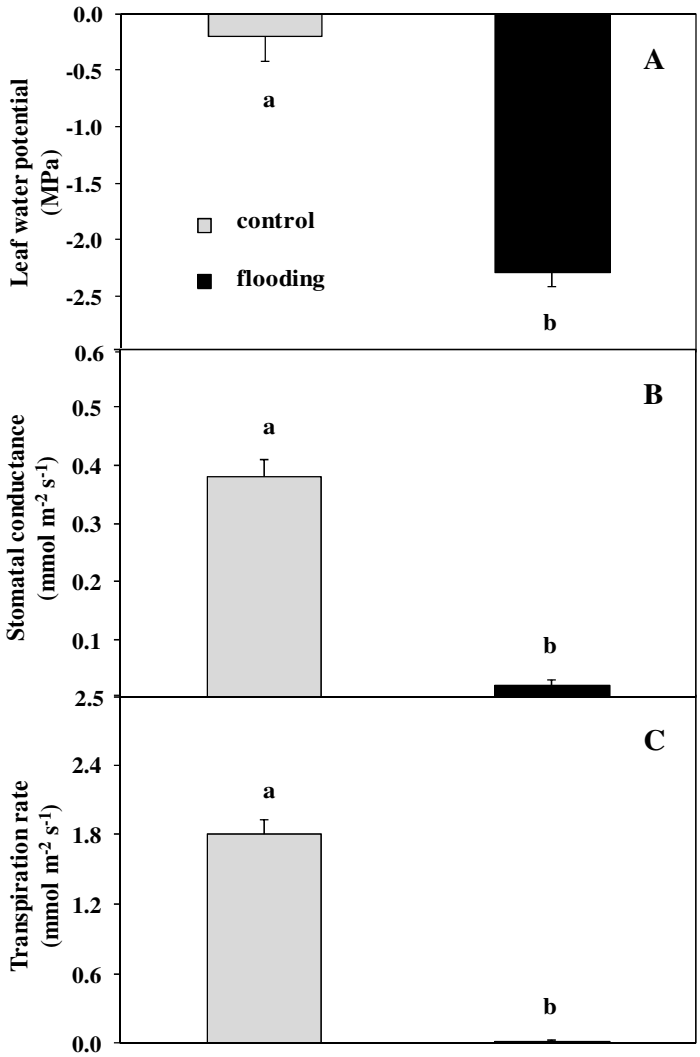

Fig 1. Leaf water potential (A), stomatal conductance (B), and transpiration rate (C) in young *Tabebuia serratifolia* plants subjected to flooding. Same letters do not show significant differences at Tukey's test ($P < 0.05$). Bars represent the mean standard errors.

reveal a reduction of 87.6 % in plants under flooding. Total soluble proteins presented a significant decrease after the flooding (Fig 3C), with control and flooded plants at 3.5 and 0.5 mg g^{-1} DM, respectively. This was a reduction of 85.7% in plants under flooding, compared with control plants. Root total soluble proteins of control and flooded plants was 3.1 and 0.3 mg g^{-1} DM, respectively, with a decrease of 96.3% in plants exposed to flooding, compared with control plants.

Responses induced by flooding on alcohol dehydrogenase and lactate dehydrogenase in leaf and root

Alcohol dehydrogenase activity presented significant differences in leaf and root after flooding (Fig 4A).

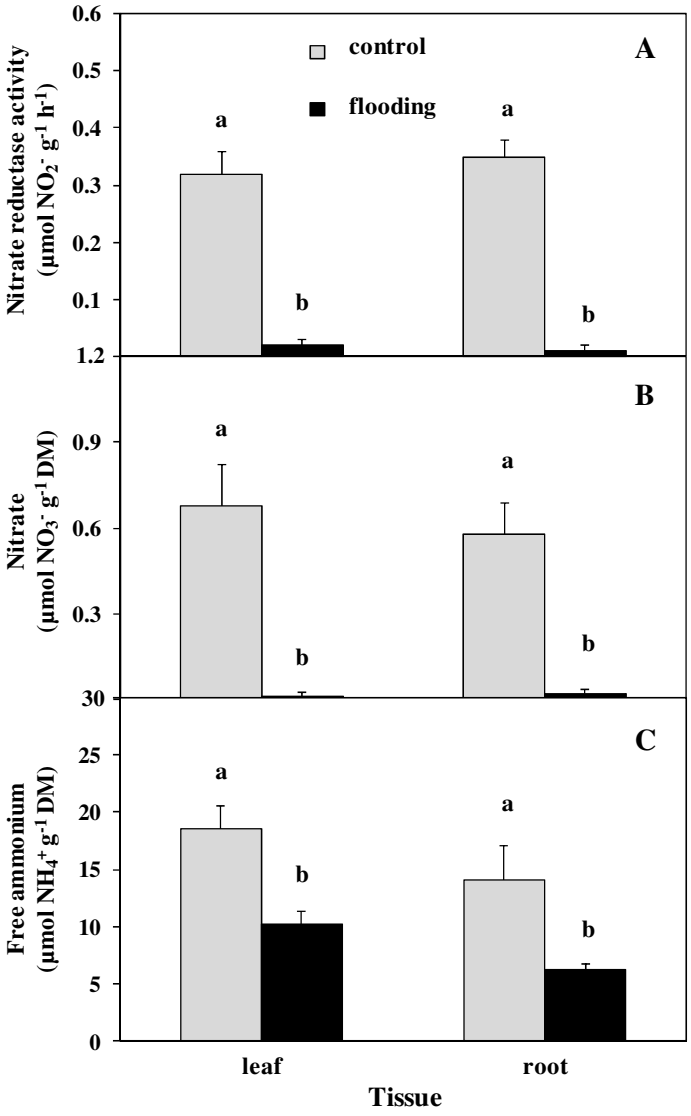

Fig 2. Nitrate reductase activity (A), nitrate (B), and free ammonium (C) in young *Tabebuia serratifolia* plants subjected to flooding. Same letters do not show significant differences at Tukey test ($P < 0.05$). Bars represent the mean ±standard errors.

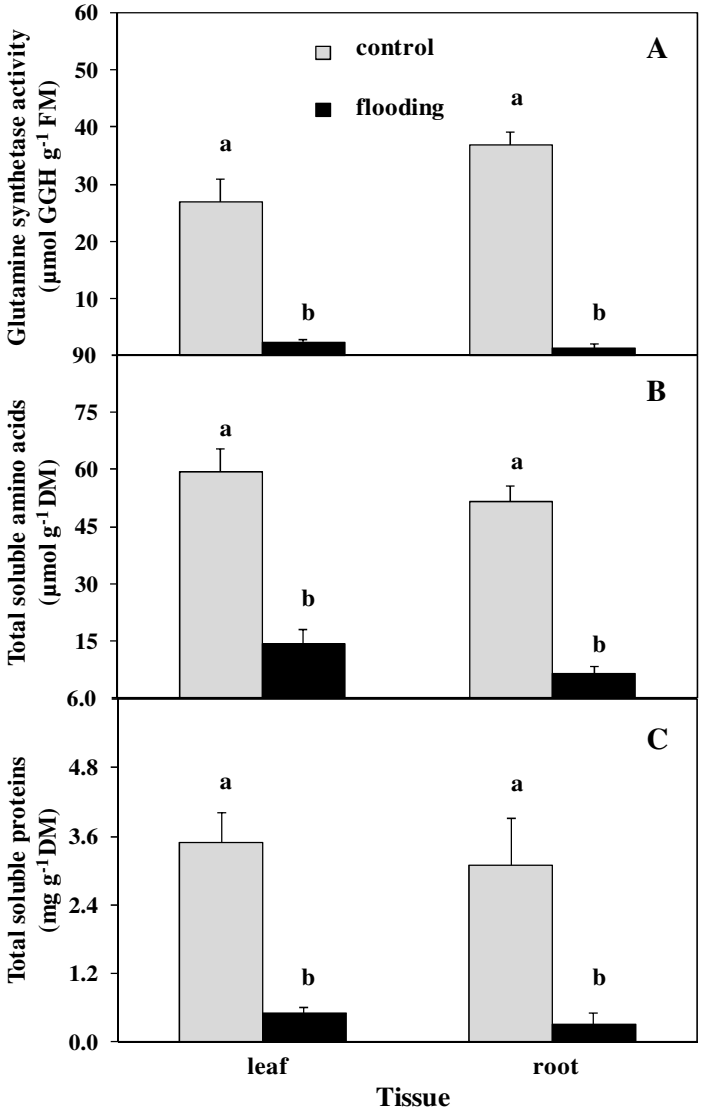

Fig 3. Glutamine synthetase activity (A), total soluble amino acids (B), and total soluble proteins (C) in young *Tabebuia serratifolia* plants subjected to flooding. Same letters do not show significant differences at Tukey test ($P < 0.05$). Bars represent the mean ±standard errors.

Activity in leaf were 0.31 and 0.60 µmol NADH g^{-1} FM in control and flooded treatments, respectively, an increase of 93.5% for the flooded plants. Activities in root were 0.80 and 1.10 µmol NADH g^{-1} FM for control and flooding, respectively. Lactate dehydrogenase activity showed significant increases in leaf and root induced by flooding (Fig 4B). An increase of 83.3% in leaf enzyme activity for flooded plants, with control and flooding treatments at 0.60 and 1.10 µmol NADH g^{-1} FM, respectively. Increase in root enzyme activity was 140% for flooded plants, with values of 0.50 and 1.20 µmol NADH g^{-1} FM in control and flooding condition, respectively.

Discussion

Leaf water potential was reduced in flooding conditions (Fig 1A), and this decrease is due to the lack of energy for physiological processes (Kerbauy, 2004), which will promote a decrease in ion absorption responsible for root development, reducing the amount of root hairs. In addition, the reduction in metabolic activity and root decomposition during the fermentation process can reduce water and mineral absorption by plants (Taiz and Zeiger, 2009). Similar results on decrease in water potential were described by Islam et al. (2010) working on *Vigna radiata* plants induced to flooding.

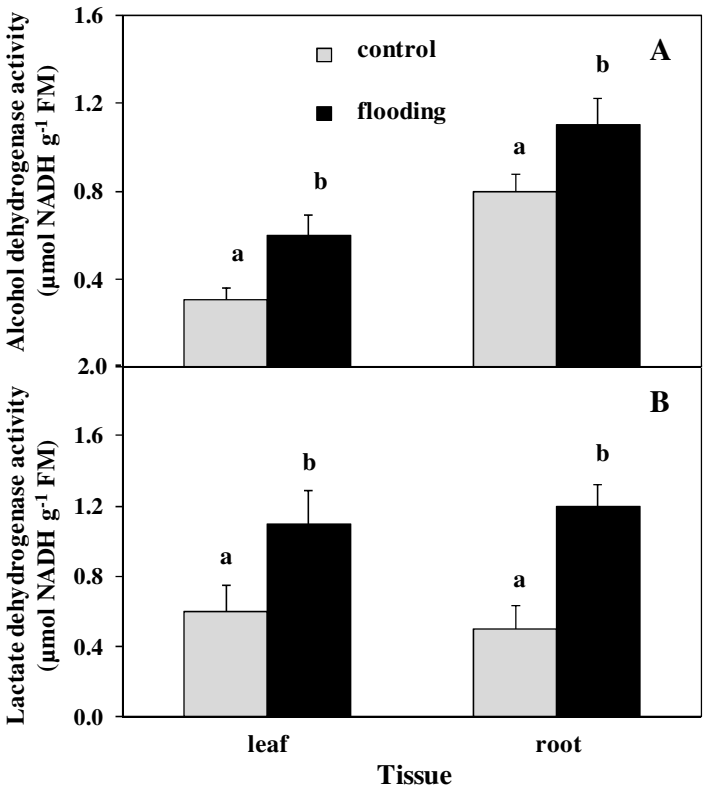

Fig 4. Alcohol dehydrogenase activity (A), and lactate dehydrogenase activity (B) in young *Tabebuia serratifolia* plants subjected to flooding. Same letters do not show significant differences at Tukey's test ($P < 0.05$). Bars represent the mean standard errors.

This study revealed a reduction in stomatal conductance of young *Tabebuia serratifolia* plants exposed to flooding (Fig 1 B), which was similar to the work reported by Batista et al. (2008), investigating *Cecropia pachystachya* plants induced to flooding. Reduction in transpiration rate is related to plant response to flooding (Fig 1C), which is corroborated by stomatal closing that can be total or partial (Matsui and Tsuchiya, 2006). This decrease in transpiration is linked to lower water supply promoted by lower amount of root hairs or function loss, causing a fall in leaf water potential (Pelacani et al., 1995). Roots under hypoxia lack sufficient energy to maintain physiological processes that are dependent of shoots (Taiz and Zeiger, 2009). Experiments have demonstrated that root deficiency to absorb nutrients and transport to shoot using xylem implicates in mineral deficit in tissues for development and expansion. Old leaves can senescence prematurely, due to re-allocation of mobile elements in phloem such as N, P, K to younger leaves. Lower permeability of root is frequently linked to a decrease in leaf water potential; however, this decrease can be temporary due to stomatal closure, avoiding more water loss by transpiration. Nitrate reductase activities were reduced in both organs, but consequences were more intense in root (Fig 2A). This decrease in plants exposed to flooding is probably due to oxygen absence in soil that will interfere in nitrate absorption from soil, provoking a nitrate decrease in leaf. Data on nitrate levels reveal reduction in leaf and root of plants exposed to flooding, to a greater extent in leaf (Fig 2 B). This reduction is related to lower activity of nitrate reductase, promoting lower nitrate absorption from substrate and transference to plant. The interaction between nitrogen and water condition is important because this nutrient is frequently responsible for early growth and development of higher plants in environments under inadequate water supply or with lower soil fertility (Silveira et al., 2003).

In plants under flooding the reduction of free ammonium in leaf is more intense, compared with root (Fig 2C). This is due to a decrease in GS-GOGAT route, with consequent reduction in glutamate generation, which to be used in glutamate dehydrogenase (GDH) pathway for future ammonium production (Oliveira Neto et al., 2009). In addition, this species presents behavior linked to the lack of tolerance to hypoxic conditions, resulting in loss of roots (data not shown). This will limit nitrogen assimilation in nitrate (NO_3^-) form, as well as in ammonium (NH_4^+) form. Other factors connected to soil nitrification process, which normally occurs with an increase in ammonium and decrease of nitrate available in soil provoked by lower oxygen availability. Plants under flooding presented a significant decrease in glutamine synthetase (GS) activity, compared to control plants, which is more intense in roots (Fig 3A). This reduction is probably linked to lower ATP concentration in cell, because this enzyme is highly dependent on energy. These ATP molecules are generated during the photochemical phase of photosynthesis. This fact is also linked to a decrease in reductase activity induced by the limited nitrate concentration and consequent lower ammonium formation under flooding condition.

In plants under flooding, a reduction in amino acids concentrations was greater in root than leaf (Fig 3B). This reduction is probably due to lower glutamine synthetase enzyme activity, which is a key enzyme and known as the precursor of formation of all amino acids in root and leaf of higher plants. Plants subjected to flooding showed a decrease in total soluble proteins in root and leaf, being more intense in root (Fig 3C). This result is probably due to decrease in protein synthesis, which can be caused by respiration reduction (Zubay, 1993). This happens because of a fall in ATP formation that provokes lower activity in nitrogen metabolism and photo-assimilates reduction induced by cellular respiration. Therefore, plants sensitive to hypoxia will enter into an auto-degradation process, aiming to survive in this inadequate situation, with severe cost linked to metabolic collapse and tissue loss. Alcohol dehydrogenase activity increased in leaf and root of plants exposed to flooding, to a greater extent in leaf (Fig 4A). These are due to hypoxic situation and consequently alcohol formation produced by fermentation process (Benz et al., 2007). During flooding, limited oxygen capitation occurs and consequently, paralyzation in electron transport and oxidative phosphorylation occurs in mitochondria. In other words, the tricarboxylic acid cycle cannot generate ATP, and this energy source will be obtained only through fermentation process. According to Moraes et al. (2001) this metabolic adaptation linked to alcohol dehydrogenase activity is necessary to avoid alcohol accumulation and other negative effects on plant tissue. Ferreira et al. (2009) described similar results linked to an increase in alcohol dehydrogenase activity of *Himatanthus sucuuba* plants exposed to flooding.

Plants subjected to flooding showed an increase in lactate dehydrogenase activity in leaf and root, and to a greater extent in root (Fig 4B). During flooding, the limitation or absence of oxygen induces fermentation, and reaction from pyruvate to lactate using lactate dehydrogenase enzyme. Therefore, plants exposed to flooding normally are exposed to lower oxygen supply, reducing ATP generation, and using

the fermentation process as a secondary route available in plant metabolism for energy production.

Materials and methods

Experimental conditions

The study was carried out in Instituto de Ciências Agrárias (ICA) of the Universidade Federal Rural da Amazônia (UFRA), Pará state, Belém city, Brazil (1º27'S and 48º26'W), during the period between January and July of 2009. This experiment was conducted in the greenhouse under natural light conditions, with air minimum, maximum and average temperatures of 22.1, 34.4, and 28.2 °C, respectively. The minimum, maximum and average air relative humidity were 53, 91, and 72 %, respectively, during the experimental period, which measured with a thermo-hygrometer (Incoterm, model 5203). The photoperiod was 12 h of light, and photosynthetic active radiation oscillated between 242 and 1100 µmol m^{-2} s^{-1} (at 12:00) depending on cloud cover.

Plant material, harvest, and storage of seed

Seeds of *Tabebuia serratifolia* (Vahl) Nicholson were harvested in the petroleum province of the Rio Urucu (City Coari, Brazil), stored in paper bags and placed in cold chamber with temperature at 5 °C and air relative humidity of 10 %.

Substrate, po, and growing of young plants

Seeds were germinated in pots with holes for water drainage in dimensions of 0.3 m for height and diameter, and capacity of 30 L. These pots were filled with substrate mix in proportion of 3:1:1 composed by substrate Plantmax®, chicken manure, and earthworm compost. After 45 days seedlings with similar aspect and size were selected. Subsequently, young plants at 60 days received 200 mL of Hoagland and Arnon (1950) nutrient solution modified for this species, and this nutritional supplementation was applied at regular intervals of 30 days until the end of the experimenton the 7th month.

Experimental design and treatments

A completely randomized block experimental designed with two water conditions (control and flooding) was used. Experiment was composed of 15 replicates and 30 experimental units, where each experimental unit was constituted by one young plant.

Flooding application

In this study, young 7-month-old *Tabebuia serratifolia* plants were used, and flooding condition was simulated through addition of distilled water in pot over a period of 9 days. Water level was kept 5 cm above the substrate superficie, with reposition of water when necessary. Large pots were used in flooding, with 0.4 m height and diameter, and capacity of 40 L without holes for water drainage. In flood implementation, pots containing young plants were transferred to large pots. However, in control plants field capacity of soil was considered to calculate the water supply, and plants were kept in pots with holes for water drainage.

Leaf water potential

Determination of leaf water potential was executed in fully expanded leaves and exposed to natural light, during the period between 9:00 and 10:00 h, using a Scholander pressure chamber (PMS Instrument Company, model 600) as described by Scholander et al. 1964, and in agreement with recommendations of Turner (1988).

Gas exchange

Stomatal conductance and transpiration were evaluated in fully expanded leaves under light, using a steady state porometer (LI-COR Biosciences, model 1600), with the gas change evaluated during the period between 10:00 and 12:00 h in all the plants.

In vivo nitrate reductase activity

Nitrate reductase enzyme (E.C. 1.6.6.1) was extracted from 200 mg of leaf and root samples. and incubated in 5 mL of extraction buffer (KH_2PO_4 at 0.1 M, KNO_3 at 50 mM, isopropanol at 1% (v/v) and pH 7.5) for 30 minutes at 30ºC, and all the procedures were carried out in the dark. The quantification of the enzyme activity was in accordance to the method of Hageman and Hucklesby (1971) with absorbance at 540 nm using spectrophotometer (Quimis, model Q798DP).

Glutamine synthetase activity

Extraction of the glutamine synthetase enzyme (E.C. 6.3.1.2) was carried out with 200 mg of leaf tissue ground in liquid nitrogen. The samples were then incubated in 5 mL of extraction mix (Tris-HCl buffer pH 7.6 containing 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 5% (w/v) PVP, and 5 mM EDTA), homogenized, centrifuged at 3.000 *g* for 10 min, and the supernatant was removed. All the procedures were carried out in the interval of 0-4°C. The quantification of the enzyme activity was carried out using the method of Kamachi et al. (1991) with absorbance at 540 nm, and g-glutamylhydroxamate (Sigma Chemicals) was used as a standard.

Alcohol dehydrogenase and lactate dehydrogenase activities

Enzymes alcohol dehydrogenase (E.C. 1.1.1.1) and lactate dehydrogenase (E.C. 1.1.1.27) were extracted from 200 mg of leaf and root tissues. Samples incubated in 2 mL of extraction mix (Tris-HCl buffer at 50 mM, Tiamina Pirofosfato at 0.5 mM, Dithiothreitol at 2 mM, EDTA at 1 mM, NaCl at 110 mM, $MgCl_2$ at 2.5 mM, with pH adjusted to 6.8). After homogenization, samples were centrifuged at 10.000 *g* for 10 min, and the supernatant was removed. All the procedures were carried out in the interval of 0-4°C. Quantification for alcohol dehydrogenase was based on the method of Bertani et al. (1980), and for lactate dehydrogenase was based on methodology described by Hoffman and Hanson (1986), with both determinations under absorbance at 340 nm, with NADH (Sigma Chemicals) as a standard.

Dehydration and sample preparation

Leaf and root were harvested and dried in an oven with forced air circulation at 70 ± 2º C by 96 h. After drying, leaf dry matter and root dry matter were triturated, with the

resulting powder kept in glass containers. These containers remained in the dark at 15ºC until they were ready for biochemical analysis.

Nitrate

For determination of nitrate, 100 mg of leaf dry matter powder was incubated with 5 ml of sterile distilled water at 100°C for 30 min, and the homogenized mixture was centrifuged at 3.000 g for 15 min at 25°C, and the supernatant was removed. The quantification of the nitrate was carried out at 410 nm in accordance with Cataldo et al. (1975), with KNO_3 (Sigma Chemical) as standard.

Free ammonium

Free ammonium was determined with 50 mg of leaf dry matter powder incubated with 5 ml of sterile distilled water at 100°C for 30 min, after the homogenized mixture was centrifuged at 2.000 *g* for 5 min at 20°C and the supernatant was removed. The quantification of free ammonium was carried out at 625 nm in accordance with Weatherburn (1967), with $(NH_4)_2SO_4$ (Sigma Chemical) as standard.

Total soluble amino acids

Determination of amino acids was performed using 50 mg of leaf dry matter powder, and incubated with 5 mL of sterile distilled water at 100ºC by 30 minutes. After incubation, the homogenized was centrifuged at 2.000 *g* for 5 minutes at 20ºC and supernatant was removed. Quantification of the total soluble amino acids was carried out at 570 nm according to Peoples et al. (1989), and L-asparagine + L-glutamine (Sigma Chemicals) was used as standard.

Total soluble proteins

Determination of the total soluble proteins was carried out with 100 mg of powder, incubated with 5 mL of extraction buffer (Tris-HCl at 25 mM and pH 7.6). This was homogenized and kept in agitation for 2 h, and centrifuged to 2.000 *g* for 10 minutes at 20ºC. Quantification of the total soluble proteins was carried out at 595 nm in accordance with Bradford (1976), with albumin bovine (Sigma Chemicals) as standard.

Data analysis

Data were subjected to variance analysis and when significant differences occurred, Tukey's test at 5% level of error probability was applied. Standard errors were calculated for all means (Steel et al., 2006). The statistical analysis was carried out with the SAS software (SAS Institute, 1996).

Conclusion

Young *Tabebuia serratifolia* plants exposed to flooding for 9 days showed significant changes in all parameters, with decreases in water relations and nitrogen compounds. However, increases were obtained in alcohol dehydrogenase activity and lactate dehydrogenase activity after flooding. In relation to organ evaluated, modifications were more intense in root for several parameters such as glutamine synthetase and lactate dehydrogenase enzymes, and leaf was more responsive only in nitrate, free ammonium, and alcohol dehydrogenase activity. Therefore, this study confirms that *Tabebuia serratifolia* plants are sensitive to flooding.

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