



**MORPHOPHYSIOLOGICAL AND BIOCHEMICAL CHANGES
IN SEEDLINGS OF *Enterolobium contortisiliquum* (VELL.)
MORONG. UNDER ABIOTIC STRESSES**

ADRIANA DOS SANTOS FERREIRA

Macaíba/RN

July of 2020

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Dissertation presented to Programa de Pós-Graduação em Ciências Florestais da Universidade Federal do Rio Grande do Norte to obtain the degree of Master in Forest Science (Area of Concentration in Forest Sciences - Research Line: Seeds, Propagation and Physiology of Forest Species).

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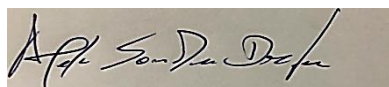
Adriana dos Santos Ferreira

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To my family, Ane dos Santos Viana, Aian dos Santos Viana, beloved children and Adanildes dos Santos Ferreira, mother.

DEDICATION

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To God, for the journey he has experienced, for his academic growth and learning and the achievement of this objective: obtaining the title of Master in Forest Sciences.

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GENERAL ABSTRACT

MORPHOPHYSIOLOGICAL AND BIOCHEMICAL CHANGES IN *Enterolobium contortisiliquum* (Vell.) Morong seedlings UNDER ABIOTIC STRESSES

Among the native species of the Caatinga, *Enterolobium contortisiliquum* (Vell.) Morong stands out as a fast-growing tree species with multiple purposes such as medicinal application, recovery of degraded areas, boat manufacturing, furniture and crates in general. The objective of this study was to evaluate the morphophysiological and biochemical changes in *E. contortisiliquum* seedlings subjected to abiotic stresses. The experience was carried out at the Academic Unit Specialized in Agrarian Sciences (UAECIA), belonging to the Federal University of Rio Grande do Norte (UFRN), Macaíba, RN - Brazil, in the first half of 2019. Water stress was established by watering suspensions of 0, 4, 8, 12 and 16 days, while salt stress was simulated at the following electrical conductivities of irrigation water (EC_w): 0.3 (control), 2, 4, 6 and 8 dS.m⁻¹. Prior to sowing, the pre-germination treatment of dormancy overcoming was performed with a sandpaper, removing part of the seed coat in the region opposite to the hilum. Substrate consisted of the mixture of common earth and aged bovine manure (1:1). The evaluated variables for water and salt stresses were: plant height, number of leaves, leaf area, stem diameter, root length, Dickson quality index, ratio between height and shoot dry mass, shoot dry mass, root dry mass and total dry mass, as well as biochemical tests (total free amino acids, proline, total soluble sugars and starch). The four-day period of water stress promotes *E. contortisiliquum* seedlings with better drought tolerance capacity. Osmotically active solutes act positively to minimize the damage caused by increased water stress, whose mechanisms may be the main determinants used by plants under this condition. The gradual increase in irrigation water salinity was detrimental to the growth and dry mass accumulation of *E. contortisiliquum* plants, with a sharp decrease from the threshold of 2 dS.m⁻¹. The biochemical mechanism of tolerance to salt stress was the increase of amino acids, proline and total sugars, concomitantly with the consumption of starch reserves.

Key words: Fabaceae, salinity, water deficit, organic solutes, osmoregulators, seedling production.

ALTERAÇÕES MORFISIOLÓGICAS E BIOQUÍMICAS EM MUDAS DE *Enterolobium contortisiliquum* (Vell.) Morong SOB ESTRESSES ABIÓTICOS

Dentre as espécies nativas da Caatinga, *Enterolobium contortisiliquum* (Vell.) Morong se destaca como uma espécie arbórea de rápido crescimento e com múltiplas finalidades como aplicação medicinal, recuperação de áreas degradadas, fabricação de barcos, móveis e engradados em geral. O objetivo deste trabalho foi avaliar as alterações morfofisiológicas e bioquímicas em mudas de *E. contortisiliquum* submetidas a estresses abióticos. A experiência foi realizada na Unidade Acadêmica de Especialização em Ciências Agrárias (UAECIA), pertencente à Universidade Federal do Rio Grande do Norte (UFRN), Macaíba, RN - Brasil, no primeiro semestre de 2019. O estresse hídrico foi constatado por rega suspensões de 0, 4, 8, 12 e 16 dias, enquanto o estresse salino foi simulado nas seguintes condutividades elétricas da água de irrigação (EC_w): 0,3 (controle), 2, 4, 6 e 8 dS.m⁻¹. Antes da semeadura, o tratamento pré-germinativo para superação da dormência foi realizado com lixa, retirando parte do tegumento na região oposta ao hilo. O substrato consistiu na mistura de terra comum e esterco bovino envelhecido (1: 1). As variáveis avaliadas para estresse hídrico e salino foram: altura da planta, número de folhas, área foliar, diâmetro do caule, comprimento da raiz, índice de qualidade de Dickson, relação entre altura e massa seca da parte aérea, massa seca da parte aérea, massa seca da raiz e massa seca total, bem como testes bioquímicos (aminoácidos livres totais, prolina, açúcares solúveis totais e amido). O período de quatro dias de estresse hídrico promove mudas de *E. contortisiliquum* com melhor capacidade de tolerância à seca. Os solutos osmoticamente ativos atuam positivamente para minimizar os danos causados pelo aumento do estresse hídrico, cujos mecanismos podem ser os principais determinantes utilizados pelas plantas nesta condição. O aumento gradual da salinidade da água de irrigação prejudicou o crescimento e o acúmulo de massa seca das plantas de *E. contortisiliquum*, com uma redução acentuada a partir do limiar de 2 dS.m⁻¹. O mecanismo bioquímico de tolerância ao estresse salino foi o aumento de aminoácidos, prolina e açúcares totais, concomitantemente com o consumo das reservas de amido.

Palavras-chave: Fabaceae, salinidade, déficit hídrico, solutos orgânicos, osmorreguladores, produção de mudas.

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ABBREVIATIONS LIST

CONABIO - National Biodiversity Commission

ECW - Electrical conductivity

H - Height of plants

IUCN - International Union for Nature Conservation

LA- Leaf area

NaCl - Sodium chloride

NL - Number of sheets

PRLN - Proline

RDM - Dry root mass

RL - Root length

SD - Neck diameter

SDM - Dry shoot weight

STA – Starch

SWRC - Field capacity

TDM - Total dry mass

TFAA - Total free amino acids

TSS - Total soluble sugars

General Introduction

1. GENERAL INTRODUCTION

Enterolobium contortisiliquum (Vell.) Morong is a species of the Fabaceae family widely distributed in forest formations from Northern to Southern Brazil, in the Atlantic Forest, Northeast, Agreste zone and Caatinga (ARAÚJO and PAIVA SOBRINHO, 2011). It is a plant that is easily adapted to environmental factors such as light, temperature and water, shows rapid growth, and can reach more than 4 m in height within two years. When adult, it reaches about 30 m in height and 1.60 m in diameter and is popularly known in Portuguese as 'tamboril', 'orelha de onça', 'timbó', 'ximbó' or 'orelha-de-macaco' (LORENZI, 2016).

The Caatinga biome extends over 800.000 km², occupying 70% of the northeastern region of Brazil, totaling 11% of the national territory (RIBEIRO FILHO et al., 2016). In this ecosystem, the arboreal shrubby physiognomy stands out as dominant, being mixed by few arboreal and herbaceous individuals commonly absent during the dry period (ARAÚJO, 2011). Under these conditions, several species, including forest species, which have adaptive characteristics, may exhibit reduced tolerance to abiotic stresses, such as water deficit and salt stress, especially in the initial growth stage (PEREIRA FILHO et al., 2013).

Precipitation in the semi-arid region is between 280 and 800 mm per year, and rains are irregularly concentrated in three or four months, while drought periods can last from eight to nine months. In addition, the high rate of potential evapotranspiration, between 1500 and 2000 mm per year, results in high water deficit over the dry season (MOURA et al., 2007). The situation in semi-arid environments is even more pronounced, since the water deficit for long periods favors the formation of superficial water table and capillary rise of salts, affecting the quality of water for irrigation and limiting the growth of plant species (ALVES et al., 2018).

The accumulation of salts in the soil intensifies water stress, causing a strong impact on crop growth and yield, since the osmotic effect caused by salts is responsible for restricting the transport and distribution of water from the medium surrounding the roots to the shoots of the plant (SILVEIRA et al., 2010). According to these authors, this causes damage to stomatal opening and photosynthetic assimilation of CO₂, as well as reduction in cell turgor, which is fundamental for adequate cell metabolism. In addition, the degree of severity of stress, salt ions in the cytosol of cells, can cause toxicity, leading the plant to death (ANJUM et al., 2011).

In situations of stress, water deficit and salt stress, the growth of most plant species is compromised, producing variations in soil pH, which reduces nutrient solubility, promotes greater aggregation of soil particles giving rise to changes in soil structure, and reduces aeration, infiltration and hydraulic conductivity to limits that are unfavorable to plants (FRANÇA et al., 2017). As a consequence, there is a decrease in the cellular expansion and

elongation of the shoots and other times of the root system (MATEOS-NARANJO et al., 2018).

Salinity and water deficit lead to a set of metabolic alterations identical to those caused by water stress, with the capacity to maintain metabolic processes, although at lower levels (MUNNS et al., 2006). In response to environmental adversities, plants can alter their growth, activate protective mechanisms and adjust osmotically to maintain cell turgor, whose function is to protect the cells in short periods of stress (PATEL et al., 2010).

In view of the above, the understanding of mechanisms and adaptive responses of *E. contortisiliquum* (Vellozo) Morong seedlings to abiotic stresses were based on identifying efficient mechanisms of tolerance to water and salt stresses that occur in the semi-arid region, based on principles and techniques of coexistence with drought.

The ecological plasticity of *E. contortisiliquum* guarantees its presence in vegetation formations, indispensable for the balance of ecosystems and recovery of degraded areas, but is also suitable for landscaping in large spaces, forage, beekeeping and also important for people from the Sertão region who use firewood for cooking food (MUNIZ and MARCHIOR, 2009). In addition, the thin cuts from its wood have better utilization in the manufacture of toys, laminates, plywood, handicrafts, roofs, packaging and crates, since the wood is light ($0.34\text{--}0.54\text{ g/cm}^3$), soft to cut, with white to slightly yellowish sapwood and pink-brown to reddish-brown heartwood, sometimes with darker vascular lines (LORENZI, 2016).

Valuing forest species stimulates their preservation, especially those of natural occurrence, besides providing the community with economic and social return, which can justify the human use and plant richness of the Caatinga.

General Objective

2. GENERAL OBJECTIVE

The objective was to evaluate the morphophysiological and biochemical changes in *E. contortisiliquum* seedlings subjected to abiotic stresses.

Literature review

3. LITERATURE REVIEW

3.1 Caatinga Ecosystem

The Northeast covers 18.27% of the Brazilian territory, with an area of 1,561,177.8 km². Of this area, 962,857.3 km² are within the Drought Polygon, of which 841,260.9 km² covers the northeastern semi-arid region, consisting of deciduous and xerophilous vegetation, covering the states of Alagoas, Bahia, Ceará, Maranhão, Pernambuco, Paraíba, Rio Grande do Norte, Piauí, Sergipe and northern Minas Gerais (ARAUJO, 2011). Caatinga has only 1% of its area covered with full protection conservation units, which is well below the 10% recommended by the International Union for Conservation of Nature (IUCN) and the National Biodiversity Commission (Conabio) (FERNANDES et al., 2017).

The very hot and seasonally dry climate has a great effect on the water regime and exerts great influence on the landscape physiognomy of the Caatinga, with presence of thorny shrubs and pioneer woody species, such as *Mimosa tenuiflora* Willd. Poir., *Croton sonderianus* Muell. Arg., *Caesalpinia bracteosa* Tul., *Bauhinia cheilantha* Bong. Steud and *Combretum leprosum* Mart. (MORO et al., 2015).

The climate in the semi-arid region has specific characteristics, high temperatures, annual averages above 20 °C, high rate of potential evapotranspiration, between 1500 and 2000 mm per year, precipitations from 280 to 800 mm concentrated in three or four months of the year, strong insolation in the remaining months depending on the atmospheric dynamics and on the rainy season, which is quite variable (ARAUJO, 2011).

Rainfall irregularity associated with high evapotranspiration further aggravates the process of soil degradation in the semi-arid region, with the phenomenon of salinization, resulting from the capillary rise of water from the water table (PEDROTTI et al., 2015). According to these authors, this water is rich in soluble salts and their deposition by high evaporation limits plant growth, besides affecting the physical properties of the soil. In addition, the practice of intensively irrigated agriculture increases the signs of soil degradation, either by the effect of salinity, by poor drainage or by lack of water (SOUZA et al., 2016).

Thus, natural and anthropic processes have led to the advance of “dry” lands, resulting in the removal of vegetation, suppressing several ecological niches, and making the mastery of knowledge about the forest resources of the Caatinga ineffective (PEDROTTI et al., 2015). The exploitation of the natural resources of this ecosystem has caused damage to the development of environmental conservation, either by the removal of soils, contamination of water resources and deforestation, with wood being used as the main fuel in the limestone

industries to produce lime and clay for ceramic manufacture, in various parts of the semi-arid region (ARAÚJO, 2011). In addition, extractive exploitation in this region has reached worrying rates, as they reach the most different production sectors, such as the extraction of bee honey, the harvest of native fruits and the reduction in the use of medicinal plants by traditional communities (ALBUQUERQUE and MELO 2018).

Studies with forest species, of natural occurrence in the Caatinga, can be a strategic instrument to ensure forest resources in the improvement of the local population, and *E. contortisiliquum* is a species whose potential for use encompasses the economic, social and ecological aspects.

3.2 General information about *Enterolobium contortisiliquum* (Vell.) Morong

Adult trees of *E. contortisiliquum* can be found in forest formations of Atlantic Forest domain, in Cerrado formations of Central Brazil and in the Northeast (Agreste and Caatinga) (ARAÚJO and PAIVA SOBRINHO, 2011). According to the authors, this species provides support of economic relevance, in the manufacture of toys, handicrafts, roofs, packaging and crates. Its ease of handling (light and soft-to-cut wood), in addition to the beauty of the heartwood, which can range from pinkish-brown to reddish-brown, sometimes with darker vascular lines of the wood, provides better utilization and yields (LORENZI, 2016).

For being a legume species, *E. contortisiliquum* establishes a symbiotic relationship with bacteria of the genus *Rhizobium* which establish in its roots and assimilate molecular N, converting it into forms that plants can absorb, using it for their nutrition (NOGUEIRA et al., 2012). According to the authors, N is accumulated in the soil and later used for fertilization and nutrition of the following species.

With regard to ecological aspects, *E. contortisiliquum* can occupy open niches in the Caatinga more quickly, increasing the resistance and resilience of ecosystems and resources for natural succession (ARAÚJO and PAIVA SOBRINHO, 2011). However, the diversity of vegetation in this ecosystem is a challenge, as some forest species may exhibit restriction of growth in the early stage, either in the field or in protected cultivation when exposed to a condition of environmental stress not tolerated by the plant (PEDROTTI et al., 2015).

3.3 Salt stress

To ensure agricultural production in demand for food, many plant species cultivated around the world are constantly exposed to biotic and abiotic stresses, which include water scarcity and salt stress (TONEL et al., 2013). Most irrigated areas worldwide, about 45

million (19.5%) of the 230 million hectares, already suffer from soil degradation due to the accumulation of salts, causing a decrease in cultivated areas (MESQUITA et al., 2015).

In Brazil, saline and sodic soils occur in Rio Grande do Sul, in the Pantanal region of Mato Grosso and predominantly in the semi-arid region of the Northeast, where approximately 25% of the irrigated areas were salinized, severely limiting agricultural production (MESQUITA et al., 2015; SOUZA et al., 2016). Saline soils are those with high contents of soluble salts (sodium, calcium or magnesium bicarbonates, chlorides, sulfates), and the increase in any of these salts constitutes one of the problems that most causes threats of irreversible degradation and desertification, as well as long-lasting contamination of downstream waters and soils (PEDROTTI et al., 2015).

Anthropic activity is the one that most influences the increase in salinization in the semi-arid region of Brazil, and the predominance of long periods of drought most of the year and the need to produce food that comes from agriculture, makes irrigation more relevant (PEDROTTI et al., 2015). In inadequate management of irrigation water, drainage control is not done or done inefficiently, affecting agricultural production, the environment and the area of forest remnants, accelerating the exploitation of marginal and fragile lands and the pollution of springs (SOUZA et al., 2016).

The increase of salts in the soil profile and surface directly exposes the plants to stress, limiting their development and their chances of survival, since the osmotic effect makes water less and less available (LARCHER, 2006). Problems of toxicity and nutritional disorder of plants also occur due to excess salts, and usually arise when soil ions are excessively accumulated in plants, thus being dissolved in the cytosol and other organelles where they end up interfering in numerous enzymatic reactions and causing damage to their growth (MUNNS and TESTER, 2008).

In general, the degree of salinity tolerance varies with the species (SILVEIRA et al., 2010). In leaves, the increase of salts is more visible in older ones, which had more time to accumulate Na^+ and experience the effects of this accumulation; notably, the leaves of some plants are more capable than others to maintain green and photosynthetic function for longer in the presence of high levels of Na^+ in tissues (NEGRÃO et al., 2017).

To avoid water loss, caused by the reduction of osmotic potential, resulting from salinity, some plant species lose their leaves in the dry season, while others reduce the leaf area, which lead to a decrease in water absorption on the transpiring surface, resulting in lower transport of Na^+ and Cl^- in the xylem and, consequently, conservation of water in plant tissues (MUNNS et al., 2006).

To avoid or not an increase in salt concentration, specific root processes are of particular importance, evidencing changes of increase and other times reduction of the root system to the detriment of the aerial part, allowing or inhibiting the capture of water in

subsurface portions of the soil (SEWELAM et al., 2016). Plant growth disorders caused by salt stress are non-specific manifestations that may represent a certain degree of severity, from the inhibition of photosynthesis and reduction of dry matter production (LARCHER, 2006).

In soils with satisfactory levels of saline ions, plants absorb and accumulate them in the vacuole of leaf cells, maintaining the saline concentration inside the cell at low levels, so the salt no longer interferes in the hydration of proteins and in the enzymatic and metabolic mechanisms of the plant. However, in excessive amount in the soils, the salt hampers the absorption of nutrients, resulting in an imbalance that is harmful to plant metabolism, in addition to producing a manifestation of non-specific disorder, such as inhibition of photosynthesis (MUNNS et al., 2006). Under extreme conditions, after exceeding the capacity of cells to compartmentalize salts in the vacuole, the salt load would accumulate rapidly in the cytoplasm and inhibit enzymatic activity, dehydrating the cell, leading to plasmolysis in plant cells (MUNNS and TESTER, 2008).

In this context, there is a need to define at what level the effects of salinity manifest themselves for each species because, in the Caatinga, vegetation is established in a more complex way as, under adverse conditions, plants adopt specific strategies to survive, shaping their metabolism, growth and development to a certain degree of resistance to salinity (SILVEIRA et al., 2010). Such resistance consists in both avoiding, through a saline regulation, excessive amounts of salt reaching the protoplasm, and tolerating its effects (LARCHER, 2006).

Salinity tolerance may be related to differences in the absorption, transfer or accumulation of Na^+ and Cl^- in the vacuole, or organic solutes in the cytoplasm, in a process called osmotic adjustment, which may allow water absorption, and cellular turgor, depending on the duration of stress (TAIZ et al., 2017).

3.4 Water deficit

Water is considered a universal solvent, whose absence imposes disorder on the processes of the soil-plant-atmosphere system (SCALON et al., 2011). In the soil, the maintenance of the amount of water is a decisive factor for the balance of the osmotic potential inside the plant. As the soil dries, permeability decreases in the plant cell, which acts by dissolving organic substances and filling gaps between the thin structures of the protoplasm and vacuoles (SILVEIRA et al., 2010). According to the authors, this process works as a means of transport to the conductive elements of xylem and phloem, responsible for extending cell walls.

The reduction of soil water potential decreases the availability of water to plants, affecting cell turgor, altering wall elasticity and consequently leading to disrupting cell homeostasis (SEWELAM et al., 2016). These authors emphasize that the more negative the water potential of the soil system, the lower the availability of water to the plant and the greater the constant loss of water by transpiration in plants, and the variations of this effect depend on the environmental conditions of temperature, insolation and humidity.

Water deficit affects the various physiological processes, increasing the resistance of stomata to the diffusion of water vapor, thus reducing transpiration and consequently the supply of CO₂ for photosynthesis (LARCHER, 2006). According to this author, to avoid drought or excessive desiccation, plants close their stomata, considered the first line of defense, even before the reduction of the water content in the leaf occurs. Since the stomatal movement mainly obeys the control of two circuits, CO₂ and H₂O (TAIZ et al., 2017).

Therefore, water is one of the determining factors for the diversity and distribution of plants, including forest species, which may exhibit growth under a certain degree of cell hydration or ability to withstand the lack of water under the conditions that prevail in the habitat (SILVEIRA et al., 2010). According to Larcher (2006), plants exposed to stress due to decreased water supply show different responses according to species and nature and severity of the stress.

As observed in a study conducted by Scalon et al. (2011) with *Guazuma ulmifolia* Lam seedlings, the growth in plant height, diameter, leaf area, shoot dry mass and root dry mass were higher at the highest water availability (100% of field capacity - FC); however, the seedlings grew satisfactorily under 50% FC and began to experience water stress below 25% FC. Oliveira et al. (2016) verified that water stress promoted morphological adaptations, such as defoliation and greater root development in *Erythrina velutina* Willd seedlings. Alves et al. (2018) found that morphological and physiological changes were not significant at 100% and 80% of the maximum water retention capacity (MWRC) in seedlings of *Allamanda blanchetii* Belongs, while under 20% MWRC there was no plant survival.

According to the aforementioned studies, it is notorious that different forest species adopt different measures in the face of water stress, morphologically altering their characteristics and reflex of adaptation to the environment (SCALON et al., 2011). These authors also reinforce that such implications of growth of plant organs under unavailability of water in the soil and transport to the transpiring surface of the leaves generally compromise the growth in plant height, causing lower number of leaves, alterations in root growth, reduction in leaf expansion rate by transpiration, avoiding dehydration.

Another mechanism of defense of plants that can contribute when the water supply in the soil decreases unfavorably is the biochemical mechanism, as the plasma membrane acts as a selective barrier between living cells and their environments (LARCHER, 2006). The

author stresses that this state of alertness to increased stress aims to stimulate in a timely manner the vital restoration of the plant after discontinuity of stress. According to the same author, although the plant responds biochemically on several occasions of stress with osmotic adjustment, especially water deficit, it can still suffer from interruption of tissue growth, with reduction in the rates of net CO₂ assimilation, higher values of leaf abscission, senescence, and reduction of transpiration and photosynthesis. It is a set of strategies that are used to adapt to the stress. Osmotic adjustment is an excellent strategy, but there is deviation of metabolic energy, which together with the lower diffusion of CO₂ affects photosynthesis and causes growth reduction.

In regard to photosynthesis, it plays a vital and early role in the detection of stress in plants, through the intersystem transport of electrons (ANJUM et al., 2011). Therefore, it is clear that any change or imbalance in chloroplast function will directly or indirectly affect other cellular functions, since the thylakoid membranes and the redox complex involved in the photosynthetic apparatus, especially the PSII of light energy capture, are very sensitive targets to various environmental stress factors (TAIZ et al., 2017).

Therefore, verifying the manner with which plants respond to water stress can be crucial for the unfavorable environment of the semi-arid climate to constitute a significant factor for plants naturally found in the Caatinga ecosystem.

3.5 Plant defense under abiotic stresses

The mechanism of resistance to drought or defense of the plant is linked to either escape or desiccation, which will be delayed by mechanisms that allow the plant to be able to maintain favorable water content regardless of the soil, before severe water shortage occurs in the face of abiotic stresses (water and salt), because the protoplasm of most plants is highly sensitive to water loss (LARCHER, 2006). In a way, the natural or anthropic processes that cause water deficit first in the soil and then in the plant can be verified under the effect of both water stress and salt stress (LISAR et al., 2012). The authors emphasize that water is osmotically retained in a saline solution, so that the concentration of salts makes it less and less available to plants, causing water deficit.

In this context, when turgor begins to decrease, osmoregulatory measures are initiated. These measured help in maintaining cell volume, delaying the loss of turgor in the mesophyll and guard cells (LISAR et al., 2012). An efficient way to maintain physiological functions under water deficit conditions is the ability of the plant to accumulate ions in the vacuole, or organic solutes in the cytoplasm, in a process called osmotic adjustment (AKHTAR et al., 2017). According to the same authors, this cell adjustment considerably increases the concentration of organic solutes or compatible solutes at cellular level (soluble

sugars, organic acids and amino acids) at the time of stress and, as an advantage, do not interfere with the functions of enzymes. This is because these solutes tend to be electrically neutral, non-ionic, ensuring membrane conformation, thus allowing cells to maintain turgor pressure despite the low water potential (MARENCO and LOPES, 2009).

The main function of compatible solutes is to prevent water loss at levels that the plant cannot tolerate, maintaining cell turgor, and once metabolites accumulate in cells, they lead to increased osmotic potential, which results in greater capacity of water capture by the roots and water savings in cells (GHOSH and XU, 2014). In addition to the function of osmoregulation, compatible solutes have other functions in plants under low water potential, such as protection of enzymes and structures and membrane integrity, protein conformation, elimination of oxygen free radicals and structural stabilization of cellular macromolecules (OKUNLOLA et al., 2016).

In relation to proteins, these are, after water, the most important compounds of protoplasm and, when accumulated, they can confer tolerance to the plant, minimizing the effects inherent to drought (LARCHER, 2006). This is due to the increased activity of the enzyme protease, which breaks down the reserve proteins with the increase of stress time, increasing the content of total soluble amino acids in order to adjust osmotically to the stressful medium (TAIZ et al., 2017). According to these authors, the increase in protein levels and enzyme activity under stress conditions can be a determining factor in the adaptability of several plant species, and this activity can be identified as a biochemical marker of stress.

Concentrations of natural osmolytes in plant cells can reach 200 mM or more under water stress, and proline concentration can reach up to 80% of the total amino acid pool in some plants (LISAR et al., 2012). These same authors emphasize that these facts contribute to the osmoregulation process, in addition to the ability to act as a regulatory or signaling molecule and activate multiple responses that are part of the adaptation process. Osmotic adjustment is not a dependent and direct response, since plants may have adjustment capacity clearly maintaining turgor under lower water potentials while other species can demonstrate just the opposite (GHOSH and XU, 2014).

In general, stress causes disorder in the structural apparatus and in the coordination of various processes at the molecular and cellular levels of the organism, which requires an additional cost; after osmotic adjustment, it often grows more slowly (KHAN et al., 2015). Therefore, the physiological responses of plant species regarding water unavailability reflect a conflict between the benefits arising from the ability to perform physiological processes according to environmental conditions and the costs associated with this capacity (TAIZ et al., 2017).

As a result of drought, some plant species promote at the cellular level the increase in the content of carbohydrates, such as starch. Reduction of this compound is a consequence of the activity of amylase, which generates a greater amount of reducing soluble sugars for the osmotic adjustment process (LISAR et al., 2012). This increase aims to avoid the dehydration of plant cells. Thus, starch consumption would be an initial response to drought, allowing the maintenance of carbon supply under a condition of reduced atmospheric CO₂ fixation (OKUNLOLA et al., 2016).

Together with sugar, proline acts as protector, serving as an energy reservoir to regulate redox potentials, as a hydroxyl radical cleaner (LISAR et al., 2012). These authors also report that these mechanisms protect macromolecules from denaturation and act as a means of reducing acidity in the cell, as well as maintaining membrane stability, preventing and protecting membrane fusion, and maintaining protein to remain functional.

Morphophysiological and biochemical mechanisms are important tools in the defense of the plant that work in association to avoid or reduce the adverse effects of the environment. Thus, it is observed that the species of the present study has the capacity to establish itself in semi-arid soils and participate in the reoccupation of degraded niches in the Caatinga ecosystem, but should be established in early stages of growth under reduced water and salt stresses.

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Chapter 1

MORPHOPHYSIOLOGICAL AND BIOCHEMICAL CHANGES IN *Enterolobium contortisiliquum* (Vell.) Morong. SEEDLINGS IRRIGATED WITH SALINIZED WATER

MORPHOPHYSIOLOGICAL AND BIOCHEMICAL CHANGES IN *Enterolobium contortisiliquum* (Vell.) Morong. SEEDLINGS IRRIGATED WITH SALINIZED WATER

ABSTRACT - The high concentration of salts is a stress factor for plants, as it reduces the osmotic potential and hampers the absorption of water by roots. The objective of this study was to verify the morphophysiological and biochemical changes in *Enterolobium contortisiliquum* (Vell.) Morong seedlings irrigated with salinized water. For this, the experiment was conducted in a greenhouse with five salinity levels: 2, 4, 6 and 8 dS.m⁻¹ and local supply water (0.3 dS.m⁻¹) as control. The experimental design was in randomized blocks, with four replicates, and the experimental unit consisted of 20 plants. At 30 days after sowing, the plants were evaluated for plant height, number of leaves, leaf area, stem diameter, Dickson quality index, stem length, root length, shoot dry mass, root dry mass and total dry mass. In addition, biochemical analyses of free amino acids, proline, starch and total soluble sugars were carried out. The data were subjected to analysis of variance and linear regression. The gradual increase in salinity of irrigation water was detrimental to the growth and development of *E. contortisiliquum* plants, with a sharp decrease from the threshold of 2 dS.m⁻¹. The biochemical mechanism of tolerance to salt stress was the increase of amino acids, proline and total sugars, concomitantly with the consumption of starch reserves.

Key words: Fabaceae, seedling production, Caatinga, salt stress, osmoregulators.

RESUMO

A alta concentração de sais é um fator de estresse para as plantas, pois reduz o potencial osmótico e dificulta a absorção de água pelas raízes. O objetivo deste estudo foi verificar as alterações morfofisiológicas e bioquímicas em mudas de *Enterolobium contortisiliquum* (Vell.) Morong irrigadas com água salinizada. Para isso, o experimento foi conduzido em casa de vegetação com cinco níveis de salinidade: 2, 4, 6 e 8 dS.m⁻¹ e água de abastecimento local (0,3 dS.m⁻¹) como controle. O delineamento experimental foi em blocos casualizados, com quatro repetições, e a unidade experimental composta por 20 plantas. Aos 30 dias após a semeadura, as plantas foram avaliadas quanto à altura da planta, número de folhas, área foliar, diâmetro do caule, índice de qualidade de Dickson, comprimento do caule, comprimento da raiz, massa seca da parte aérea, massa seca da raiz e massa seca total. Além disso, foram realizadas análises bioquímicas de aminoácidos livres, prolina, amido e açúcares solúveis totais. Os dados foram submetidos à análise de variância e regressão linear. O aumento gradual da salinidade da água de irrigação foi prejudicial ao crescimento e desenvolvimento das plantas de *E. contortisiliquum*, com uma diminuição acentuada a partir do limiar de 2 dS.m⁻¹. O mecanismo bioquímico de tolerância ao estresse salino foi o aumento de aminoácidos, prolina e açúcares totais, concomitantemente com o consumo das reservas de amido.

Palavras-chave: Fabaceae, produção de mudas, Caatinga, estresse salino, osmorreguladores.

INTRODUCTION

Enterolobium contortisiliquum (Vell.) Morong, Fabaceae family, is a tree species known in Portuguese as 'tamboril' or 'orelha de macaco'. It is a pioneer, deciduous and fast-growing species that can reach 30 m in height (LORENZI, 2016). This species stands out mainly for its potential use in the recovery of degraded areas, for its ability to nodulate with diazotrophic bacteria, in the process of symbiosis, causing it to acquire part of the atmospheric nitrogen, which promotes an ecological advantage over non-nodulating species (SOUSA *et al.*, 2016).

The high salinity accentuated by high evapotranspiration, combined with low rainfall, hampers the establishment of several plant species in the dry forest of Caatinga, including the trees (CUNHA *et al.*, 2013). Thus, the accumulation of soluble salts in the soil causes a reduction in osmotic potential, leading to water stress, toxicity disorders, and nutritional deficiency in plants, directly influencing cell turgor and reducing yield (NASCIMENTO *et al.*, 2015).

Recent studies have shown that salinity greater than 5.0 dS.m⁻¹ negatively interfered with the growth of *Talisia esculenta* (MELO FILHO *et al.*, 2017), *Erythrina velutina* (LOPES *et al.*, 2019) and *Combretum leprosum* (LEAL *et al.*, 2019). Inadequate fertilizer management also leads to soil salinization, and this was observed in *Mimosa scabrella*, which, despite being subjected to slow-release fertilizer (Basacote Plus 9M – 16-8-12 + 2 Mg + 5 S + 0.4 Fe + 0.02 B + 0.02 Zn + 0.05 Cu + 0.06 Mn + 0.015 Mo), showed reduced growth as a function of the negative soil osmotic potential (-0.3 and -0.4 MPa) (AVRELLA *et al.*, 2019).

Several organs and tissues of a plant may have different resistances in relation to the combination of stresses. This imposed combination is related to the deviation from an optimal situation for a certain time (LARCHER, 2006). In this context, the stresses caused to plants can promote morphological changes in the growth of their shoots and root system. In addition, there are biochemical changes such as the accumulation of organic solutes, responsible for the osmotic adjustment of the cell in an attempt to mitigate the effects of the damage (MITCHELL *et al.*, 2013). Salt stress can still alter the normal cellular metabolism of plants and cause overproduction of reactive oxygen species, affecting the balance of the redox system through the oxidation of biomolecules (MORAIS *et al.*, 2018).

Increase of salinity levels in irrigation water results in plant growth losses, and biochemical osmoregulators can probably mitigate the implications of this stress. Studies on this subject are scarce, particularly when associated with biochemical responses of plants.

In this context, the objective of this study was to evaluate the morphophysiological and biochemical changes in *E. contortisiliquum* seedlings irrigated with salinized water.

MATERIAL AND METHODS

The study was conducted in laboratory and greenhouse at the Federal University of Rio Grande do Norte, Campus Macaíba, RN, Brazil (5°53'10.0" S, 35°21'52.5" W and 56 m altitude), with seedlings of *E. contortisiliquum*, produced from seeds provided by the Center for Ecology and Environmental Monitoring of the Federal University of the São Francisco Valley, Petrolina, PE (9°19'10.47" S, 40°33'48.91" W and 375 m altitude).

The plants were subjected to the effects of five levels of electrical conductivity of irrigation water: 2, 4, 6, 8 dS.m⁻¹ and 0.3 dS.m⁻¹ (corresponding to the control, without addition of salts). To obtain the salt concentrations of the irrigation water, portions of sodium chloride (NaCl) of 50, 100, 160, 220 g of common iodinated salt were dissolved in local supply water in a polyethylene container with capacity of 60 L. From the measurement of the initial electrical conductivity, the other higher levels were prepared.

The design was in randomized blocks, with five treatments and four replicates, with each experimental unit composed of 20 seedlings. The experimental units consisted of black polyethylene bags with capacity of 1.5 dm³. These bags, filled with substrate resulting from the mixture of soil and cattle manure (1:1), were placed directly on the soil in the greenhouse and watered with local supply water. Prior to sowing, the seeds were manually scarified in the region opposite to the hilum using sandpaper number 100. Two seeds were sown in each container in moist substrate (total saturation). At 15 days after sowing, thinning was performed, leaving one plant per container. At 30 days after sowing, when the plants reached approximately 35 cm in height, the treatments began to be applied.

To control the irrigation with the respective saline waters, the daily volume supplied to each container was approximately 80 mL, in order to maintain the substrate moisture, applied using a graduated container, and shoots of the plant were not wetted.

At the end of the experiment, the chemical and physical analyses of the substrate were performed (Table 1).

Table 1 - Chemical and physical characteristics of the substrates as a function of salinity levels

Samples dS.m ⁻¹	N g. kg ⁻¹	P mg. dm ⁻³	Cations (cmol _c . dm ⁻³)				pH H ₂ O	EC dS. m ⁻¹	ESP %
			K	Mg	Ca	Na			
0.3	1.89	241.1	2.72	1.3	2.60	1.66	7.5	1.36	20
2.0	2.10	231.8	3.05	1.7	2.40	1.62	7.5	1.53	19
4.0	1.82	181.4	2.47	1.9	1.40	2.73	7.6	1.21	32
6.0	1.54	270.3	2.39	2.1	1.50	2.44	7.5	1.38	29
8.0	1.68	190.0	3.05	0.9	2.90	3.68	7.7	2.20	35

Soil Analysis carried out by the Laboratory of Soil Fertility and Plant Nutrition, UFERSA

Plant growth was evaluated weekly until 28 days, before the beginning of the treatments, based on the number of leaves (NL); plant height (H), measured from the ground level to the tip of the highest leaf, with a graduated ruler (0.1 mm); and stem diameter (SD), measured using a digital caliper (0.01 mm).

Leaf area was determined by measuring 10 leaflets, randomly collected at 6:30 a.m., before the destruction of the plants. The leaves were measured by length (L) and width (W), in repetition of all treatments using a digital caliper (0.001 centimeters). The length corresponded to the distance between the distal base of the petiole and the final tip, while the width corresponded to the longest distance perpendicular to the axis of the length, both expressed in cm, according to Equation 1.

Equation 1: $A = L.W$, expressed in (cm²); where: L = length in cm; W = width in cm, in the median position.

For root length (RL), the length of the largest root was measured. To quantify the dry masses of shoots (SDM), roots (RDM) and total (TDM), the organs were separated, individually placed in paper bags, and dried in an oven at 65 °C until reaching constant weight. The materials were weighed on a precision analytical scale (0.001 g) and the results were expressed in g plant⁻¹. Dickson quality index (DQI) was determined following the methodology proposed by Dickson *et al.* (1960) (Equation 2).

Equation 2: $DQI = TDMP \div \left[\frac{H}{D} + \frac{SDMP}{RDMP} \right]$; where: TDMP = Total dry mass production (g); H = Height of the shoots (cm); D = Stem diameter (mm); RDMP = Root dry mass production (g); SDMP = Shoot dry mass production (g).

Biochemical determinations were performed using 20 grams of fresh leaves of each treatment, which were stored in an ultra-freezer (-20 °C). These were used to determine the contents of free amino acids, proline, starch and total soluble sugars. Starch and sugar contents were quantified according to Morris (1948) and Yemm and Willis (1954), respectively, using the anthrone reagent and reference curve from known glucose concentrations, expressed in mg g⁻¹ of fresh matter of leaves.

Amino acids were determined by the ninhydrin method, according to Yemm *et al.* (1955) using glycine as standard. Proline content was quantified by the method proposed by Bates *et al.* (1973), and the results were expressed in μmol of proline g⁻¹ of fresh mass.

The data were subjected to analysis of variance and polynomial regression, testing the mathematical models. The model that showed significant difference by the F test at 5% probability level was adopted and, in case of significance, the response of the best biological expression was considered. Statistical analysis was carried out using the statistical program Agroestat® (BARBOSA; MALDONADO, 2010).

RESULTS AND DISCUSSION

The results of the analysis variance indicated a significant effect ($p < 0.01$) with the increase in irrigation water salinity, and *E. contortisiliquum* seedlings were drastically affected under the highest salinity level (Table 2).

Table 2. Summary of the analysis of variance for plant height (H), number of leaves (NL), leaf area (LA) and stem diameter (SD) of *Enterolobium contortisiliquum* (Vell.) Morong. seedlings irrigated with water of different salt concentrations.

Source of variation	DF	H	NL	LA	SD
Treatment	4	166.36**	11.65**	0.11**	1.53**
Blocks	3	9.72	0.36	0.0	0.02
Residual	12	9.63	0.46	0.0	0.02
CV (%)	-	10.47	13.38	10.25	8.12

** significant at 1% probability level.

The growth variables decreased linearly as a function of the increase in electrical conductivity of irrigation water (Figure 1).

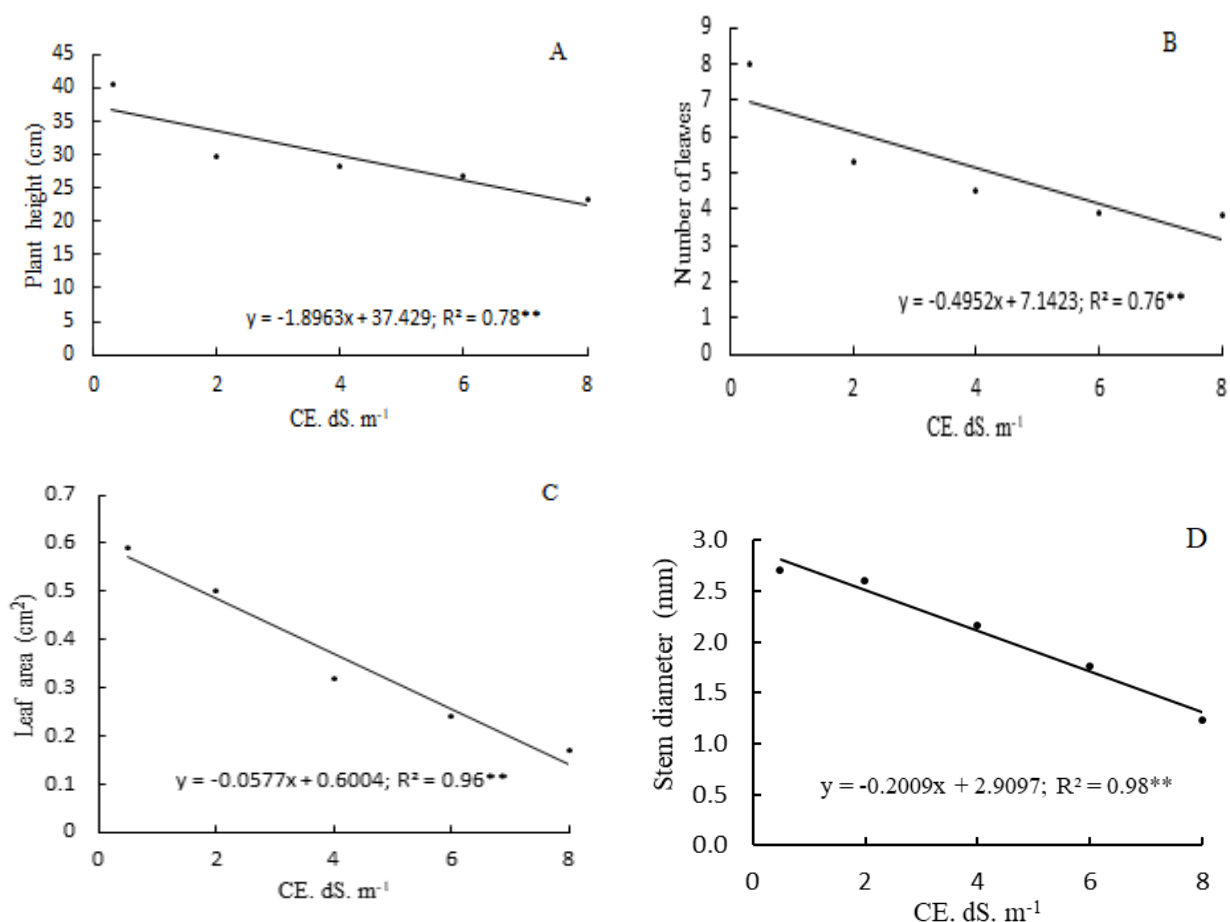
E. contortisiliquum plants irrigated with salinized water reacted to increased electrical conductivity (EC_w), decreasing their growth in height. According to the regression analysis, this reduction was equal to 18.9 and 37.8% in plants irrigated with the waters of lowest and highest salinity, 2 and 8 dS.m⁻¹, respectively, compared to the control (0.3 dS.m⁻¹) (Figure 1 A). It is verified that when these plants are irrigated with low-salinity water (2 dS.m⁻¹) they may exhibit acceptable response of growth in height when grown in soils at an early stage of degradation. As observed here, the studies of Lima *et al.* (2015) with *Albizia lebeck* (L.) Benth., Lopes *et al.* (2019) with *Erythrina velutina* Willd and Leal *et al.* (2019) with *Combretum leprosum* Mart. found similar results regarding the reduction of plant height with the increase in salt stress.

As verified for the growth of seedlings in height, salt stress reduced the number of leaves, with a variation from 7.0 leaves at the lowest salinity to 3.2 leaves at the highest salinity, resulting in a total loss of 54.2% in plants subjected to the highest salinity level (Figure 1 B).

It is possible that the loss in the number of leaves up to a certain limit is a strategy to minimize transpiration by the leaves, in view of the reduction in the osmotic potential caused by the salt. The number of leaves in *Schinopsis brasiliensis* Engler seedlings grown in substrate washed after application of fertilizer salts was higher than that in plants grown in

unwashed substrate, with an increase of 19.34% (BRITO *et al.*, 2018). On the other hand, *Malpighia emarginata* seedlings had a reduction of 10 leaves at the highest salinity level (4.3 dS.m⁻¹) compared to those subjected to 2.3 dS.m⁻¹ (MELO *et al.*, 2018).

Figure 1 - Growth variables of *Enterolobium contortisiliquum* (Vell.) Morong seedlings irrigated with water of different salt concentrations. A - Plant height; B - Number of leaves; C - Leaf area; D - Stem diameter.



The estimated mean values of leaf area of *E. contortisiliquum* plants were inversely proportional to the increase in NaCl concentrations. When comparing the effect between the lowest and the highest salt concentrations of salinized waters, 2 and 8 dS.m⁻¹, it was possible to note reductions in leaf area of 17 and 65% between the ECw levels, respectively (Figure 1 C). At low salinity levels, the water flow in the soil and to the plant was little affected.

In this context, the leaf area of *Talisia esculenta* (A. St.-Hil.) Radlk. seedlings was lower with the increase of ECw in the substrate solution and the time of exposure to stress (MELO FILHO *et al.*, 2017). According to these authors, at the highest level of ECw (4 dS.m⁻¹), the reduction was equal to 79 and 82% at 60 and 90 days after sowing, respectively. The

authors also verified that the highest values of leaf area were obtained by seedlings irrigated using waters with lower EC_w (1 dS.m⁻¹), with maximum values of 18 and 23 cm² at 60 and 90 days after sowing, respectively.

The stem diameter of *E. contortisiliquum* seedlings showed decreasing linear behavior as a function of the increase in irrigation water salinity, with variation from 2.8 to 1.3 mm, at salinity levels of 0.3 and 8.0 dS.m⁻¹, respectively, so that seedlings irrigated using water with higher salinity showed a reduction of 54.3% (Figure 1 D).

Under a condition of low salinity (2 dS.m⁻¹), it was found that plants showed twice the stem diameter compared to those subjected to the highest level of salt stress (8 dS.m⁻¹), 1.4 mm. In this context, Larcher (2006) points out that plants affected by the presence of soluble salts in the soil solution, up to certain thresholds, may show lower expense with the support structure as an artifice, reducing the amount of lignified tissues through the peripheral arrangement of the stem. This fact is reinforced by the results obtained by Lopes *et al.* (2019) with *Erythrina velutina* Willd and Avrella *et al.* (2019) with *Mimosa scabrella* Benth, besides being in agreement with those obtained in the present study. These authors report that the seedlings of these species had the stem diameter significantly reduced when they were subjected to the highest level of salinity.

The variables root length (RL), shoot dry mass (SDM), root dry mass (RDM), total dry mass (TDM) and Dickson quality index (DQI) were significantly affected by the increase in irrigation water salinity at 1% probability level (Table 3).

Table 3 - Summary of the analysis of variance for Dickson quality index (DQI), root length (RL), shoot dry mass (SDM), root dry mass (RDM) and total dry mass (TDM) of *Enterolobium contortisiliquum* (Vell.) Morong. seedlings irrigated with water of different salt concentrations.

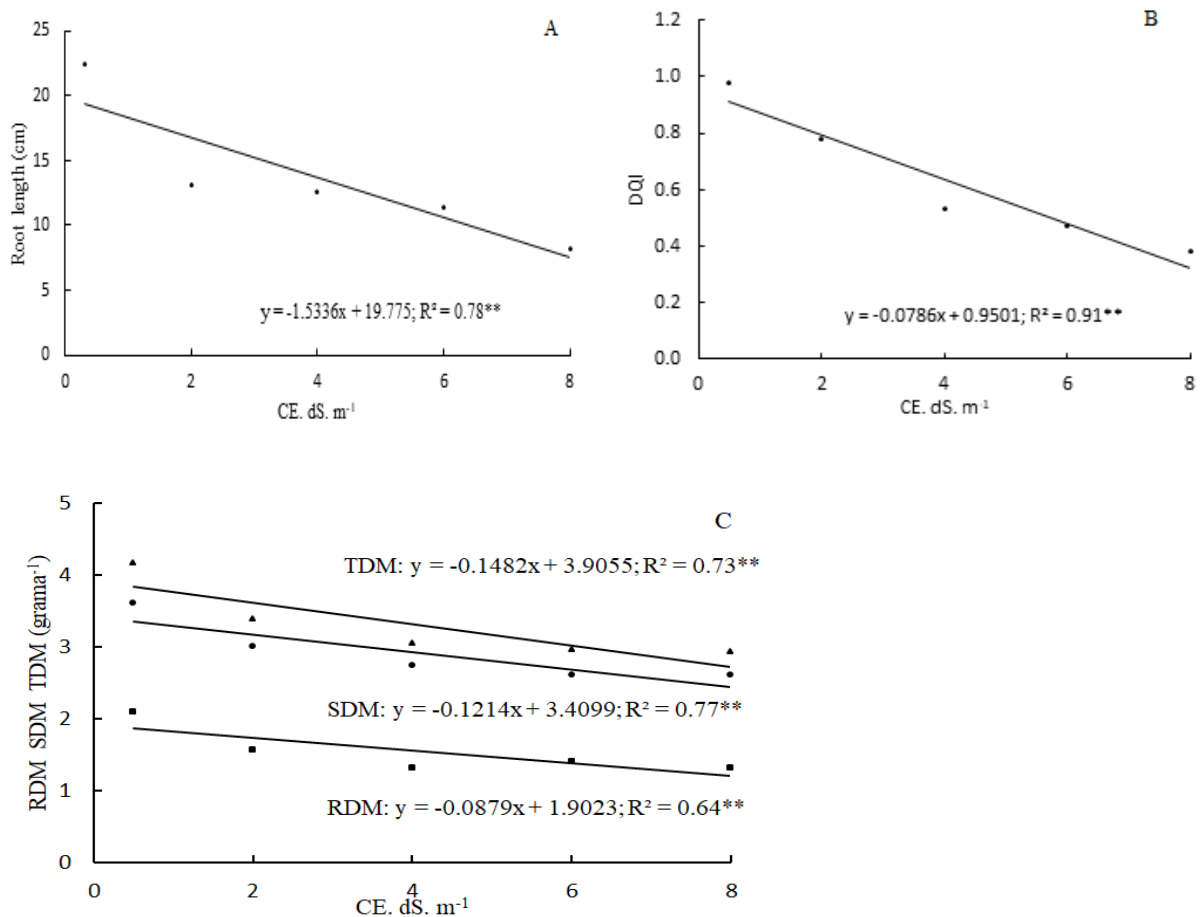
S. V.	DF	DQI	RL	RDM	SDM	TDM
Treatment	4	0.24**	113.26**	0.43**	0.68**	1.07**
Blocks	3	0.00	6.7	0.03	0.09	0.11
Residual	12	0.02	5.51	0.04	0.11	0.14
CV (%)	-	23.07	17.33	13.27	11.81	11.56

SV: source of variation; **: significant at 1%

The values of root length from salinity levels of 2 and 8 dS.m⁻¹ resulted in dimensions of 12.8 cm and 8.6 cm, respectively, with a reduction of 32.8% in comparison to the lowest salinity level (Figure 2 A). The better root growth, in length, under a condition of low salinity, is due to the greater osmotic potential of the soil solution, which in turn promotes greater efficiency in the transport of water and minerals to the other organs of the plant, promoting a better development. Thus, in part, the acquisition of resources (water, nutrients) by plants is associated with the ability to explore the subsurface environment. Results similar to those

found in this study were also found by Al-Huqail *et al.* (2019) with seedlings of *Acacia gerrardii* Benth. These authors verified that the negative effects of NaCl caused changes in nutrient uptake, as well as reduction of 43% in root length at the highest salt concentration (200 mM) compared to the control. Similarly, Leal *et al.* (2019) found that the length of *Combretum leprosum* Mart. seedlings, subjected to NaCl solution (5.5 dS.m⁻¹), decreased dramatically.

Figure 2 - Growth variables of *Enterolobium contortisiliquum* (Vell.) Morong plants irrigated with water of different salt concentrations. A - Root length; B - Dickson Quality Index (DQI); C - Shoot dry mass (SDM), root dry mass (RDM) and total dry mass (TDM).



The best Dickson quality index (DQI) occurred for *E. contortisiliquum* seedlings subjected to ECw of 2 dS.m⁻¹, with DWI of 0.8, whereas at the most intense salt concentration (8 dS.m⁻¹) this index was 0.4 (Figure 2 B). The lower addition of salt in the irrigation water caused lower osmotic tension, thus favoring the absorption of water by the roots and, consequently, the production of shoot and root biomass. According to Gomes *et al.* (2002), the higher the DQI, the better the quality standard and field survival of the seedlings,

so DQI > 0.2 is recommended. Results similar to those found here were also verified by Oliveira *et al.* (2017) with seedlings of *Artocarpus heterophyllus* Lam., which even subjected to the highest level of salt stress (4 dS.m⁻¹) showed DQI greater than 0.2. The DQI of *Malpighia emarginata* seedlings was also gradually reduced according to the increase in ECw, which was 1.3, 2.3, 3.3 and 4.3 dS.m⁻¹, and those that came subjected to lower levels of electrical conductivity were acceptable (MELO *et al.*, 2018).

The results obtained for the dry masses of shoots (SDM), roots (RDM) and total (TDM) show that these variables were linearly reduced with the increase in the electrical conductivity of irrigation water, with percentage losses of 3.6, 4.7 and 3.8% per unit increase in salinity, so that at the highest level of salinity there were total losses of 27.7, 36.1 and 29.5%, for SDM, RDM and TDM, respectively, in seedlings at the highest salinity level (8 dS.m⁻¹) (Figure 2 C).

The reductions of dry biomass (SDM, RDM and TDM) is largely due to the formation of lower biomass as the salt stress increases. Similar results are pointed out by Lima *et al.* (2015) in seedlings of *Albizia lebbbeck* (L.) Benth., whose highest and lowest accumulations of dry mass were observed in seedlings at salinity levels 0.68 and 4 dS.m⁻¹, with a reduction of 13% between these treatments. Likewise, Avrella *et al.* (2019) with *Mimosa scabrella* Benth. and Leal *et al.* (2019) with *Combretum leprosum* Mart. verified that the phytomass of the seedlings of these species decreased with the increase in salt concentration in the irrigation water and that, in the case of *C. leprosum*, this reduction was 100% for plants irrigated with salinized water of 6.5 dS.m⁻¹.

The analysis of variance pointed to a significant effect of the electrical conductivity of irrigation water on the contents of free amino acids, proline and starch ($p < 0.01$), as well as total soluble sugars ($p < 0.05$) in *Enterolobium contortisiliquum* (Vell.) Morong seedlings (Table 4).

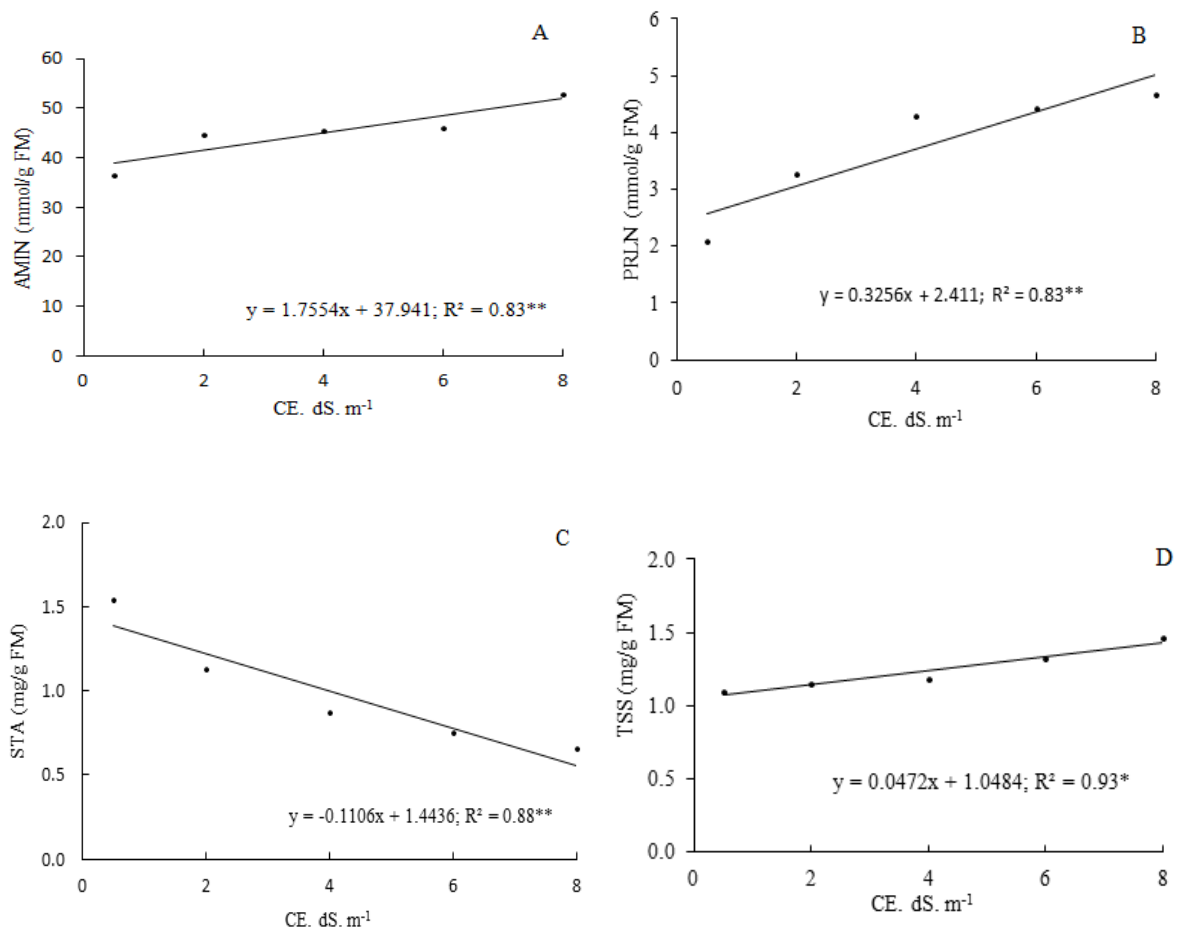
The concentrations of free amino acids (AMIN) (Figure 3A), proline (PRLN) (Figure 3B) and total soluble sugars (TSS) (Figure 3C) were linearly and positively affected by the increase in the electrical conductivity of irrigation water, so that the highest values were obtained in seedlings irrigated with water of highest salinity (8 dSm⁻¹), with maximum values of 51.9 mmol g⁻¹ of fresh mass for AMIN, 5.0 mmol g⁻¹ of fresh matter for PRLN and 1.4 mg g⁻¹ for TSS, corresponding to accumulated increases of 35.1, 99.9 and 34.2% for AMIN, PRLN and TSS, respectively (Figures 3A, 3B and 3C).

Table 4 - Summary of the analysis of variance for the quantifications of free amino acids, proline, starch and total soluble sugars in leaves of *Enterolobium contortisiliquum* (Vell.) Morong seedlings irrigated with water of different salt concentrations.

Source of variation	DF	Free amino acids	Proline	Starch	Total soluble sugars
Treatment	4	188.51**	4.31**	0.50**	0.27*
Blocks	3	13.99	0.27	0.03	0.01
Residual	12	23.96	0.50	0.02	0.05
CV (%)	-	11.00	18.23	16.74	19.24

*, **; significant at 5% and 1% probability levels by the F test, respectively.

Figure 3 – Contents of A - Free amino acids (AMIN), B - Proline (PRLN), C - Starch (STA) and D - Total soluble sugars (TSS) quantified in the leaf extracts of *Enterolobium contortisiliquum* (Vell.) Morong irrigated with water of different salt concentrations.



The waters of lowest and highest salt concentrations, 2 and 8 dS.m⁻¹, respectively, resulted in accumulation of amino acids in the leaf tissue of 43 and 53 mmol g⁻¹ of fresh mass, and this accumulation was 10 times greater for the seedlings under condition of greater stress (Figure3 A). This accumulation of amino acids shows that there was protection of plant cell against possible damage caused by salt stress. According to Soares and Machado (2007), plants under stress trigger several responses, which start intracellularly by

gene expression and cellular metabolism and result in variations in growth rate and biomass production. Also according to these authors, this change in the pattern of protein expression may be due to the increased activity of proteases which have the function of breaking down reserve proteins as the stress time increases and, consequently, increasing the content of total soluble amino acids in order to regulate intracellular osmotic potential and accelerate the recovery of plants under adverse conditions (LECHINOSKI *et al.*, 2007). In seedlings of *Moringa oleifera* Lam., Silva *et al.* (2017) found that amino acid accumulation was higher under salt stress at 120 mM of NaCl L⁻¹ at 15 days after sowing.

The progressive increase in proline content is a good biochemical indicator, as this demonstrates the plant's ability to invest in organic solutes inside the cell as a way of surviving stress situations. According to Lehmann *et al.* (2010), the increase in proline is related to the relief of post stress, in which there is rapid breakdown of proline, enabling reducing agents, which participate in mitochondrial oxidative phosphorylation, to produce ATP for recovery and restoration of stress-induced damage.

The increase of proline due to the increase in the level of salinity is considered common, because this metabolite is directly related to plant stress, as evidenced by Cunha *et al.* (2013) with *Jatropha curcas* L., Monteiro *et al.* (2014) with *Cajanus cajan* (L.) Millsp. and Nascimento *et al.* (2015) with *Hymenaea courbaril* L. This result has been recently observed by Hesami *et al.* (2020) with *Ziziphus spina-christi* (L.) Willd seedlings after 10 months of cultivation under EC_w of 1.8, 5.8, 10.2 and 16.2 dS. m⁻¹. These authors verified the significant decrease in the growth of all vegetative parameters, while the proline content was increased with the increase of salt concentration in the soil.

Irrigation with salinized water at different levels caused reduction in leaf starch contents. There was a greater reduction of starch at the EC_w of 8 dS.m⁻¹, equal to 57% in comparison to the control plants (0.3 dS.m⁻¹) (Figure 3 C). The decrease in biomass, especially leaf biomass, resulted in lower carbon assimilation in the leaves as a function of the increase in stress. According to Mitchell *et al.* (2013), as a way to protect the photosynthetic apparatus from the accumulation of reactive oxygen species and provide carbon for the maintenance of cellular processes, there is consumption of starch (raffinose and sorbitol), and accumulation and/or maintenance of soluble sugars, as verified in the present study. Corroborating these results, Dietze *et al.* (2014) claim that starch synthesis and degradation are controlled by interdependent regulatory networks that ultimately make it possible to balance carbon supply via photosynthesis with the use of carbon for other metabolic activities.

The contents of soluble sugars in the leaves of *E. contortisiliquum* under salinized waters gradually increased from 2 dS.m⁻¹, with 1.2 mg g⁻¹ fresh mass, followed by accumulation of 0.1 mg g⁻¹ per increase in EC, and culminating with 1.5 mg g⁻¹ of fresh mass

at the highest EC_w (8 dS.m⁻¹) (Figure 3 D). The increase in the biosynthesis of total soluble sugars in leaves occurs under a condition of low water potential in the soil and due to the osmotic effect of the salt, leading to the greater establishment of this carbohydrate. Thus, hexoses released from sucrose hydrolysis can be used in anabolic or catabolic processes, as well as providing reducing sugars for the osmotic adjustment process (CHAVES FILHO; STACCIARINI-SERAPHIN, 2001). According to these authors, this is because in most plants, sucrose is the main sugar exported from the sites of synthesis (leaves) to the regions of consumption (stem, vegetative buds, roots), where it will be used for growth and/or storage.

The results of soluble sugar contents obtained in the present study are consistent with those found by Silveira *et al.* (2015) in seedlings of *Sebastiania membranifolia* Müll. Arg. The authors verified that the contents of this carbohydrate in plants under flood stress increased linearly and were significantly higher than those of the control in the period of 12 and 36 days. In addition, in the partial immersion of plants, there was an increase in the consumption of carbohydrates, expressed by the higher values of total soluble sugars and starch degradation.

CONCLUSIONS

The gradual increase in irrigation water salinity is detrimental to the growth and dry mass accumulation of *E. contortisiliquum* plants, with decrease from the threshold of 2 dS.m⁻¹.

The biochemical mechanism of tolerance to salt stress occurs by the accumulation of amino acids, proline and total sugars, concomitantly with the consumption of starch reserves.

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Chapter 2

CHAPTER 2

MORPHOPHYSIOLOGICAL AND BIOCHEMICAL CHANGES IN *Enterolobium contortisiliquum* (Vellozo) Morong SEEDLINGS UNDER WATER DEFICIT

**Morphophysiological and biochemical changes in *Enterolobium contortisiliquum*
(Vellozo) Morong seedlings under water deficit**

ABSTRACT: Water stress is one of the main factors that limit plant development in semi-arid regions. Thus, the objective was to evaluate the morphophysiological and biochemical changes in *E. contortisiliquum* seedlings subjected to different periods of water deficit. The study was conducted at the Federal University of Rio Grande do Norte, Macaíba, RN, Brasil. The experimental design used was randomized blocks, with four replicates, with 20 plants per plot. The treatments consisted of different periods of water deficit imposed to the seedlings: 4, 8, 12 and 16 days, with plants in the control treatment being irrigated daily. After these periods, the seedlings were subjected to the following evaluations: plant height, number of leaves, leaf area, stem diameter, Dickson quality index, plant height to shoot dry mass ratio, root length and total dry mass, in addition to biochemical variables, amino acid and starch contents. The data were subjected to analysis of variance and, in case of significance, subjected to polynomial regression analysis. Water deficit affects the initial development and metabolic processes of *E. contortisiliquum*, which indicates that this species has an osmotic adjustment mechanism, under water stress conditions.

Key words: Caatinga, forest species, water unavailability, organic solutes

Alterações morfofisiológicas e bioquímicas em mudas de *Enterolobium contortisiliquum* (Vellozo) Morong sob déficit hídrico

RESUMO: O estresse hídrico é um dos principais fatores que limitam desenvolvimento vegetal, sobretudo em regiões semiáridas. Com isso, objetivou-se avaliar as alterações morfofisiológicas e bioquímicas em mudas de *E. contortisiliquum* submetidas a diferentes períodos de déficit hídrico. A pesquisa foi conduzida na Universidade Federal do Rio Grande do Norte, Macaíba, RN, Brasil, em delineamento experimental em blocos casualizados, com quatro repetições, sendo 20 plantas por parcela. Os tratamentos foram constituindo de diferentes períodos de submissão das mudas ao déficit hídrico: 4, 8, 12 e 16 dias, sendo as do tratamento controle irrigadas diariamente. Após esses períodos, as mudas foram submetidas às seguintes avaliações: altura de plantas, número de folhas, área foliar, diâmetro do caule, índice de qualidade de Dickson, relação altura de planta e massa seca da parte aérea, comprimento da raiz e massa seca total, bem como as determinações bioquímicas de teor de aminoácidos e amido. Os dados foram submetidos à análise de variância e, em caso de significância, submetidos à análise de regressão polinomial. Mudas de *E. contortisiliquum* obtiveram crescimento inicial maior em ambiente com disponibilidade de água. O período de quatro dias de estresse hídrico é o limite para produção de mudas de *E. contortisiliquum* com melhor qualidade. Os solutos osmoticamente ativos atuam para minimizar os danos provocados pelo aumento do estresse hídrico, cujos mecanismos podem ser os principais determinantes utilizados pelas plantas nesta condição.

Palavras-chave: Fabaceae, Caatinga, Déficit hídrico, Osmorregulação

INTRODUCTION

Enterolobium contortisiliquum (Vell.) Morong, Fabaceae family, is known in Portuguese as 'tamboril', 'timbaúba' or 'orelha de macaco' and can be used for purposes such as toy making, handicrafts, civil construction, packaging and crates (Lorenzi, 2016). In addition, this species has a natural occurrence throughout the Caatinga biome, constituting an excellent indication for reforestation of degraded areas, mainly due to its rapid initial growth (Araújo; Paiva Sobrinho, 2011).

The Caatinga is quite diverse in terms of landscape and vegetation, whose area of 734,478 km² has been progressively affected by deforestation, associated with the natural phenomenon of drought (Oliveira et al., 2017). Despite that, some plant species maintain strategies for growth and survival under water stress conditions, whose occurrence is considered critical at the initial stage of growth for most plants (Lúcio et al., 2017).

When exposed to low water availability, the metabolic balance of the cell of some plant species may show rapid osmotic adjustment, assisting in the action of mechanisms of tolerance to this adverse condition (Moura et al., 2016). Therefore, investigations are needed to elucidate these mechanisms of tolerance to water stress aiming at plant survival, such as metabolic responses and abilities to perceive extreme environmental signs. Thus, studies have been carried out with different tree species under water stress conditions, such as those of Costa et al. (2015) with 'aroreira' (*Myracrodruon urundeuva* Allemão), França et al. (2017) with seedlings of 'guanandi' (*Calophyllum brasiliense* Cambess), Lúcio et al. (2017) with 'mulungu' (*Erythrina velutina* Willd.) and 'tamboril' (*Enterolobium contortisiliquum* (Vell.) Morong), Alves et al. (2018) with 'mororó' (*Bauhinia divaricata* L.) and Campelo et al. (2018) with the woody species 'Gonçalo-Alves' (*Astronium fraxinifolium* Schott ex Spreng), 'guanandi' (*Calophyllum brasiliense* Cambess.), 'ipê-amarelo' (*Handroanthus serratifolius* (Vahl.)), 'ipê-roxo' (*Handroanthus impetiginosa* (Mart.) Matos), 'marupá' (*Simarouba amara* Aubl.) and mahogany (*Swietenia macrophylla* King.). These studies highlight the strategic difference of each species in the fight against water stress but emphasize that the gradual increase of soil water deficit causes changes in physiological processes, culminating in stoppage of development at a certain level of stress.

In view of the above, the objective was to evaluate the morphophysiological and biochemical changes in *E. contortisiliquum* seedlings when subjected to different periods of water deficit.

MATERIAL AND METHODS

The experiment was conducted in laboratory and greenhouse at the Federal University of Rio Grande do Norte (UFRN), Macaíba, RN, Brazil (5°53'10.0" S, 35°21'52.5" W and 56 m altitude) in the first half of 2019. The climate of the region is semi-arid, with dry summer and rainy season in the period from March to July, with little occurrence in the other months of the year. The seeds were collected from parent trees located in the municipality of Petrolina, PE (9°19'10.47" S, 40°33'48.91" W and average altitude of 375 m) and donated by the Federal University of the São Francisco Valley (UNIVASF), Petrolina, PE.

Prior to sowing, the seeds were scarified in the region opposite to the embryonic axis with an abrasive sandpaper no. 100. Then, two seeds were sown per polyethylene container (24 cm x 15 cm) at the depth 2 cm. The substrate used was a mixture of compost of subsoil and aged bovine manure (1:1 proportion), which were irrigated daily in quantity that ensured the saturation of the substrate. The chemical analysis of the substrate was performed by the Laboratory of Soil Fertility and Plant Nutrition of the Federal University of the Semi-Arid (UFERSA), which indicated the following characteristics: pH = 7.5; Ca = 2.6 cmolc dm⁻³; Mg

= 1.3 cmolc dm⁻³; Na = 1.66 cmolc dm⁻³; K = 2.7 cmolc dm⁻³; N = 1.89 g kg⁻¹, P = 241.1 mg dm⁻³, EC = 1.36 (dS.m⁻¹), ESP = 20%.

At 15 days after sowing, thinning was performed, leaving only one plant per container and maintaining irrigation daily for more 15 days. Then, with the seedlings formed and uniform (30 days after sowing), the treatments consisting of water restriction periods of 4, 8, 12 and 16 days, with daily irrigation only for control treatment, began to be applied.

The experimental period lasted 46 days, with a randomized block design, composed of five treatments and four replicates, and the experimental plot consisted of twenty plants.

The morphophysiological evaluations of plant height, number of leaves and stem diameter were performed twice, every seven days, for 15 days. Plant height was obtained using a millimeter ruler (cm), measured from the base of the stem to the insertion of the last leaf, and stem diameter was measured with a digital caliper. The counting of the number of leaves of each plant per treatment considered those that were fully expanded.

Before the destruction of the plant material, leaf area was determined using 10 leaflets per plant randomly collected in the morning. These were placed in white polyethylene containers and immediately measured according to the method of linear dimensions (Equation 1), using a digital caliper (0.01 mm).

Equation 1: $A = L.W$, expressed in (cm²), where: L = length in cm; W = width in cm (median position).

Plants were separated into stem, leaf and root at 46 days, and root length was measured using a millimeter ruler (cm).

The total dry mass of the plants of each treatment was placed in paper bags and dried in an oven at 65 °C until reaching constant weight.

Seedling quality was evaluated based on the Dickson Quality Index (DQI) (Dickson et al., 1960), according to equation 2:

$$\text{Equation 2: } DQI = TDM \div \left[\frac{H}{D} + \frac{SDM}{RDM} \right]$$

In what: TDM = total dry mass (g); H = shoot height (cm); D = stem diameter (mm); RDM = root dry mass (g); SDM = shoot dry mass (g).

For biochemical evaluations, leaf material was stored in an ultra-freezer and the amino acid and starch contents were subsequently determined. For amino acids, the ninhydrin method was used, with glycine as the standard (Yemm et al., 1955). The concentrations were expressed in mmol of amino acids g⁻¹ fresh mass.

Starch content was quantified according to Morris (1948) and Yemm & Willis (1954), using the anthrone reagent and the reference curve from known concentrations of glucose, expressed in mg g⁻¹ of fresh mass.

The data were subjected to analysis of variance and polynomial regression, testing the mathematical models. The model that showed significant difference by the F test at 5% probability level was adopted and, in case of significance, the response with best biological expression was considered. Statistical analysis was performed using the statistical program Agroestat® (Barbosa; Maldonado, 2010).

RESULTS AND DISCUSSION

According to the analysis of variance, there were significant effects at 1% probability level for plant height, number of leaves, leaf area, stem diameter, Dickson quality index, root length and total dry mass. It was also found that the height/shoot dry mass ratio was significantly affected at 5% probability level, indicating that the different periods of water deficit interfered with the morphological characteristics of *E. contortisiliquum* seedlings (Table 1).

Table 1. Mean squares for plant height (H), stem diameter (SD), number of leaves (NL), leaf area (LA), Dickson quality index (DQI), root length (RL), plant height/shoot dry mass ratio (H/SDM) and total dry mass (TDM) of *Enterolobium contortisiliquum* (Vell.) Morong seedlings under water deficit.

S. V.	DF	H	NL	LA	SD	DQI	H/SDM	RL	TDM
Periods	4	42.20**	5.03**	0.15**	0.17**	0.15**	1.43*	34.21**	33.75**
Blocks	3	1.05	0.29	0.00	0.00	0.00	0.56	4.84	4.13
Residual	12	3.39	0.15	0.00	0.00	0.01	0.34	7.58	4.41
CV (%)	–	6.39	6.41	12.30	2.65	19.77	13.69	18.15	22.88

S. V: Source of variation; *, **: respectively significant at 5% and 1% probability levels by F test

In response to water deficit, there was a progressive decrease in plant height. The treatment without water restriction resulted in seedlings with an average height of 33 cm, while the water restriction for four days resulted in a reduction of 3 cm, with an average reduction of 9%. Plant height showed a gradual linear decrease with the increase in water restriction, with the period of 16 days without irrigation leading to mean height values close to 25 cm, i.e., an average reduction of 25.5% in height between 0 and 16 days without irrigation (Figure 1 A).

The greatest lengths observed in plants without water restriction, with subsequent decrease as the restriction increased, are related to the guarantee of water availability to the

roots. In addition, there is the organic increment of the substrate used, which enhances its moisture retention capacity, favoring the absorption and distribution of water to the plant. This is evidenced by Campelo et al. (2018), who stated that water stress reduces the synthesis in protein metabolism and causes the interruption of cell division, affecting the speed of the mitotic process and, thus, extension growth of plants. In this context, plants that develop under condition of deficit water supply exhibit morphological, physiological and anatomical adaptations to maintain functional metabolism (Larcher, 2006). Water is very important for the photosynthetic metabolism. Plants with restriction of this resource tend to close their stomata to avoid dehydration and CO₂ absorption is thereby limited, hampering biomass production.

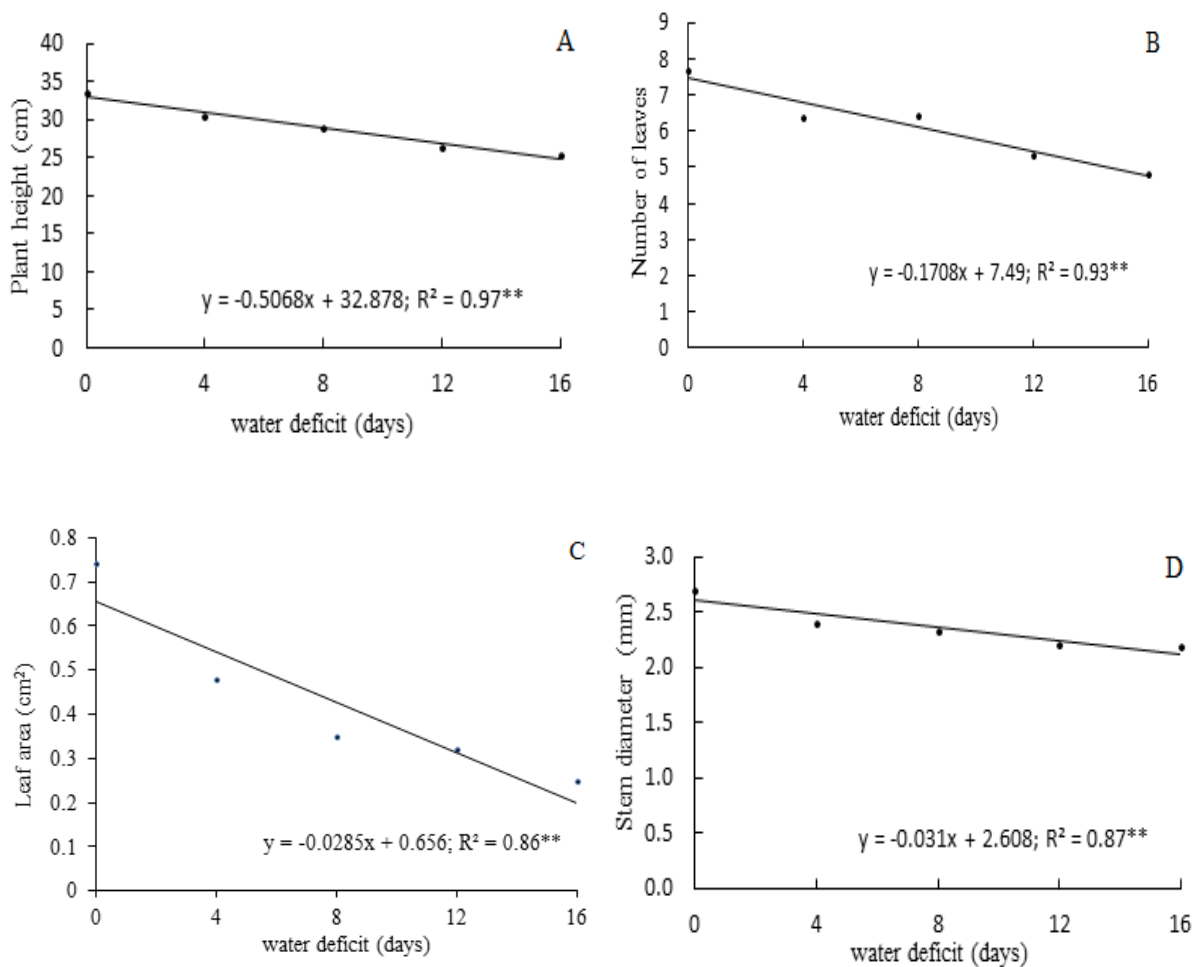


Figure 1. Plant height (A), number of leaves (B), leaf area (C), stem diameter (D) of *Enterolobium contortisiliquum* (Vell.) Morong seedlings subjected to different periods of water deficit.

In a study similar to this, in *Jatropha curcas* L. seedlings, Padilha et al. (2016) applied water regimes of 20, 40, 60, 80 and 100% of soil water retention capacity (SWRC) in

Latossolo Vermelho distrófico (Oxisol) in Dourados, MS. These authors found that 20% SWRC caused limitation in plant height growth throughout the evaluations, while the treatments of 60 and 100% SWRC led to the highest values of plant height. Thus, the results observed in *J. curcas* corroborate those found in *E. contortisiliquum*, associating the reduction of growth with the water deficit. Similarly, Tognon (2010) also observed that this is the main symptom of the reduction in water availability, because it is directly linked to cell expansion and elongation, since this only occurs due to cellular turgor promoted by water availability and that the restriction causes the stoppage of these physiological processes in the plant. According to Alves et al. (2018), the reduction of growth was one of the main symptoms of water deficit in *Bauhinia divaricata* L. seedlings under water regime of 80% of field capacity (FC). These seedlings grew satisfactorily up to 40% FC, entering into a state of water stress in regimes lower than this.

The number of leaves per seedling was inversely proportional to the periods in which the plants remained subjected to water deficit. It was verified that *E. contortisiliquum* seedlings under daily irrigation had on average 7.4 leaves, while seedlings subjected to water restriction for 16 days had, on average, 4.6 leaves, thus representing a reduction of 36.9%. For the periods of 4, 8 and 12 days without irrigation, the average values were 6.7, 6.0 and 5.3 leaves per seedling (Figure 1B). In this context, it is verified that the intensity of water stress causes marked leaf senescence and restriction to the appearance of new leaves (Padilha et al., 2016).

Two common species of the Caatinga ecosystem (*Erythrina velutina* Willd. and *Enterolobium contortisiliquum* (Vell.) Morong) were subjected to regimens of 450 (control), 225 (moderate stress) and 112.5 mm (severe stress) at 20 and 40 days after the start of treatments (DAT) (Lúcio et al., 2017). These authors found that at 20 DAT there was no effect of water stress on the leaves of *E. contortisiliquum* and, for *E. velutina*, the reduction was observed only under severe conditions and, at 40 DAT, the reduction in the number of leaflets occurred in both species. The results obtained for the number of leaves corroborate those obtained by Alves et al. (2018), with *B. divaricata*, who found that the production of new leaves became slower only under severe water stress, in the treatment of 20% field capacity.

Reduction in the number of leaves was proportional to the increase in water deficit, directly affecting the dimensions of the leaflets. The four-day period without water resulted in leaf area of 0.5 cm², being even more severe in plants subjected to 16 days of water stress (0.3 cm²), with mean values of 0.2 cm². The initial treatment, without water restriction, led to values around 0.68 cm². By comparing the estimated values between the shortest and longest period of water deficit, it was observed that leaf reduction was 70.5% (Figure 1 C). This trend was also verified by França et al. (2017) in *Calophyllum brasiliense* Cambess seedlings because, as a strategy to tolerate water stress, they decrease leaf water potential,

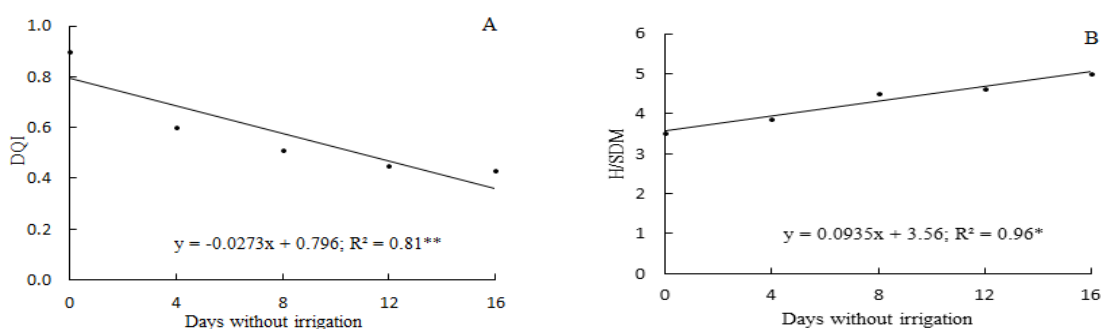
regulate stomatal opening, and thereby reduce photosynthesis and transpiration. In agreement with the findings of Campelo et al. (2018), the balance between CO₂ assimilation and leaf water potential is an important factor that expresses the direct relationship between photosynthesis and yield.

For stem diameter (SD), there was a linear reduction with the increase in the water deficit period from the initial treatment. Plants cultivated without water restriction showed mean SD of 2.6 mm, while seedlings subjected to the longest period without irrigation had lower SD (2.1 mm), thus corresponding to a reduction of 19.2% (Figure 1 D).

This type of response indicates that stem diameter may vary according to the availability of water in the soil to which the plant is subjected. Water imbalance alters the highly organized structures of the plant cell, affecting the growth of the vascular cambium, which is responsible for the formation of new layers of phloem and xylem and, consequently, increase in stem diameter (Larcher, 2006).

The reduction of available water in the soil limited the growth in the stem diameter of *B. divaricata* in a study similar to this one conducted by Alves et al. (2018). According to these authors, plants in the control treatment (100% FC) obtained the highest values of stem diameter, followed by those in the treatments 80, 60, 40% FC, while plants subjected to 20% FC showed a reduction of 54.51% when compared to those of the initial treatment. Results found by Silva et al. (2019), evaluating the initial vegetative growth of the forest essences of *Khaya ivorensis*, *Tuberous spondias*, *Eucalyptus urophylla* x *Eucalyptus grandis*, *Toona ciliata* and *Anadenanthera colubrina*, subjected to irrigation depths of 10, 50, 100 and 150% FC based on the estimated evapotranspiration, found that the lowest irrigation depth caused reductions in stem diameter of about 67, 73, 76, 78 and 85% for these species, respectively, compared to the highest depth applied.

The highest value of the Dickson quality index (DQI) was obtained in the absence of water deficit, with gradual reduction of seedling quality as the periods without irrigation increased. It was verified for the shorter period of water deficit (four days) that the quality of *E. contortisiliquum* seedlings was considerably higher than the values obtained in subsequent treatments (Figure 2 A). Thus, the DQI represented the quality of seedlings, since this index correlates with relevant characteristics of vigor and phytomass production (Freitas et al., 2017).



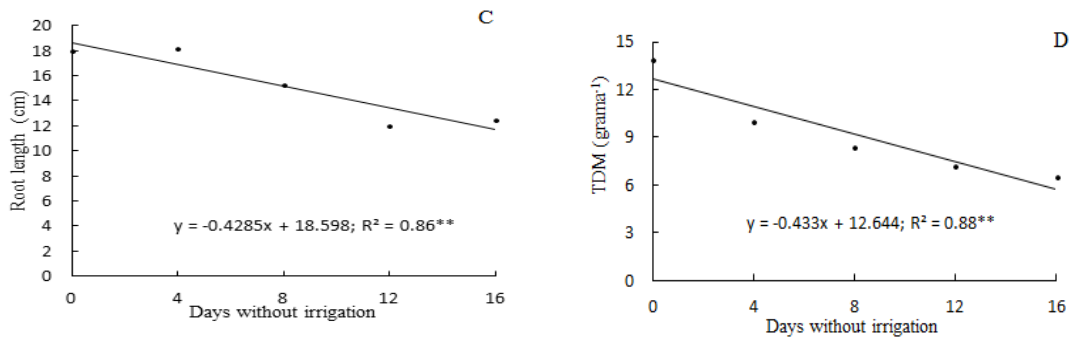


Figure 2. Dickson quality index (A), H/SDM ratio (B), root length (C) and total dry mass (D) of *Enterolobium contortisiliquum* (Vell.) Morong seedlings subjected to different periods of water deficit.

Reduction in the values of Dickson quality index as a function of water deficit were also verified in seedlings of *Peltophorum dubium* (Sprengel) Taubert., whose quality gradually decreased, according to the decrease in soil water availability (Carvalho et al., 2015).

For the H/SDM ratio, the lower the value of this allometric ratio, the more lignified the seedling will be and the greater its survival capacity in the field should be (Gomes; Paiva, 2013). Seedlings of *E. contortisiliquum* show a linear increase with the increase in the periods of water deficit and, in the absence of water deficit, there was a lower H/SDM ratio (Figure 2 B).

Root growth (cm) was linearly affected with the increase in water deficit, so that the highest values occurred in the absence of water restriction (18.6 cm) and decreased considerably in seedlings subjected to the 16-day water restriction period (11.7 cm), corresponding to a total reduction of 36.8%. It is also observed that plants subjected to a period of four days without irrigation had an average length of 16.9 cm, a value close to that obtained for the control treatment, with a loss of 9.2% (Figure 2C).

It can be noted that the roots of plants subjected to a period of four days without irrigation, had an average length around 18 cm, similar to the value obtained for the control treatment. Thus, it is possible to indicate tolerance of this species to this period without irrigation, showing a minimization of water stress, since both treatments promoted a more developed root system. Root architecture and its ability to explore the deepest and wettest layers of the soil are important characteristics to escape from water deficits (Scalon et al., 2011). The biometric parameter of the root length of *B. divaricata* was only affected under field capacity of 20%, with a reduction of 51.8% in comparison to the control (Alves et al., 2018).

For the total dry mass (TDM), there was reduction with the increase in the non-irrigated periods (Figure 2 D). The highest dry mass accumulation was observed in plants of the treatment without water restriction (12.6 g plant⁻¹), while the lowest TDM values were obtained for a period of 16 days (5.7 g plant⁻¹).

It is inferred that the greater phytomass in *E. contortisiliquum* seedlings comes from the better water advantage, since there was a decreasing change in dry mass production under intense stress, affecting root morphology. When the protoplasm dehydrates due to water deficit, photosynthetic activity decreases along with cell volume (Larcher, 2006). Research conducted by Alves et al. (2018) confirmed that the biomass of *B. divaricata* decreased with the decrease in FC, from 11.16 g at 100% FC to 9.72, 8.88, 5.67 g at 80.60 and 40% FC, respectively. The biomass of plants under 20% FC was further reduced.

The result of the analysis of variance for amino acid and starch contents in fresh leaf tissue showed significant effects at 5% and 1% probability levels, respectively, indicating that the water deficit interferes in the contents of these components in the plant (Table 2).

Table 2. Summary of the analysis of variance for the quantification of amino acids and starch in leaves of *Enterolobium contortisiliquum* (Vell.) Morong seedlings under water deficit.

Source of variation	Degrees of freedom	Amino acids	Starch
Periods	4	68.95*	0.18**
Blocks	3	30.71	0.01
Residual	12	20.67	0.02
CV (%)	-	9.60	12.5
Mean	-	47.34	1.25

Quantitative data of starch statistically transformed $\sqrt{(x+k)}$, where x represents the variable and K= 0. Significant at 1% and 5% probability levels by F test. *, **, respectively.

Amino acid contents increased linearly in response to the increase in the water restriction period in *E. contortisiliquum* seedlings, so that the period of 16 days without irrigation resulted in higher accumulation of amino acids in the fresh leaf mass (52.0 mmol g⁻¹ FM), while the absence of water stress led to lower concentration of amino acids (42.6 mmol g⁻¹ FM), representing an increase of 22.0% (Figure 3A).

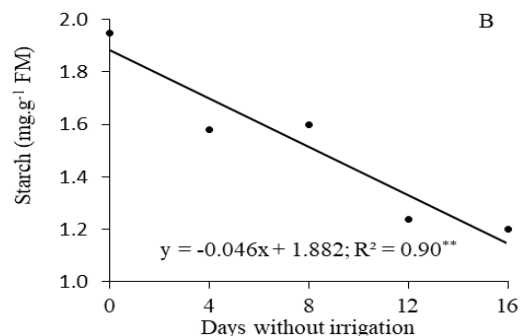
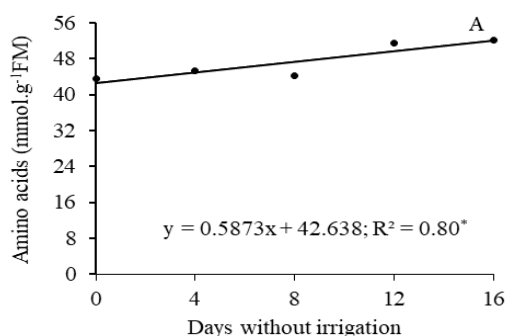


Figure 3. Amino acid (A) and starch (B) contents in the leaves of *Enterolobium contortisiliquum* (Vell.) Morong seedlings subjected to different periods of water deficit.

Regarding the defense mechanisms of plants under water deficit conditions, there are usually changes in metabolic behavior, increasing the activity of proteolytic enzymes, and transformation of proteins into amino acids, which accumulate by synthesis or degradation of other organic compounds, reducing the damage caused by water scarcity (Silva et al., 2013). Several studies have demonstrated accumulation of osmotically active solutes caused by water deficit (Dichio et al., 2009; Nio et al., 2011), and the amount and type of solute accumulated directly depend on the plant species and on the duration of the deficit period. In this context, plants of *Myracrodruon urundeuva* Allemão were subjected to 12 days of water deficit and there was a progressive increase in leaf concentrations of total amino acids from the sixth day (Costa et al., 2015). According to these authors, on the last day of deficit the values obtained under this condition were 3.2 times higher than those of plants in the control treatment.

Absence of water for four days in the plants resulted in starch concentration of 1.6 mg. g⁻¹ FM, while in those subjected to water stress for 16 days, this concentration was 1.1 mg. g⁻¹ FM, representing a reduction of 31.25% between periods. The reduction of this carbohydrate in leaves when compared to control plants may have contributed to the osmotic adjustment of tissues, as a result of immediate starch consumption, functioning as a strategy to escape from water stress (Anderson; Kohorn, 2001). This result emphasizes that osmotically active metabolites are involved in the drought tolerance mechanism, as highlighted by Nascimento et al. (2019) in young plants of *Hevea brasiliensis* Willd. Ex A. Juss. Müll. Arg., which showed reduction in starch in contrast to the increase in the concentration of soluble sugar. These same authors also report that the consumption of starch and accumulation or maintenance of soluble sugar act by providing carbon for the maintenance of cellular processes and survival. These findings were observed by Mitchell et al. (2013), analyzing hydraulic dysfunction and carbohydrate depletion during the mortality of trees.

CONCLUSIONS

1. *E. contortisiliquum* seedlings obtain higher initial growth in environment with water restriction. The four-day period of water stress is the maximum limit for the production of *E. contortisiliquum* seedlings with good quality.

2. The increase of amino acid and decrease of starch contributed to osmoregulation and tolerance to water deficit.

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ANNEX 1 - Rules of NEW FORESTS (Chapters 1 and 2)

INSTRUCTIONS FOR AUTHORS

General

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All manuscripts must be submitted in standard English. It is the responsibility of the author to assure the manuscript is written in standard English. Manuscript submitted in poor quality and non-standard English will be rejected without review.

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Manuscript Submission

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- A clear indication and an active e-mail address of the corresponding author
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Trial registration number, date of registration followed by “retrospectively registered”

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Funding (information that explains whether and by whom the research was supported)

Conflicts of interest/Competing interests (include appropriate disclosures)

Availability of data and material (data transparency)

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Authors' contributions (optional: please review the submission guidelines from the journal whether statements are mandatory)

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Ethics approval (include appropriate approvals or waivers)

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References

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Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 1998; Medvec et al. 1999, 2000).

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Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. *Eur J Appl Physiol* 105:731-738. <https://doi.org/10.1007/s00421-008-0955-8>

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. *N Engl J Med* 341:325–329

- Article by DOI

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- Book

South J, Blass B (2001) *The future of modern genomics.* Blackwell, London

- Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) *The rise of modern genomics*, 3rd edn. Wiley, New York, pp 230-257

- Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. <http://physicsweb.org/articles/news/11/6/16/1>. Accessed 26 June 2007

- Dissertation

Trent JW (1975) *Experimental acute renal failure.* Dissertation, University of California

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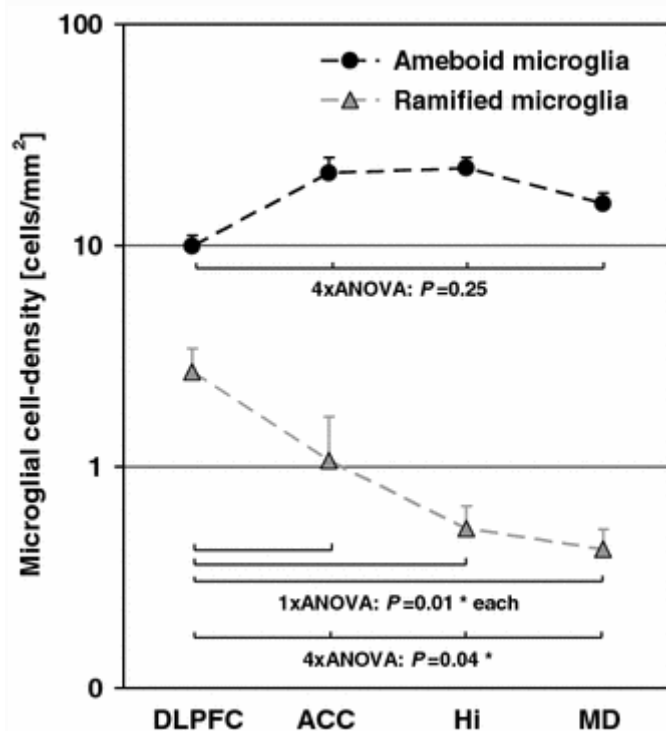
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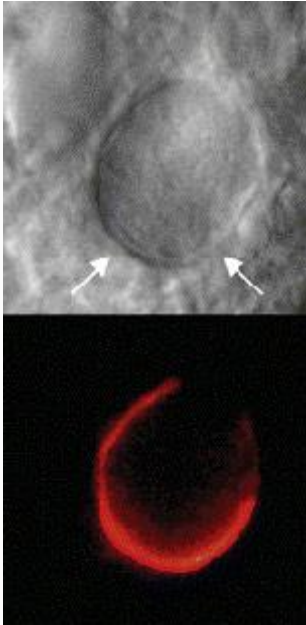
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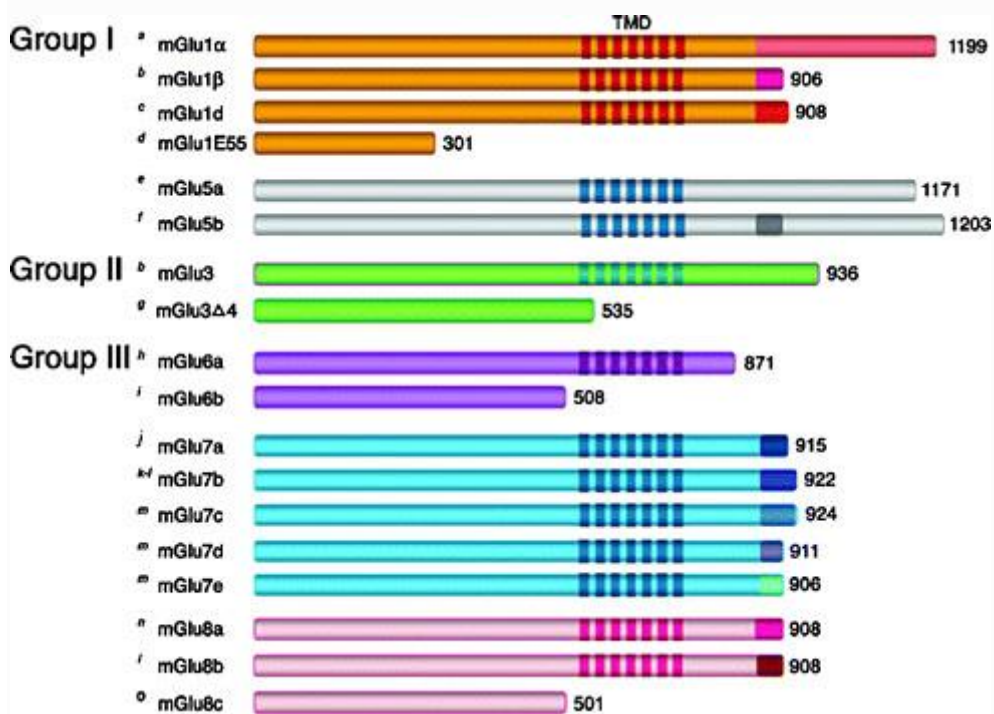
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