TOLERANCE MECHANISMS IN *Hymenaea courbaril* L. AND *Jatropha curcas* L. plants AS A RESPONSE TO WATER DEFICIT AND CONTAMINATION BY OIL DERIVATIVES

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ABSTRACT – Accidents that occur during the transport of oil and its derivatives have been one of the primary causes of environmental pollution in recent decades. Studies on changes in plant physiology caused by these pollutants are scarce, and the effects on plant metabolism are little known. As such, the aim of the present study was to analyze the effect of soil contaminated with diesel fuel on the physiology of young *Hymenaea courbaril* L. and *Jatropha curcas* L. plants. The following variables were analyzed: gas exchanges, photosynthetic pigments, chlorophyll index (SPAD) and protoplasmic damage. The treatments used were: T0 – control (100% of maximum soil water holding capacity – contamination-free soil), T1 and T2 (50% of maximum soil water holding capacity + addition of 23 mL and 46 mL of diesel fuel, respectively) and two assessment times (3h and 192h after contamination). Significant differences in gas exchanges were observed for both species 3h after diesel fuel application. For photosynthetic pigments and the chlorophyll index, increases were found 3h and 192 h after imposed stress, for both species. A significant rise in electrolyte leakage was observed, more pronounced in *J. curcas* plants after 192h, for treatments T1 and T2 compared to T0. These findings show the potential of species to acclimate themselves to adverse conditions, and should be considered in recovery programs for environments degraded by oil derivatives.

Keywords: Stomatal behavior, Ddiesel fuel; Soil contamination.

MECANISMOS DE TOLERÂNCIA EM PLANTAS DE Hymenaea courbaril L. E Jatropha curcas L. EM RESPOSTA AO DEFICIT HÍDRICO E CONTAMINAÇÃO POR DERIVADOS DE PETRÓLEO

RESUMO – Acidentes que ocorrem no processo de transporte do petróleo e de seus derivados têm sido um dos principais motivos de poluição ambiental nas últimas décadas. Pesquisas sobre alterações ocasionadas por estes poluentes na fisiologia de plantas ainda são escassos, sendo pouco conhecidos os efeitos ocasionados no metabolismo vegetal. Diante do exposto, o objetivo do presente estudo foi analisar o efeito do solo contaminado com óleo diesel na fisiologia de plantas jovens de **Hymenaea courbaril** *L. e* **Jatropha curcas** *L. As variáveis analisadas foram: trocas gasosas, pigmentos fotossintéticos, índice de clorofila (SPAD) e danos protoplasmáticos. Os tratamentos utilizados foram: T0 – controle (100% da capacidade máxima de retenção de água no solo – solo sem contaminação), T1 e T2 (50% da capacidade máxima de retenção de água no solo + adição de 23 mL e 46 mL de óleo diesel, respectivamente) e 2 épocas de avaliação (3h e 192h após contaminação). Diferenças significativas foram evidenciadas após 3h de aplicação do óleo diesel para as trocas gasosas para ambas as espécies. Para os pigmentos fotossintéticos e o índice de clorofila, foram verificados incrementos após 3h e 192h de imposição do estresse, para as duas espécies. Verificou-se elevados acréscimos para o* *percentual de vazamento de eletrólitos, sendo mais pronunciando nas plantas de* ***J. curcas****, após 192h, para os tratamentos T1 e T2 em relação ao tratamento T0. Estas observações evidenciam o potencial de aclimatação das espécies às condições adversas, devendo ser consideradas em programas de recuperação de ambientes degradados por contaminação com derivados de petróleo.*

Palavras-chave: Comportamento estomático; Óleo diesel; Contaminação do solo.

1. INTRODUCTION

The industrial revolution transformed the world, changing the economy, politics, society, culture and environment. Mankind progressed with new inventions and technologies and the resulting industrialization saw pollution increase, primarily from the burning of fossil fuels which continue to drive the world economy (Aguiar et al., 2012).

In the 20th century, the growth of the petrochemical sector saw an increase in oil production and its derivatives, including diesel fuel, widely used in modern industry for everything from propelling cars to energy generating units. However, repeated accidents during the transport of oil and its derivatives have been a major cause of environmental pollution in recent years (Araújo et al., 2015).

The oil industry engages in activities that pose a danger to the environment, since they contaminate a number of media (land, aquatic and atmospheric) with different organic compounds, triggering a set of transformations in their biota. An area impacted by these pollutants compromises the quality of the environment as a whole, restricting the use of soil and water resources (Rosa, 2006).

Furthermore, after contamination, plants can adsorb the contaminant (Tan et al., 2005), in addition to promoting changes in the physical and chemical properties of the soil (Penã-Castro et al., 2006), since soils contaminated with hydrocarbons lead to a lack of water, oxygen and nutrients (De Jong, 1980; Roy et al., 2003; Merkl et al., 2005), resulting in so-called combined or multiple stress.

Moreover, Njoku et al. (2008) warn against contamination by oil and its derivatives, since they have negative effects on ecosystems and contribute indirectly to compromising other parameters. This occurs when post-spill recovery is slow. With no vegetation, the surface of the soil is exposed to several agents, preventing the establishment of plant cover that protects the ecosystem.

In an attempt to control damage to nature, a number of decontamination methods for areas that have suffered or are suffering from contamination caused by oil and its derivatives are used, such as digging, incineration, extraction with solvent, and redox, among others.

Silva et al. (2014) studied strategies to recover areas contaminated by solid residues. These methods are ecologically appropriate when compared to existing physical and chemical techniques that pose a secondary contamination risk, thereby increasing the costs of reversing these polluting processes. For this reason, recent years have witnessed a shift to more economical methods that have less impact on the environment. In this respect, phytoremediation and bioremediation have emerged as promising alternatives to complement an array of financially viable environmental technologies (Balliana, 2015).

Mechanisms and techniques such as phytoremediation seek to minimize the impacts caused by these events. Numerous studies have been conducted in this area, but few involved *Hymenaea courbaril* L (jatoba). and *Jatropha curcas* L. (Barbados nut). This suggests that research on the performance of native species growing in diesel fuel-contaminated soil, from germination to plant development, will be of significant ecological importance, contributing to recovery plans through direct sowing.

Accordingly, the aim of the present study was to assess the effect of diesel fuel-contaminated soil on the physiology of young *Hymenaea courbaril* L. and *Jatropha curcas* L. plants submitted to water deficiency and different contamination times and the possible phytoremediation effect of these species in areas degraded by oil derivatives.

2. MATERIAL AND METHODS

The experiment was conducted in the greenhouse of the Laboratory of Plant Physiology, Department of Biology at the Federal Rural University of Pernambuco

(UFRPE). Seedlings used were from the species *Hymenaea courbaril* (jatoba) and *Jatropha curcas* (Barbados nut) originating from sexual propagation with seeds obtained in the municipality of Areia, located in the Brejo Paraibano microregion and the experimental campus of the Agrarian Science Center of the Federal University of Alagoas (UFAL), respectively. The soil used in the experiment was sandy-clayey-silt, classified as Dystrophic Yellow Argisol (Simões Neto et al., 2012) and was collected at the Advanced Campus of UFRPE – Experimental Sugarcane Station of Carpina (EECAC/ UFRPE), located in the municipality of Carpina, North Forest Zone of Pernambuco.

After seedlings germinated, they were selected for their health and uniform height, at two months old and approximately 22 cm high. They were then transferred to polyethylene pots, with a capacity for 3 kg of soil, one plant per pot. During the 60-day acclimation period, the pots were kept at capacity (100%), previously determined by the gravimetric method, according to methodology described by Souza et al. (2000). The amount of diesel fuel used was based on Rezende (2006) and Silva (2006), for both percentage of water retained in the soil (50% of Maximum Water Holding Capacity - MWHC) and the amount of oil per kg of soil (46.2 mL).

The experimental design was a factorial scheme, consisting of 2 species (*H courbaril* and *Jatropha curcas*), 3 treatments, namely T0 – control (100% of maximum soil water holding capacity – contamination-free soil), T1 (50% of maximum soil water holding capacity + addition of 23 mL of diesel fuel per kg of soil) and T2s (50% of maximum soil water holding capacity + 46.2 mL of diesel per kg of soil), in accordance with Li et al. (1997) and Muratova et al. (2003), and 2 assessment times (3 and 192h after soil contamination), with 5 repetitions per treatment. Assessment times complied with criteria for early and late stress, that is, when the plants have yet to exhibit visible symptoms and when they start to show visible signs of toxicity, respectively.

Air temperature (Tair ºC) and relative humidity (%RH) inside the greenhouse were measured daily. After treatments were differentiated, the mean Tair ºC at first (3h) and second collection (192h) between 11:00 and 13:00h were 37.26ºC and 39.24ºC, varying from 32.62ºC to 39.34ºC and 36.1ºC to 40.7ºC, respectively. The mean RH was 44% and 40%, varying from 38% to 57% and 36% to 48%, respectively. These high temperatures and low humidity reflect the high light intensity and evaporative demand.

Before the treatments were differentiated, the net photosynthesis rate was measured every 2h. After assessment, measurements were taken between 11:00h and 13:00h, the time of greatest stomatal opening (Figure 1), using a portable infrared CO_2 gas analyzer (IRGA, ADC LCi Pro, Hoddesdon, UK).

To assess gas exchange, net photosynthesis (A), transpiration (E) and stomatal conductance (gs) were analyzed in mature, fully expanded leaves, located in the upper third of the plants, using the portable infrared CO_2 analyzer described above.

The chlorophyll index of the leaves was measured 30 minutes before gas exchanges using the SPAD-502 portable chlorophyll meter (Minolta Camera Co, Osaka, Japan). Ten readings were carried out in the leaves used for gas exchanges.

These same leaves were collected to determine photosynthetic pigment contents. Readings were conducted in a spectrophotometer, at wavelengths of 662 nm, 645 nm and 470 nm (extractor: pure acetone), to determine chlorophyll *a*, *b* and carotenoids, respectively, according to methodology described by Lichtenthaler and Buschmann (2001).

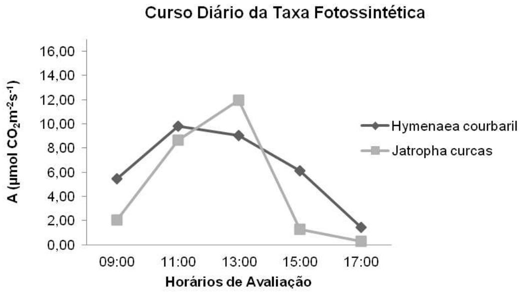

Figure 1 – Diurnal course of net photosynthetic rate of young plants of Jatobá (*Hymenaea courbaril* L.) and Pinhão-Manso (*Jatropha curcas* L.) grown in a greenhouse.

Figura 1 *– Curso diário da taxa de fotossíntese liquida de plantas jovens de jatobá (**Hymenaea courbaril** L.) e Pinhão-Manso (**Jatropha curcas** L.) cultivadas em casa de vegetação.*

The degree of membrane integrity was estimated by electrolyte leakage analysis, as per Alves et al. 2009, with changes. Although the authors used 100 mg of fresh mass, in this study 10 discs of leaf fresh mass with an area of 0.5 cm^2 were employed. The percentage of electrolyte leakage, proportional to plasma membrane damage, was estimated by the following formula:

%MD (C1/C2) x 100

where %MD is percentage membrane damage; C1 electrical conductivity of the extract (after incubation in a water bath at 25ºC for 24h) and C2 electrical conductivity of the extract (after incubation in a water bath at 100ºC for 1h).

The data obtained were submitted to analysis of variance (ANOVA) and the means compared by Tukey's test at 5% probability, using the Assistat 7.5 beta software (2008).

3. RESULTS

Analysis of gas exchanges for *H. courbaril* and *J. curcas* plants shows that after 3h of imposed stress, the net photosynthesis rate (A), transpiration (E) and stomatal conductance (gs) exhibited similar behavior, with significant increases in gas exchanges (A, E and gs) in treatments T1 and T2, for *H. courbaril* and *J. curcas*, of 127% and 169%, 96.24% and 190%, and 401.5% and 651.87%, respectively, in relation to controls (T0). Even though plants showed high rates for these variables in the first hours after stress application, stomatal closing was inevitable (stomatal conductance = 0 mol $H_2O.m^{-2}.s^{-1}$), occurring after 192h for the same treatments. The following values were obtained for the species *H. courbaril* and *J. curcas*: 0.1797 µmol of CO_2. $m^{-1}.s^{-1}$ and 0.4477 µmol of CO_2. $m^{-1}.s^{-1}$ and 0.6602 µmol of CO_2. $m^{-1}.s^{-1}$ and 0.3495 µmol of CO_2. $m^{-1}.s^{-1}$ for photosynthesis; 0.3395 mmol of $H_2O.m^{-1}.s^{-1}$ and 0.1764 mmol of $H_2O.m^{-1}.s^{-1}$ and 0.2016 mmol of $H_2O.m^{-1}.s^{-1}$ and 0.2155 mmol of $H_2O.m^{-1}.s^{-1}$ for transpiration, respectively (Figures 2A, B, C, D, E and F).

For the chlorophyll index, measured with a portable chlorophyll meter, significant differences were observed 3h and 192h after stress application (Figure 3). After 3h of exposure to diesel fuel, *H. courbaril* plants in treatment T1 showed the best mean chlorophyll index (49.03), while the lowest means (44.26 and 41.30) were found in treatments T0 and T2 (Figure B), a decline of approximately 10% and 16%, respectively, in relation to T1 plants. *J. curcas* plants exhibited no significant differences in the first hours after imposed stress (Figure 3A). The opposite was seen after 192h, when *H. courbaril* and *J. curcas* plants displayed significant increases in chlorophyll indices. For *H. courbaril*, a rise of 22.12% was found for treatment T2 compared to treatment T0 (Figure 3B). For *J. curcas*, significant differences were observed for treatment T1 (39.06) and T2 (39.10) in relation to treatment T0, which obtained the lowest mean (30.50) (Figure 3A).

After 3h of imposed stress, no significant differences in chlorophyll *a* were detected in any of the treatments. By contrast, after 192h *H. courbaril* plants exhibited a rise in chlorophyll *a* for treatment T2, with increases of around 150% in relation to treatment T0 (Figure 4B).

For chlorophyll *b* and total chlorophyll, after 3h of applied stress, only *H. courbaril* plants showed significant differences between treatments. Increases of 87.93% and 81.91%, respectively, were obtained for treatment T2 compared with treatment T0. After 192h *H. courbaril* and *J. curcas* exhibited a rise of 111.71% and 122.52% and 54.27% and 26%, respectively, for chlorophyll *b* and total chlorophyll for treatment 2 in relation to treatment T0 (Fig. 4C and D, E and F).

For carotenoids, significant differences were found for *H. courbaril*. These differences were observed after 3h and 192h of imposed stress, with increases of 142.20% and 81.64% for treatment T2, compared to treatment T0 (Fig. 4H). Comparison between times (3h and 192h) with respect to chlorophyll *a* (Figure 4A and B), chlorophyll *b* (Figure 4C and D), total chlorophyll (Figure 4E and F) and carotenoids (Figure 4G and H), showed a significant difference for treatment T2 in *H. courbaril* plants and for treatments T1 and T2 in *J. curcas*, which displayed a rise in chlorophyll contents of 62.54%, 38.01% and 45.54%, respectively in relation to the first assessment. Chlorophyll *b* and total chlorophyll behaved in a similar manner. Treatment T2 showed an increase of around 49.17% and 53.15 for *H. courbaril* and 57.26% and 78.29% and 44.13% and 55.70% for *J. curcas* in treatments T1 and T2, respectively. With respect to carotenoids, only *H. courbaril* exhibited significant differences for treatment times, with percentages of 97.34%, 55.82% and 48% for treatments T0, T1 and T2, respectively.

In regard to protoplasmic damage, after 3h of exposure to the contaminant, the species showed

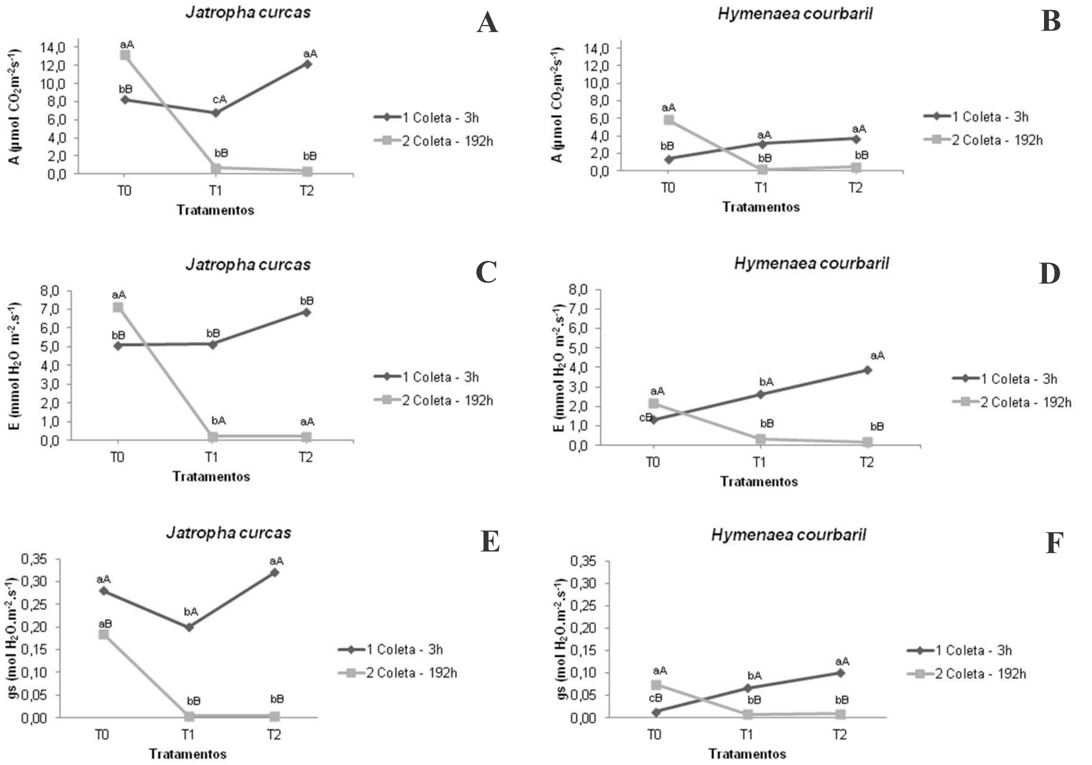

Figure 2 – Photosynthesis (E), transpiration (E) and stomatal conductance (gs) in seedlings Jatobá (***Hymenaea courbaril*** L.) and Pinhão-Manso (***Jatropha curcas*** L.) under different diesel oil concentrations in the soil. Equal letters, lower case compare treatments and upper case compare time of evaluate (3h e 192h), after contamination with diesel oil. Averages followed by the same letter do not differ by Tukey's test ($p<0.05$).

***Figura 2** – Fotossíntese (A), transpiração (E) e condutância estomática (gs) em mudas de jatobá (**Hymenaea courbaril** L.) e Pinhão-Manso (**Jatropha curcas** L) submetidas a diferentes concentrações de óleo diesel no solo. Letras iguais minúsculas comparam os tratamentos e letras iguais maiúsculas comparam as épocas de avaliação (3h e 192h) após contaminação com óleo diesel. Medias seguida de mesma letra não diferem entre si pelo teste Tukey (p<0.05).*

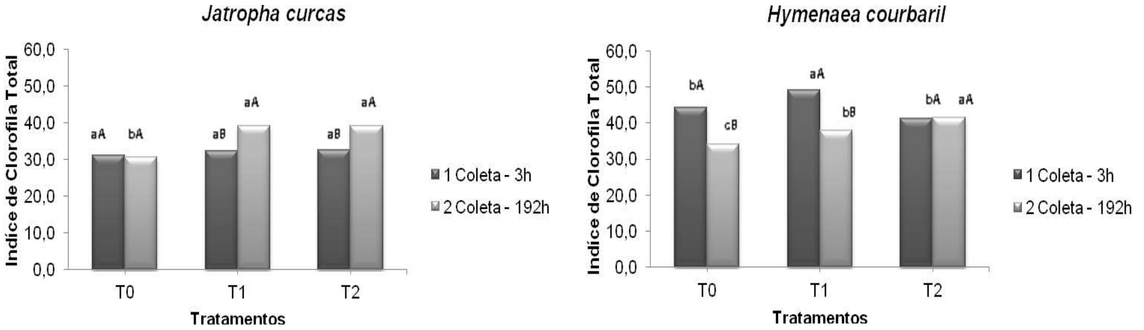

Figura 3 – Índice de clorofila SPAD (medidor portátil de clorofila-MPC) em mudas de Jatobá (*Hymenaea courbaril* L). e Pinhão-Manso (*Jatropha curcas* L.) submetidas a diferentes concentrações de óleo diesel no solo. Letras minúsculas e iguais comparam os tratamentos e letras maiúsculas e iguais comparam as épocas (3h e 192h) após contaminação com óleo diesel. Medias seguida de mesma letra não diferem entre si pelo teste Tukey ($p<0.05$).

***Figure 3** – Chlorophyll index SPAD (portable measuring chlorophyll-MPC) in seedlings Jatobá (**Hymenaea courbaril** L.) and Pinhão-Manso (**Jatropha curcas** L.) under different diesel oil concentrations in the soil. Equal letters, lower case compare treatments and upper case compare time of evaluate (3h e 192h), after contamination with diesel oil. Averages followed by the same letter do not differ by Tukey's test (p<0.05) .*

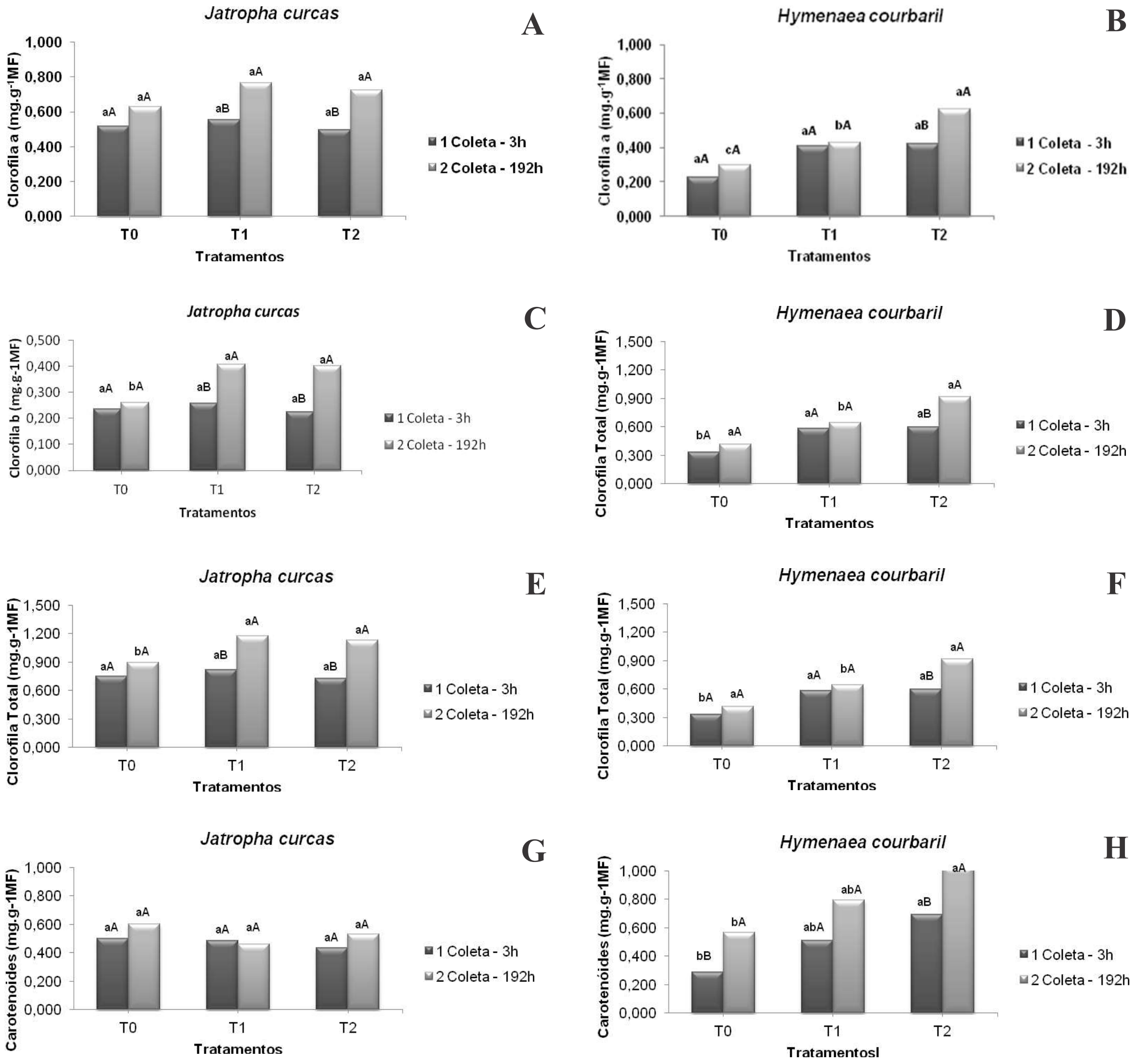

Figure 4 – Quantification of photosynthetic pigments in seedlings Jatobá (*Hymenaea courbaril* L.) and Pinhão-Manso (*Jatropha curcas* L.) under different diesel oil concentrations in the soil. in seedlings Jatobá (*Hymenaea courbaril* L.) and Pinhão-Manso (*Jatropha curcas* L.) under different diesel oil concentrations in the soil. Equal letters, lower case compare treatments and upper case compare time of evaluate (3h e 192h), after contamination with diesel oil. Averages followed by the same letter do not differ by Tukey's test ($p<0.05$).

***Figura 4** – Quantificação dos teores de pigmentos fotossintéticos em mudas de Jatobá (**Hymenaea courbaril** L.) e Pinhão-Manso (**Jatropha curcas** L.) submetidas a diferentes concentrações de óleo diesel no solo. Letras minúsculas e iguais comparam os tratamentos e letras maiúsculas e iguais comparam as épocas (3h e 192h) após contaminação com óleo diesel. Medias seguida de mesma letra não diferem entre si pelo teste Tukey ($p<0.05$).*

significant differences in percentage electrolyte leakage in relation to treatment T0, despite treatment T1 plants of both species displaying higher means, albeit not significant (Figure 5). With prolonged stress, statistical differences were only observed for *J. curcas* plants, which exhibited increases of 90.90% and 54.48% for treatments T1 and T2 compared to T0 (Figure 5A), respectively. When times (3h and 192h) were compared, the treatments showed a percentage rise in electrolyte leakage with prolonged stress of around 111.36% and

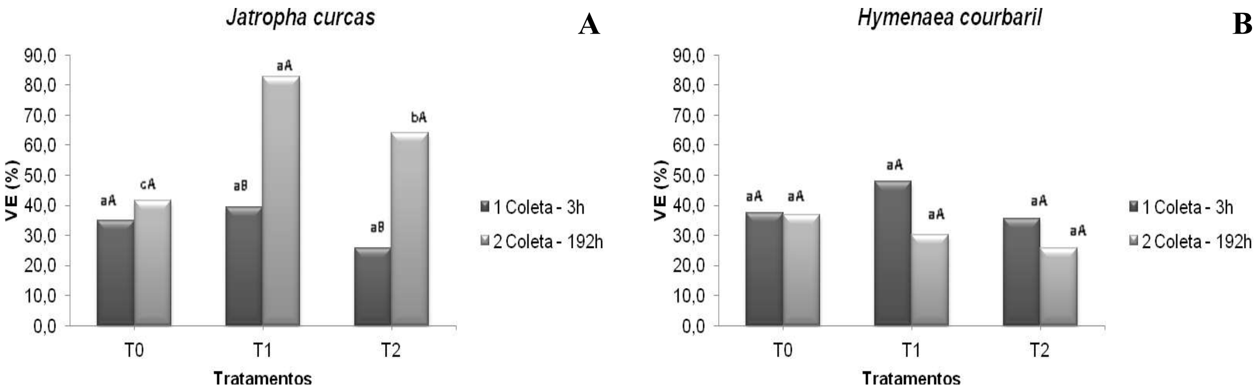

Figure 5 – Percentage of electrolyte leakage in seedlings Jatobá (*Hymenaea courbaril* L.) and Pinhão-Manso (*Jatropha curcas* L.) under different diesel oil concentrations in the soil. Equal letters, lower case compare treatments and upper case compare time of evaluate (3h e 192h), after contamination with diesel oil. Averages followed by the same letter do not differ by Tukey's test ($p<0.05$).

***Figura 5** – Percentual de vazamento de eletrólitos em mudas de Hymenaea courbaril L. (A) e Jatropha curcas L.(B) submetidas a diferentes concentrações de óleo diesel no solo. Letras minúsculas e iguais comparam os tratamentos e letras maiúsculas e iguais comparam as épocas (3h e 192h) após contaminação com óleo diesel. Medias seguida de mesma letra não diferem entre si pelo teste Tukey ($p<0.05$).*

149.45%, respectively, for *J. curcas* plants. An interesting fact is that *H. courbaril* plants, despite not differing statistically, showed higher electrolyte leakage percentages in the first 3h after application of diesel oil for treatment T1 (Figure 5B). With prolonged stress (192h), these values declined, showing a possible late tolerance mechanism in these plants (Figure 5B).

4. DISCUSSION

It is important to underscore that control plants (T0) showed significant reductions in gas exchanges compared to the other treatments at initial assessment. This reduction may be attributed to the climatic conditions such as temperature and relative humidity (37.26ºC and 44%), respectively, at the assessment times. According to Taiz and Zeiger (2013), in natural and cultivable conditions, plants are often exposed to climatic fluctuations. While temperature and humidity become stressful in a few minutes, factors such as water availability in the soil can take days or even weeks to manifest themselves, and mineral deficiencies in the soil take months.

One possible explanation for the high photosynthetic rates in the first hours after diesel fuel application may be related to its elevated concentrations of hydrocarbons. Given that it is an oil-based fuel, composed basically of hydrocarbons, carbon atoms, hydrogen and low concentrations of sulfur, nitrogen and oxygen, and given its hydrophobic character, (Viana (2008)), diesel fuel may have made the water molecules more available to plants in the first hours, representing an obstacle between the soil/air interface. This fact provides plants with a momentary water supply, where gas exchanges are not expected to be initially affected.

However, with prolonged stress, hydrocarbons were likely adsorbed by root hairs, suppressing the amount of water adsorbed by the roots, causing water, respiratory and nutritional deficiency in plants (Baker, 1970). According to Larcher (2006), when water availability in the soil is less than that required by the plant, all metabolic processes are compromised, resulting in leaf withering and a decline in turgor pressure, paralyzing the mechanical entry of water into the cell, leading to a reduction in cell volume. The effect of low water availability on plant development depends on the magnitude and duration of stress, as well as the genetic ability of plants to respond to environmental changes (Silva and Nogueira, 2003).

Assessment of chlorophyll content in leaves is an important aspect for plant physiology studies, since it could be an indicator of leaf senescence, nitrogen nutritional status, in addition to expressing the possible changes as a response to environmental stress (Silva et al., 2016).

The results show that plants did not suffer from nitrogen (N) deficiency, and closed their stomata to impede the escape of water vapor (transpiration), due to the unavailability of water absorption by the roots. Given that it contains nitrogen, the application of diesel oil increased the concentration of this element in the soil, and was absorbed by the plant. Nitrogen (N) is an essential element, required in large amounts by plants, and a constituent of chlorophyll and many compounds, including highly mobile proteins and nucleic acids. As leaves aged, symptoms such as chlorosis (yellowing of leaves) and necrosis were observed, with consequent foliar abscission. These symptoms indicate nitrogen deficiency (Lacerda, 2006), and given that assessments were always conducted in the upper third of the leaf, no decline in the chlorophyll index was found using the portable chlorophyll meter, due to the significant mobility of this element from old to young leaves (Taiz and Zeiger, 2013).

The results demonstrate that plants showed no degradation in photosynthetic pigments. This behavior may be explained by the short experimental period. A drop in chlorophyll concentration in plants grown in soil contaminated by oil and its derivatives was observed in *Canavalia ensiformes* plants submitted to different doses of diesel fuel (Balliana, 2015).

However, *H. courbaril* and *J. curcas* plants showed an increase in chlorophyll *a*, *b*, total chlorophyll and carotenoids. Behavior similar to that observed in the present study was reported by Oliveira (2008), investigating *Schinus terebinthifolius* with soil from an area where an oil spill of approximately 4 million liters occurred, 700 thousand liters of which seeped into the soil. The author observed a rise in chlorophyll *a*, *b* and total chlorophyll in plants growing in oil-contaminated soil.

Electrolyte leakage is directly linked to cell death, since it is triggered by ionic toxicity, which destabilizes the plasmic membrane and leads to cytoplasmic electrolyte leakage (Mengel and Kirkby, 2001). This may explain the much more evident visual symptoms (blackish spots and subsequent necrosis) in *J. curcas* plants compared to their *H. courbaril* counterparts.

As such, in the short term, the use of young plants instead of seeds in areas contaminated by diesel fuel was much more efficient in recovery efforts. More phytophysiology studies are needed to determine the specific effects of different contaminants on plant metabolism, in order to understand their synergetic effects.

5. CONCLUSIONS

Hymenaea courbaril and *Jatropha curcas* displayed significant alterations in gas exchanges, with an increase in means 3h after diesel addition for treatments T1 and T2, and subsequent decline with prolonged stress (192h);

Soil contaminated with diesel fuel exhibited an increase in photosynthetic pigment levels and chlorophyll index for *Hymenaea courbaril* and *Jatropha curcas* plants in treatments T1 and T2, 192h after imposed stress, but these results did not reflect in higher photosynthetic rates;

Jatropha curcas plants showed more significant symptoms of leaf injury than *Hymenaea courbaril* plants, evidenced in the variable electrolyte leakage percentage (protoplasmic membrane damage);

All the variables studied (gas exchanges, chlorophyll index, photosynthetic pigments and electrolyte leakage) were good indicators of the degree of tolerance displayed by *Hymenaeae courbaril* and *Jatropha curcas* plants to the stress caused by diesel fuel.

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