

Nitrate reductase activity (Nra) and levels of nitrogenous compounds in leaves of crabwood (*Carapa guianensis* Aubl.) under three water regimes and suspension of the stresses

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Abstract

The crabwood has high phenotypic plasticity due to its adaptability to different environments. The aim of this work was to evaluate the content of nitrate, free ammonium, nitrate reductase activity (Nra), and the levels of total soluble amino acids, proline, glycine betaine in young plants of crabwood (*Carapa guianensis* Aubl.) under to three water regimes and subsequent recovery process through the stresses suspension. The experiment was carried out on a greenhouse from Universidade Federal Rural da Amazônia, Capitão Poço city, PA State. A completely randomized design on factorial scheme 3×5 was used (three water conditions: irrigated control, water deficit and flooding; and five evaluation times: 0 day, 10 days, 20 days, 30 days and suspension of water stress after 72 hours from the 30th day) with five repetitions, totalizing 75 experimental units. The results showed that the water potential, nitrate concentration and the Nra decreased significantly in plants under water stress, as well as in plants under flooding, with average of -3.46 MPa, 0.6 NO₃⁻/kg DM, 0.1294 μmoles of NO₂⁻/g FM/h (water potential, nitrate and Nra, respectively). The ammonium (NH₄⁺) levels increased in the plant under water stress. The increasing level was more significant in the plants under water deficit, averaging 4.0 of mmol of NH₄⁺/kg of DM. The amino acids levels, proline and glycine betaine had a significant increase in plants with water stress, especially in the plants under water deficit. The plants subjected to flooding did not show any significant result. After the suspension of the water stress, only the contents of free ammonium and levels and total soluble amino acids recovered to their initial values. To others variables, the stress suspension period was not enough for plant's total recovering. The young plants of crabwood subjected to water stress promoted changes in nitrogen metabolic pathways. These changes showed a bigger tolerance of the plants of crabwood to flooding.

Keywords: Flooding; Free ammonium level; Nitrogen metabolism; Rehydration, Water Suspension.

Abbreviations: AA_ aminoacids, ADP_Adenosine diphosphate, AIMEX_Associação das Indústrias Exportadoras de Madeira do Estado do Pará, ATP_Adenosine triphosphate, DM_Seedling dry matter, EBPs_Biodiversity Studies Laboratory in Higher Plants, ROS_Reactive Oxygen Species, Gb_Glycine Betaine levels, N_Nitrogen, NH₄⁺_Ammonium, NO₃⁻_Nitrate, Nra_Nitrate Reductase Activity, MPa_Mega Pascal, SAS_Statistical Analysis System, UFRA_Universidade Federal Rural da Amazônia, Ψ_{am}_Predawn Water Potential, Ψ_x_ Xylem potential.

Introduction

The crabwood (*Carapa guianensis* Aubl.) is one of the species with big timber exploitation and non-timber potential in the Amazon (Tonini et al., 2009). Belonging to the

Meliaceae family, *Carapa guianensis* Aubl. is the most widespread species among the 27 species of the genus

Carapa, only found on the American continent (Kenfack, 2011).

The population density of reproductive individuals is relatively high in natural populations with more than five individuals per hectare (Martins et al., 2012). Its seeds produce oil with cream color, sour (oil-carapa) and high acidity (Camargo and Marengo, 2012). It is considered as promising specie to enrich secondary forests, agroforestry and recovering of degraded wetlands areas (Ferraz et al., 2002). Therefore, the crabwood has promising potential to acclimate to environmental changes predicted in the near future for most of the Amazon region (Camargo and Marengo, 2012). The species is quite elastic which gives morphological differences and ability to occupy different niches, living from the sea level, in permanently flooded areas (igapó) or periodically flooded (paddy fields), to the high forests not subject to flooding (350 - 1400 m of altitude). This confirms the morphological differences according to their habitat (Leite, 1997; Boufleuer, 2004).

Stress is a deviation from the optimum conditions during development of plant, which can be changed and responded in some tissues or whole plant. At first, these changes may be reversible but depending on the length and stress, it might be irreversible, leading to permanent damage or even death (Pimentel, 2004). The process of opening and closing of stomata is mainly related to the intensity of light and the hydration status of plant. The functioning of stomata and leaf area influence the productivity of the plant because it controls the absorption of CO₂ and determines the light interception (Costa and Marengo, 2007).

According to Larcher (2000), the photosynthetic capacity is an intrinsic characteristic of each plant species. The gas exchange alters during the individual development cycle and depends on the annual course and even the daily course of environmental fluctuations (light, temperature, etc.) around the vegetable.

The water deficit is an abiotic stress, which is one of the largest agricultural limitations due to negative interference in relation to the growth and development of the plant (Endres et al. 2010).

Among the responses of plants to stress by flooding, the stomatal closure, partial or total, is one of the first answers on the top area (Kozłowski and Pallardy, 2002). Studies show that organic solutes protect plants from stress through different mechanisms, including osmotic adjustment, removal of reactive oxygen species, "protection integrity of cell membranes" and "stabilization of proteins and enzymes" (Pompelli, 2010; Turkan, 2011).

Generally, low molecular weight metabolites such as proline (Pérez-Pérez et al., 2009) and glycine betaine (Bajji et al., 2001) are commonly observed as metabolic responses of higher plants to water deficit, and may occur in agreement with the changes in the content of sugars and amino acids (Pérez-Pérez et al., 2009).

Concurrent to this, the assimilation of nitrogen (N) is one of the interesting factors to be considered before their significance in plant metabolism (Parent, 2008; Li et al., 2013). The deficiency of this element is the most common limitation of plant development after the water stress (Li et al., 2013).

The different forms of N (ammonium - NH₄⁺ and nitrate - NO₃⁻) affect some physiological and morphological characteristics of the plants. For example, root growth, photosynthetic rates, mesophyll conductance, total N content and free amino acids, as well as the amount sugars are affected. In addition to these factors, alteration in absorption of mineral ions, the accumulation of nutrients in plants and

produce different effects on reactive oxygen species (ROS) and the levels of antioxidant enzymes may be occurred (Li et al., 2013). Studies on the effects of abiotic stress in the development of plants serve as subsidies for agricultural and forestry researches, and become potential indicators of the mechanisms of plant tolerance to adverse conditions (Silva, 2013).

Therefore, the aim of this work was to evaluate the nitrogen metabolism disorders in young plants of crabwood subjected to water deficit and flooding and subsequent recovery.

Results and Discussion

Predawn water potential (Ψ_{am})

The predawn water potential (Ψ_{am}) of plants under water stress and flooding were significantly reduced with the increase in the number of days of water stress. However, after suspension of water stress, the plant did not recover (Fig 1A). The values for the plants under drought were -0.48; -0.89; -1.94, -3.46 and -0.95 MPa. For the plants under flooding values are -0.47, -0.68, -1.07, -2.85 and -0.77 MPa in time evaluation of 0 day, 10 days, 20 days, 30 days and suspension of water stress after 72 hours from the 30th day, respectively. These results suggest that the decrease in Ψ_{am} in plants under water deficit is related to the time that the young plants were kept under stress (without receiving water).

Plants with water deficit had a greater reduction of water potential in relation to the flooded plants, with a smaller decline in the 30th day of the experiment established. The decrease of water potential is possibly due to the increased tension in xylematic vessels. The more negative the Ψ_w , the larger the strength that plant absorbs the water from soil transported to the top layer. This transport may be impaired depending on the severity of water stress in *C. guianensis* Aubl. The transport after stress was partially affected because when rehydrated, species have not recovered their initial potential which was -0.48 MPa, obtaining a recovery of 82.29%.

In plants subject to flooding, the decrease in water potential should be related to the low concentration of O₂, although the roots are completely submerged in water. The plants showed a water deficiency in the leaves. However, this decrease should have promoted a change in the behavior of the plant metabolism, such as oxidative phosphorylation to the phosphorylation of ADP to ATP on reactions that occur exclusively in glycolysis and fermentation. This enables plant to change the synthesis of 36 ATP from aerobic respiration, to only 2 ATP through fermentation (Oliveira Neto, 2010).

The plants subjected to flooding have shown a recovery of 85.24% after only 72 h of water deficit suspension, compared to control plants. Gonçalves et al., (2009) studied the young plants of *Carapa guianensis* Aubl. confirming that the young plants of crabwood satisfactorily tolerated periods of 15 to 30 days of water deficit. The same author noted that during this period water stress decreased leaf water potential to values between - 2.0 and - 3.0 MPa, similar to observation of this study.

Nitrate levels in the leaves

The results show that the nitrate levels decreased considerably in crabwood leaves in both treatments with more pronounced results on the 30th day of water stress. However, the most affected were the plants under water deficit with values of 0.88 to 0.40 $\mu\text{moles of NO}_3^- / \text{kg DM}$

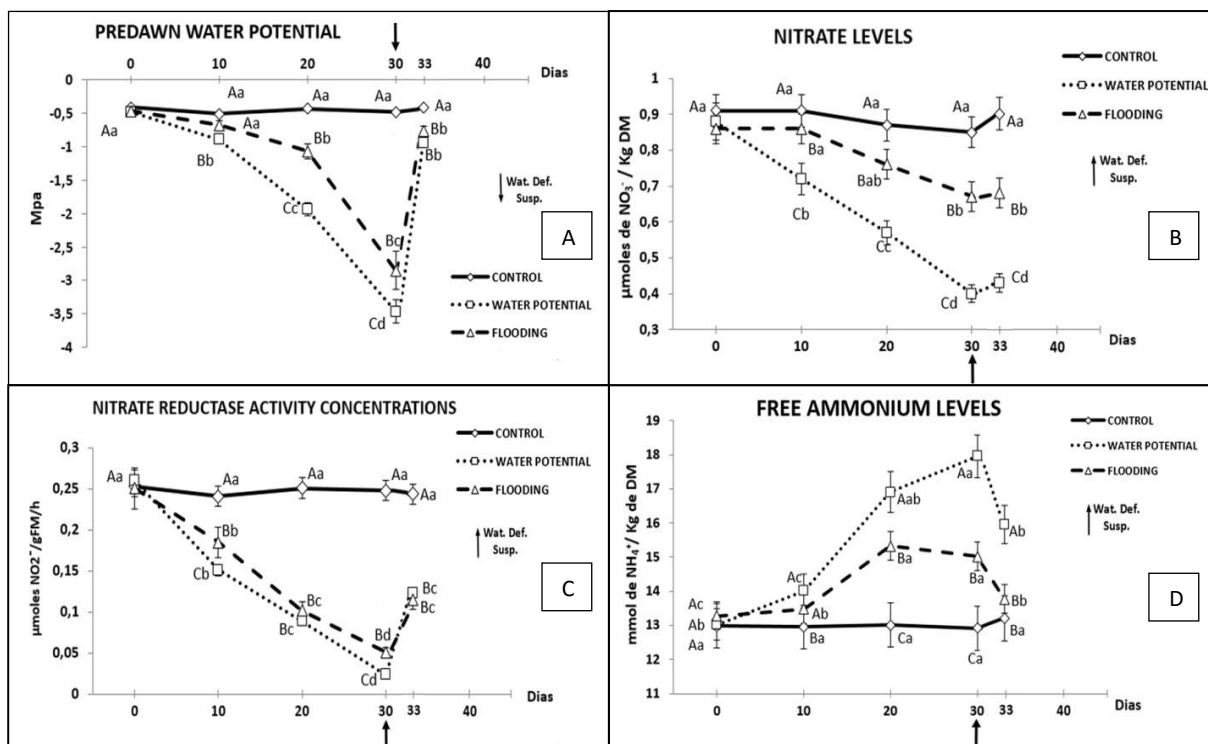


Fig 1. Predawn water potential (A), nitrate levels (B), nitrate reductase activity (C) and free ammonium levels (D) in young plants of crabwood subjected to water deficit and flooding. Capital letters show statistical differences between the treatments and the lowercase letters show differences between the collection days of the same treatment.

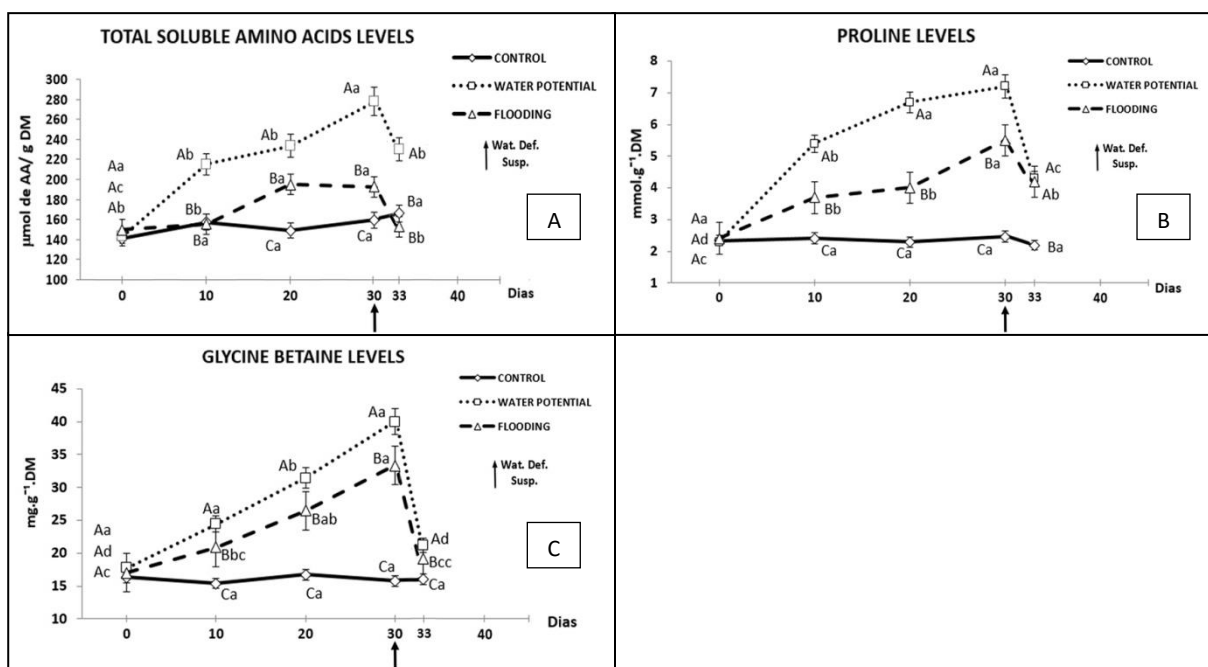


Fig 2. Total soluble amino acids levels (A), proline levels (B) and glycine betaine levels (C) in young plants of crabwood subjected to water deficit and flooding. Capital letters show statistical differences between the treatments and the lowercase letters show statistical differences between the collection days of a same treatment, compared by Tukey's test at 5% of probability, where the means followed by the same capital letters or lowercase letters do not differ, bars represent the standard deviation of the means.

while the plants under flooding had values from 0.86 to 0.67 $\mu\text{moles of NO}_3^- / \text{kg DM}$, compared to control plants with values from 0.91 to 0.90 $\mu\text{moles of NO}_3^- / \text{kg DM}$.

The results can be elucidated by the involvement of metabolic pathways to explain the consumption of nitrate and nitrate transport to the plant tops. For plants with water deficit, the gradual decrease of water in the soil may have promoted the reduction of soil nitrate absorption by the roots, which possibly cause reduction of transport to the leaves through transpiration stream (xylem), decreasing the nitrate reductase activity enzyme, which is highly dependent on its substrate that is the NO_3^- contributing to the significant reduction for plants under water stress (Shaner and Boyer, 1976). After the suspension of water stress, the plants under deficiency showed a recovery of 5.88% as compared to the control plants, not recovering its initial value.

For the flooded plants, reductions in nitrate concentration correspond to a reduction of energy of roots (breathing). Therefore, the nitrate active absorption process by the roots was also decreased; thus, the flow in to the leaves declined. Furthermore, the nitrate reductase enzyme, even low (Fig 1C), used the nitrate concentration of the vegetable plant tissue, reducing the nitrate content in leaves (Fig 1B). After the suspension of water stress, plants obtained a percentage of 3.76% recovery, not recovering the initial nitrate levels in 72 hours of suspension of water stress from the 30th day.

Results found by Freitas (2014) confirm the decrease in nitrate levels in the leaves of plants subjected to water stress on young plants of *Acapu* (*Vouacapoua americana* Aubl.).

Nitrate reductase activity (Nra)

The nitrate reductase activity (Nra) was also affected in both treatments. However, plants under water deficit were the most declined in 30 days of water stress. The values were 0.253 to 0.248; 0.26 to 0.024; 0.251 to 0.051 $\text{NO}_2^- \text{g FM}^{-1} \text{h}^{-1}$ to control plants under water deficit and flooded, respectively. During this period, young plants of *C. guianensis* subjected to water deficit reduced the Nra to more than half of the initial activity. The young plants subjected to flooding showed an inferior reduction than plants under water deficit (Fig 1C). Recovery after the suspension of water stress was not satisfactory, with values of 43.23% and 31.68% for plants under water deficit and plants under flooding, respectively. However, the decreasing activity of this enzyme to the water deficit and flooding treatment may probably be due to the constant presence of nitrate (NO_3^-) from the vacuole reserve. Therefore, its activity was maintained low or could be due to its re-synthesis again (Andrade Netto, 2005). Thus, nitrate reductase, main enzyme responsible for nitrogen assimilation by plants, may have its activity influenced by the availability of water (Sharma and Dubey, 2005), and can be used as an indicative parameter to environmental stresses (Carelli et al., 1991; Oliveira et al., 2005).

Free ammonium concentrations

The free ammonium concentrations showed a significant increase in plants subjected to water stress (Fig 1D). The values were 13.02 to 17.96 $\text{mmol of NH}_4^+ / \text{kg of DM}$ for plants exposed to water deficit, and 13.28 to 15.02 $\text{mmol of NH}_4^+ / \text{kg of DM}$ for flooded plants. The control plants showed continuous values of 12.98 to 12.92 $\text{mmol of NH}_4^+ / \text{kg of DM}$ during the 30 days of treatment. For plants under water deficit and flooding, the suspension of stress was not successful, since the plants under water deficit showed a recovery of 40.16% and the plants under flooding had a more

significant recovery because the recovery rate values reached to 61.27%, compared to control plants.

This increase, for the plants under water stress can be linked to other routes for formation of the same, promoting an accumulation of ammonium by a mismatch between deamination, photorespiration, catabolism of amino acids (Ferreira et al., 2002), which pointed the photorespiration as one of the routes for the formation of NH_4^+ . According to Oliveira Neto (2010), the increase in ammonium concentration also occurred due to possible reduction of photosynthesis, as it keeps the metabolism of nitrogen through the energy supply (ATP) and the formation of reducing powers (NADPH, FADH and NADH).

Total soluble amino acids level

The significant increase in amino acid levels was significant in plants under water deficit with values of 143.02 to 278.05 $\mu\text{mol of AA/g DM}$, while in the flooded plants the values were 149.58 to 192.52 $\mu\text{mol of AA/g DM}$, compared to control plants which showed values 140.54 to 159.54 $\mu\text{mol of AA/g DM}$, (Fig 2A). After suspension of water stress, the plants under water deficit did not reach their initial values. They only achieved the values of 34.87%, and the plants flooded showed significant values of 76.53%. However, both plants subjected to water stress, did not reach a relevant concentration when compared to control plants, not restoring their initial values with the suspension of stress.

After the introduction of water stress (semi-moderated or moderate) the increase of free amino acids is notorious (Silva et al., 2010). Among the various amino acids in vegetables, proline is an indicator of sensitivity to water stress and is considered a reliable physiological variable to evaluate the levels of plant drought tolerance. In addition, to the role in the osmotic regulation, proline acts in the protection of membranes and proteins from the harmful effects of extreme temperatures and high concentrations of inorganic ions (Turkan, 2011).

Proline level

The increase of the rate of proline levels was evident since the 10 days of stress, intensifying on the 30th day in both water deficit treatments (Fig 2B). The values were 2.3 to 7.2 $\text{mmol g}^{-1} \text{DM}$ for plants under water deficit, and 2.41 to 5.5 $\text{mmol g}^{-1} \text{DM}$ for flooded plants. The control plants maintained continuous values from 2.34 to 2.46 $\text{mmol g}^{-1} \text{DM}$. The stress suspension period was not enough for full recovery of the specie, recovering a percentage of 59.67% and 41.14% for plants under water deficit and flood, respectively. The proline accumulation under tension in many plant species has been correlated with water deficit tolerance, and its concentration is usually higher in tolerant plants than in sensitive plants to stress (Ashraf and Foolad, 2007). In plants under water deficit, the increase of the proline levels can be possibly related to the increase of the activity of proteolytic enzymes promoting greater availability of free amino acids (Fig 2A) to protect plant tissues against this stress by serving as a reserve of nitrogen. In plants under flooding, no significant decrease in free amino acids was occurred. After suspension of the stress, the species tries to restore, reaching average values of 4.1 $\text{mmol g}^{-1} \text{DM}$, but cannot return to its initial value, possibly because the species need more time to fully recover.

Nogueira et al. (2005) and Lisar et al., (2012) showed that proline accumulation will not be indicated as a resistance factor but rather an indicator of acquired tolerance. In several

experiments the increase in proline in the plant cells classed as tolerant has been observed. This has not been observed in sensitive plants.

The synthesis and degradation of proline processes are considered beneficial to plants when subjected to adverse conditions. Proline biosynthesis is activated and its catabolism is inhibited during dehydration, while the rehydration triggers opposite regulation. The degradation can provide reducing required for mitochondrial electron transport and the generation of ATP for the recovery and repair of damage induced by stress (Szabados and Savoure, 2010).

Glycine betaine level

The results showed an increase in glycine betaine levels (Gb) in plants under flooding, as under water deficit compared to control plants. The concentrations of Gb was found 17.78 to 40.02; 17.02 to 33.35; 16.35 to 15.78 mg g⁻¹ DM in plants under water deficit, under flooding and control plants, respectively (Fig 2C). After suspension of stress, stressed plants failed to recover to their initial levels, showing a 79.55% recovery rate in plants under water deficit and 83.82% for flooded plants.

In plants under water deficit, this accumulation may have occurred to protect the metabolism of the plant, since glycine can maintain the water balance between the plant cell and the environment, contributing to better absorption and soil water transport for the top area. Additionally, the glycine betaine value increased due to the probable formation of amino acids from the breakdown of proteins and high ammonium levels from possibly photorespiration and deamination processes (McNeil et al., 1999).

With the resumption of irrigation, there may be an immediately contribution for recovery of water status in plant. The metabolism of proline can occur in the cytosol in different ways such as conversion into other amino acids, used in protein synthesis, and especially conversion of intermediates of the Krebs cycle, with stimulation respiration to the synthesis of ATP (Szabados and Savoure, 2010). In wetland plants, the increase of Gb in leaves seems also relates to osmotic adjustment of the hyaloplasm plants subjected to stress situations (Jaleel et al., 2007). Also, the accumulation of Gb could contribute to a reduction of Ψ_x in cell during periods of osmotic stress (Taiz and Zeiger, 2013), favoring the absorption and soil water transport to top part of the plant. Thus, this can protect the plant tissues and physiological processes, improving plant tolerance to abiotic stress. Oliveira Neto (2010) observed an increase of 59.37 and 84.88% of Gb in leaves and roots of young plants of *Hymenaea courbaril* L. submitted to flooding for 30 days. Contrarily, Alves (2010) noted that the flooding has dramatically reduced the concentration of Gb in young plants of *Tabebuia serratifolia*, 86.2 and 82% in roots and leaves, respectively. They suggested that this decline seems to demonstrate that Gb do not work as osmoprotectors.

Materials and Methods

Study's localization

The experiment was conducted in a greenhouse at the Universidade Federal Rural da Amazônia-UFRA, Campus Capitão Poço – PA (Latitude 01° 44' 47'' and Longitude 47° 03'34'') the laboratory tests were performed in Plant Physiology Laboratory in the Universidade Federal Rural da Amazônia - UFRA.

Obtaining young plants

The crabwood young plants from seeds were provided by AIMEX (Associação das Indústrias Exportadoras de Madeira do Estado do Pará) at four months of age and were placed in plastic pots with a capacity of 20 L.

Substrate and its compounds

The substrate consisted of dystrophic yellow latosol more tanned cattle manure in the proportion of 3: 1, respectively. Before the start of treatment, all plants were placed under 50% sombrite, irrigated daily for a month to keep them at field capacity and acclimatization (Fernandes and Sykes, 1968). A 200 mL of macro and micronutrients was added every 15 days until the beginning of the experiment, according to Hoagland and Arnon (1950), modified for Biodiversity Studies Laboratory in Higher Plants (EBPs), UFRA.

Experimental design

The experimental design was completely randomized in a factorial 3 × 5 (three water conditions: control, water deficit and flooding, and five evaluation times 0, 10, 20, 30 days and suspension of water stress after 72 hours from the 30th day with 5 repetitions, totaling 75 experimental units.

Predawn water potential (Ψ_{am})

Predawn Water potential (Ψ_{am}) was determined between 4:30 and 5:30 am, by means of a pressure pump type Scholander (M670, PMS Instrument Co., Albany, USA) as described by Pinheiro et al. (2007). As strains, mature and fully expanded leaflets were selected from the second or third pair of mature leaves from the apex, detached with the aid of a razor blade, between 9:00 and 11:00, stored in wet polyethylene bags and transported on ice to the laboratory for analysis in a maximum time of 60 minutes and immediately frozen (approximately -20 °C) for subsequent biochemical analysis.

Determination of the concentrations of nitrate

50 mg of previously lyophilized leaves were weighted. Then 5% salicylic acid solution in concentrated sulfuric acid was added to total extract. The concentration of nitrate was obtained from a standard curve with increasing concentrations of NO₃⁻ (0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 μmol ml⁻¹). The method was according to (Cataldo et al., 1975).

Determination of nitrate reductase activity

A 200 mg of leaf disks of 0.5 cm diameter were weighted. The samples placed in test tubes containing phosphate buffer isopropanol from a standard curve obtained with KNO₂ p.a. (Sigma) according to *in vivo* method recommended by Hageman and Hucklesby, (1971).

Free ammonium concentrations

50 mg of lyophilized leaves weighted and placed in test tubes containing the total extract, solution A and solution B after shaking. The free ammonium concentrations were estimated from the standard curve constructed with (NH₄)₂SO₄ p.a. (Sigma) according to method described by (Weatherburn, 1967).

Total soluble amino acid concentrations

50 mg of previously lyophilised leaves weighted then buffered solution and reagent ninhydrin were added. The total free amino acid levels were determined based on a standard curve adjusted from increasing concentrations of a standard mixture of L-glutamine according to the method described by Peoples et al. (1989).

Determination of proline levels

50 mg of lyophilised leaves weighted and placed in the test tubes containing total extract, ninhydrin acid and glacial acetic acid. It was determined through a calibration curve proline and proline result expressed in mmol g⁻¹ dry matter (DM) according to Bates et al. (1973).

Glycine betaine content determination

25 mg of lyophilised leaves weighted and H₂SO₄ 2N added into the test tubes and KI-I₂ iced. A standard curve was used of Glycine – Betaine according to the method of Grieve and Grattan (1983).

Data analysis

The analysis of variance was applied to the results and when there was a significant difference, the means were compared by Tukey test at 5% significance level. Moreover, the standard deviations were calculated for each treatment, and statistical analyzes performed by the program SAS - Institute, 1996.

Conclusions

Crabwood young plants subjected to water stress promoted changes in metabolic nitrogen routes. For free ammonium content and total soluble amino acids, the period of 72 hours of water stress suspension from the 30th day was enough for the resumption of initial water values. The results showed that the plant of crabwood is more tolerant to flooded soils, which is consistent with the climate of the Amazon region with heavy rainfall in certain areas. This condition may favor the development and expansion of this species for the recovery of riparian forests.

Acknowledgments

The authors are grateful to the Biodiversity Studies Laboratory in Higher Plants of Universidade Federal Rural da Amazônia for the collaboration of researchers. We also acknowledge the CAPES (Coordenação de aperfeiçoamento de pessoal de nível superior) for the scholarship granted that enabled the completion of the dissertation.

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