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Physiological modifications resulting from chemical and mechanical hardening of *Hymenaea courbaril* L. seedlings[1]

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ABSTRACT

Hardening is the process of exposing seedlings gradually to field conditions. It improves seedling quality through several strategies, including stem bending and the use of plant regulators. Thus, the objective of the experiment was to quantify the physiological changes resulting from mechanical and chemical hardening in *Hymenaea courbaril* L. seedlings as a function of seedling growth stages. The experiment was conducted at Marechal Cândido Rondon, PR, in a shade house. The design used was a completely randomized, consisting of three treatments (control, methyl jasmonate and stem bending), with seven replications of 16 seedlings. At the end of the hardening treatments we quantified stem bending stiffness, lignin, phenolic compounds, loss of electrolytes and chlorophyll. The phenolic compounds quantified in the leaves increased with the application of both treatments in seedlings of stages I and II. Furthermore, mechanical hardening favored an increase in chlorophyll at all stages. *H. courbaril* seedlings from stages II and III would be the most recommended for hardening treatments. Mechanical hardening was the most suitable for *Hymenaea courbaril* L. seedlings.

Keywords: phenolic compounds; flexural stiffness; lignin; stages; treatments.

INTRODUCTION

Planting of wood species is used for revegetation of degraded areas, soils, and springs as well as the provision of green manure and increased nutrient soil cycling due to the vegetation cover (De Souza *et al.*, 2018). Additionally, in the long term, planting trees will result in other economic and environmental benefits (De Oliveira *et al.*, 2017). Choice of wood species will depend on the objective as well as the soil and climate conditions of the planting site.

Hymenaea courbaril L. popularly known as jatoba belongs to the Fabaceae family, subfamily Caesalpinioideae. The species can be described as an opportunistic semi-deciduous, light demanding and selective xerophytic when in water-restricted environments. Jatoba is native to the Amazon Forest, with its hardwood used in civil construction and furniture manufacturing with high prodution potential and tolerance to atypical environmental conditions (Carvalho, 2003; Costa *et al.*, 2011).

Because of wood properties, the listed jatoba in the group of the ten most valuable species worlwide. Studies have been developed mainly regarding the production of seedlings since the species has some limitations in the germination process, offset by a large number of seeds (Barbieri Junior *et al.*, 2007).

After outplanting, seedlings of wood species will be

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subjected to numerous stresses of biotic or abiotic origin. The stressful effects can be mitigated with hardening practices during final stages of propagation in the nursery.

Seedling hardening aims to increase the tolerance of seedlings, attenuating the “post-planting shock”. Hardening is likely to activate the defensive responses of plants to non-ideal post-planting conditions (Jacobs & Landis, 2009; Pinto-Zevallos *et al.*, 2013).

Hardening or acclimatization process consists of a transition phase from an environment with suitable characteristics for plant development to another without control or stressfull (Dumroese *et al.*, 2009). Hardening protocols include reduction of fertilization, water, and light regime as well as application of stem bending and plant regulators such as methyl jasmonate (MeJA) and salicylic acid (SA).

MeJA, in addition to regulate metabolic activities, can signal against biotic and abiotic stresses and therefore, as a hardening seedling agent. The process is triggered after the regulator sends the message and triggers the signal transduction process. The responses obtained will vary according to the characteristics of the species and the relationship between plant hormones, and may be synergistic or antagonistic (Zhang *et al.*, 2012; Deuner *et al.*, 2015; Pereira-Neto, 2019). Furthermore, recent results show that salicylic acid and jasmonic acid can be promising compounds to reduce the sensitivity of crops to abiotic stresses, because under certain conditions they attenuate the adverse effects produced by different stressful environmental factors such as water deficit. The exogenous application of jasmonates to plants produces effects such as the closure of stomata under stress conditions and increases plant resistance to pathogen infections (Sanchez, 2008; Pereira-Netto, 2019).

In addition to plant regulators, seedling hardening can be induuced by stem bending resulting in reduction of aboveground growth and increase of stem diameter and root system. Furthermore, stem bending induces physiological and biochemical changes that can trigger a series of defensive responses (Jaffe, 1973; Volkweis *et al.*, 2014; Dranski *et al.*, 2015).

Another important point is the age or growth stage at which seedlings are shipped out for planting. Quite oftern it is based on assumptions and empirical knowledge or even adaptation from other species. Most of those recommendations are based only on morphometric characteristics, which may underestimate the real responses of seedlings to stressful conditions (Costa & Streck, 2018; Gonzaga *et al.*, 2018; Ataíde *et al.*, 2019).

The application of chemical and mechanical hardening will affect growth and development depending on seedling age or stage. However, this modification is interesting as, depending on the intensity of application and the period that the treatments will be imposed, they may promote an increase in the seedling tolerance to stresses, resulting in a seedling better prepared for the post-planting shock.

Seedling hardening with plant regulators derived from the salicycate and jasmonate groups, as well as the use of mechanical stimuli, is still very incipient, especially for woody species, as the internal and external changes in those plants need to be detailed and quantified. Therefore, the objective of the experiment was to quantify physiological alterations of *Hymenaea courbaril* L. seedlings hardened with methyl jasmonate and stem bending as a function of growth stage as well as seedling survival after outplanting.

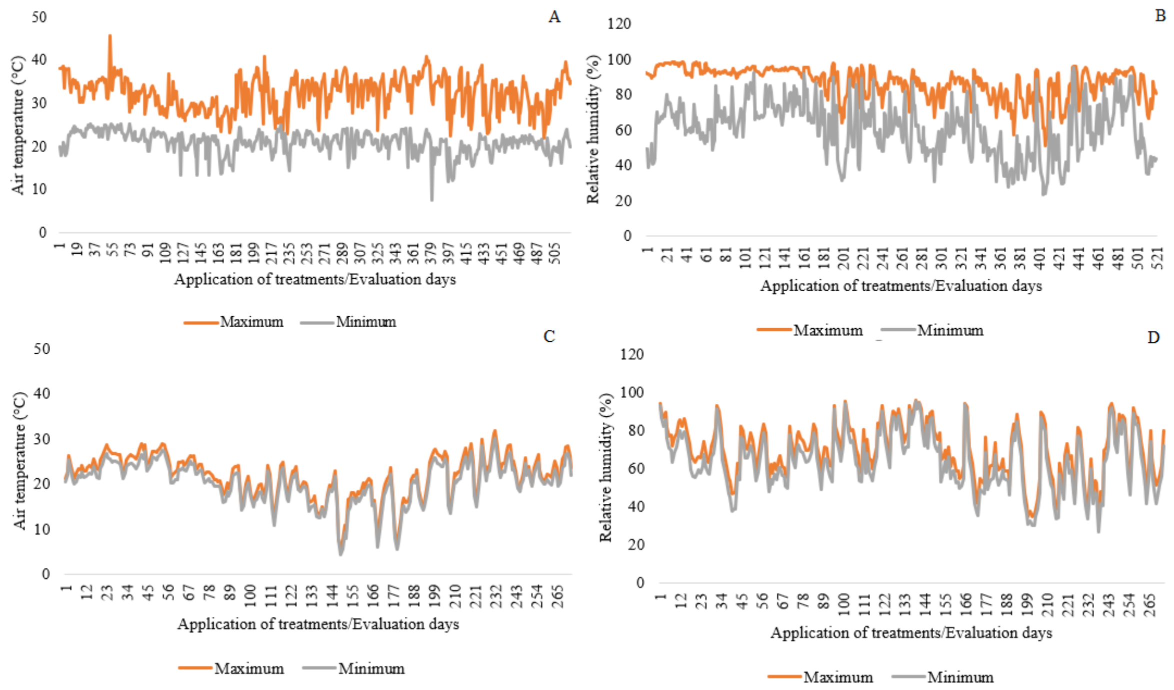
MATERIAL AND METHODS

The experiment was conducted in Marechal Cândido Rondon-PR, with geographic coordinates of 24° 33’ 24” S, 54° 05’ 67” W and altitude of 420 m. According to the climatological classification of IAPAR and Köppen the region climate is a subtropical Cfa type (Alvares *et al.*, 2013) with average temperatures between 22 and 23 °C, good distribution of rainfall during the year characterized by hot summers (Nitsche *et al.*, 2019).

Daily relative humidity and air temperature (Figure 1A e 1B) were obtained with a datalogger (KlimaLogg Smart®) throughout the experiment.

Seedlings were propagated with seeds acquired from Rede de Sementes (Portal Amazônia) in a shade house with a 150 micron thick anti-UV low-density polyethylene film equivalent to 20% shading. Jatoba seeds were collected from approximately 28 adult trees between the counties of Carlinda and Nova Guarita, state of Mato Grosso, from planted and spontaneous forest areas. Seeds were stored at 16 °C and 40% humidity in a seed storage chamber for approximately 2 months and subsequently scarified and sown.

Jatoba seeds were scarified in the opposite side of the embryonic axis with sandpaper (n° 36) to overcome tegumentary dormancy and disinfected with 10% sodium hypochlorite for 30 minutes followed by washing with tap water. Afterword, seeds were submerged in water for 48 hours at 25 °C in a BOD-type germination chamber and sown in a 290 cm^3 plastic plug filled with Humusfertil® vermicompost based on pine bark, sand, and vermiculite



Source: Rocha (2022).

Figure 1: Air temperature (A) and humidity (B) in a shade house during the hardening of *Hymenaea courbaril* seedlings and temperature (C) and humidity (D) of the air in the field after the expedition of seedlings.

with electrical conductivity of 1.5 mS cm^{-1}, density of 480 kg m^{-3}, pH of 6.5, maximum humidity and retention capacity by mass/mass equal to 60%.

Before hardening, seedlings were fertilized with a nutrient solution of 50.2 grams of Osmocote® plus (slow-release fertilizer), 50.6 grams of urea and a second solution formed by 80.4 grams of NPK (10-15-15) both diluted together in 2 L of water. The solutions were prepared separately, but the application was performed simultaneously every 15 days. The amount was 20 mL in a volume of 10 liters of water (fertilization was adjusted from pre-tests). Fertilization was interrupted as soon as the hardening treatments were started.

Jatoba seedlings were subject to chemical hardening (100 µmol L^{-1} of methyl jasmonate) mechanical hardening (through pendulum movement of the seedlings stem) and control (without hardening). The design used was a completely randomized design with three treatments, seven replications (16 seedlings each) totaling 336 seedlings per growth stage.

Chemical hardening was applied weekly while mechanical hardening was applied daily. Both treatments were applied for 30 days in seedlings at 50, 80 and 110 days after emergence-DAE (growth stages). Depending on the seedling growth stage treatments were applied from November 2019 to February 2020.

The growth stages were defined based on the analysis of seedling growth, defining as initial (50 DAE), medium (80 DAE) and advanced (110 DAE) growth stage, in order to determine in which moment would be ideal for hardening treatments (Rocha *et al.*, 2022).

Chemical hardening used a solution with methyl jasmonate at 100 µmol L^{-1}, deionized water and a non-ionic surfactant (Agral-Syngenta®) at a proportion of 30 mL in 100 L of water to increase the absorption by the leaves. The solution was applied with a manual sprayer between 6:00 and 6:30 pm in a volume equivalent to 100 L ha^{-1}.

Mechanical hardening consisted of 20 daily stem bending at a constant speed of 0.10 m s^{-1} for 4 weeks at the same time according to Volkweis *et al.* (2014) and the model proposed by Jacobs & Landis (2009).

Throughout the experiment, the necessary cultural treatments were carried out. Irrigation used micro-sprinkler in five daily 10 minutes irrigation periods in summer (07:00am, 10:00am, 01:00pm, 04:00pm and 06:00pm) and in three 10 minute daily periods during winter (09:00am, 01:00pm and 05:00pm). The equivalente water table obtained with irrigation was 4 mm determined by three rain gauges.

After the hardening treatments, we quantified flexural stiffness (Lima *et al.*, 2020) as well as chlorophyll pigments, and determined lignin content from stems and roots (Van Soest, 1994), phenolic compounds (CF) from leaves and roots (Georgé *et al.*, 2005) and performed the test of electrolyte loss from root tissues (Wilner, 1955).

Determination of stem flexural stiffness used 42 randomly selected seedlings per growth stage. In this analysis, we used an equipment according to Lima *et al.* (2020). From the measurement of mass and distance, the data were transformed into N cm^{-1}.

Chlorophill pigments were determined by the biochemical method (Arnon, 1949) with the suppression of the grinding and centrifugation phases (Barbieri Junior *et al.*, 2010) and with chlorophyll meter (Minolta Chlorophyll Meter®) in four leaves of the lower third of jatoba seedlings foliage.

After hardening, an alleatory sub-sample of seedlings was outplanted to quantify survival and growth in an area with coordinates of 24.532116 S and 54.025485 W. The design was a randomized block, with three treatments (similar to those used in the shade house), seven replications of each of the treatments, distributed in three blocks totaling 21 seedlings per growth stage.

Morphometric evaluations were performed at 90 days after outplanting and survival at 180 days after outplanting. The quantified morphometric parameters included height and stem diameter. In young jatoba seedlings, the persistence of leaves was observed throughout the evaluation, since it is characteristic of the species to shed leaves depending on environmental conditions and stress levels. Climatic data (Figure 1C e 1D) were obtained from the meteorological station located close to the planting site by accessing the historical data on the website of the National Institute of Meteorology (INMET, 2022).

Seedlings from each growth stage were outplanted according to the end of the hardening from February 28 to October 31, 2021. We used planting holes of 27 cm in diameter and 60 cm in depth spaced 1 m between rows and 1 m between seedlings.

Soil in the planting area is classified as typical dystroferric RED LATOSOL, with a very clay texture (Dos Santos *et al.*, 2013). Cultural practices throughout the experiment involved manual weeding of spontaneous plants. We irrigated 2 L per seedling every 3 days for 20 days after outplating to make the transition from the nursery to the planting site. Ant control was achieved by mechanical (cones of milk cartons surrounding the stems, with diameter between 8 and 10 cm) and chemical (commercial baits, distributed between the lines weekly) means.

Data was tested to confirm the existence of statistical assumptions of normality and homogeneity followed by analysis of variance. It ANOVA showed differences treatment means were unfolded and tested by the Tukey test at 1 and 5% probability as a function of seedling growth stage.

RESULTS AND DISCUSSION

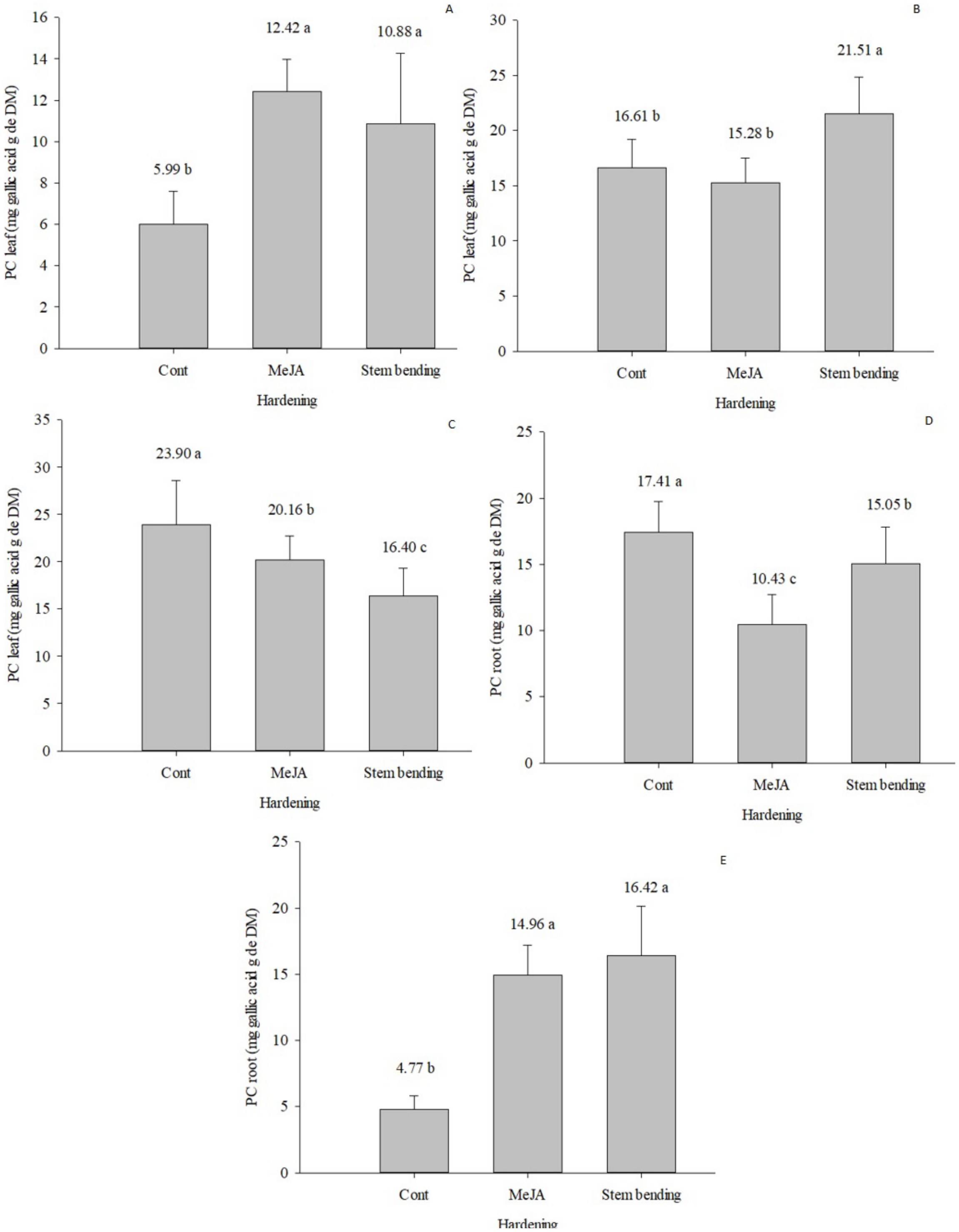
Stem flexural stiffness of *Hymenaea courbaril* seedlings of the three growth stages did not result in differences ($P \leq 0.05$) between hardening treatments. However, with increasing DAE, the stem stiffness strength increased reaching values greater than 2 N cm^{-1} in stage III seedlings. The values of stem flexural stiffness in seedlings of stages I, II, and III were 0.67713; 1.08216; and 2.35858 N cm^{-1}, respectively.

The analysis of the results did not detect differences ($P \leq 0.05$) as a function of the hardening treatments regarding lignin content of stems and roots. Contrary to our findings, Heberle *et al.* (2018) reported differences in stem lignification of *Patagonula americana* L. seedlings with jasmonic acid application. The above authors concluded that the doses used were not able to significantly differentiate or increase lignin in root tissues.

Jatoba seedlings from growth stages I (50 DAE) and II (80 DAE) showed that variation in the concentrations of phenolic compounds was altered ($P < 0 .05$) because of hardening treatments. In stage I seedlings (50 DAE) the concentrations of those compounds increased more than 50% in seedlings treated with MeJA ranging from 5.99 to 12.42 mg of gallic acid per g of DM (Figure 2A).

One of the functions of phenolic compounds is their action as an antioxidant agent and the ability to bind free radicals, making these less harmful to plant cells. The signal transmission process is triggered by the activation of enzymes and proteins linked to plant defense, which will culminate in the capture of electrons and the formation of non-ionic molecules that are harmful to plant development (Pereira & Cardoso, 2012; Sharma & Singh, 2013).

During the experiment, variations in temperature and air humidity (Figure 1) occurred, which intensified the stresses induced by the hardening treatments. Treatment with stem bending resulted in an increase in antioxidant compounds (Figures 2A and 2B). On the other hand, stage III seedlings (110 DAE) showed an inverse response where



Source: Rocha (2022).

Figure 2: Concentration of phenolic compounds in *Hymenaea courbaril* seedling leaves as a function of hardening methods and growth stages I (A), II (B) and III (C) and roots at stage I (D) and III (E). The means followed by the same letter do not differ statistically from each other by the Tukey test at 1 and 5% probability.

the control treatment yelded the highest means. In the case of seedlings with higher DAE, the stem bending performed for one month did not stress the seedlings as drastically as the seedlings in the early stages of development (Figure 2C).

In leaves, because of the exposure and sensitivity to the environment, the results of induced stresses are more intense. In younger seedlings, where the growth system is prioritized, the defense apparatus may react slowly to those stresses induced by hardening processes (Biddington, 1986).

Root tissues are considered the main organs responsible for the release of phenolic compounds. Consequently, root tissues can use their allelopathic potential to reduce intraspecific and interspecific competition (De Oliveira, 2017). Furthermore, it is possible that, if stimulated, seedlings distribute these compounds among other tissues to signal any alteration resulting from external stimuli. This response was evidenced with *Hymenaea courbaril* seedlings hardened at stage III (110 DAE) where an increase of those contents (244%) was observed in the roots of the control seedlings compared to seedlings submitted to stem bending (Figure 2E).

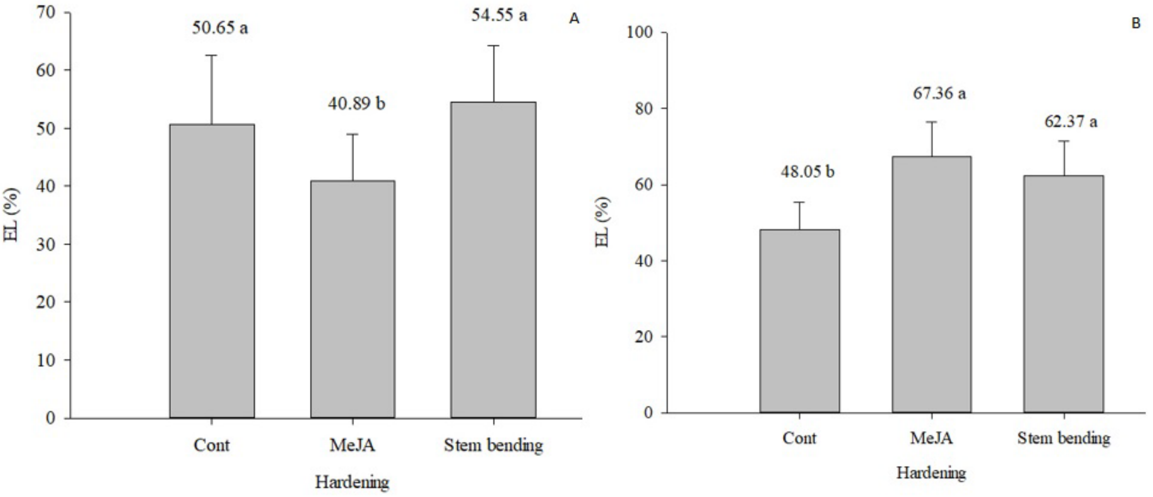
Jatoba seedlings from stage I (Figure 2D) hardened with MeJA expressed the lowest mean phenolic compounds (10.43 mg gallic acid g DM). There was an inverse response when comparing the amount of phenolic compounds from leaves and roots in stage III seedlings, where the increments were decreasing in leaves, while in the roots they were increasing. These results indicated that part of those components, instead of being translocated, were deposited in the root tissues.

The lowest value of the electrolyte loss (EL) test of root tissues was obtained in seedlings from state I that received MeJA (Figure 3A). The EL test is correlated with the stress level in seedlings of wood species (Oro *et al.*, 2012; Volkweis *et al.*, 2014; Cadorin *et al.*, 2015; Dranski *et al.*, 2017; Cadorin *et al.*, 2021).

Seedlings receiving MeJA did not compromise the integrity of the root membranes, reducing the amount of leached ions. The reduction in EL values may be linked to the deposition of osmotic regulators through the signaling promoted by MeJA, neutralizing the effect of toxic ions released into the cell environment (Guo *et al.*, 2010). On the other hand, seedlings of stage II (80 DAE) showed different responses. The seedlings that expressed symptoms of stress because of high EL value were those treated with MeJA compared to control seedlings (Figure 3B).

Cadorin *et al.* (2015) evaluated the loss of electrolytes in the roots of *Cordia trichotoma* (Vell.) Arrab. ex Steud and reported the lowest means regardless of hardening (chemical or mechanical) with a reduction of 40% compared to the control seedlings.

The SPAD index did not reveal differences ($P > 0.05$) between hardening treatments from stage I seedlings with values of 31.11; 30.91; 32.91 µg cm^{-2} for the control,



Source: Rocha (2022).

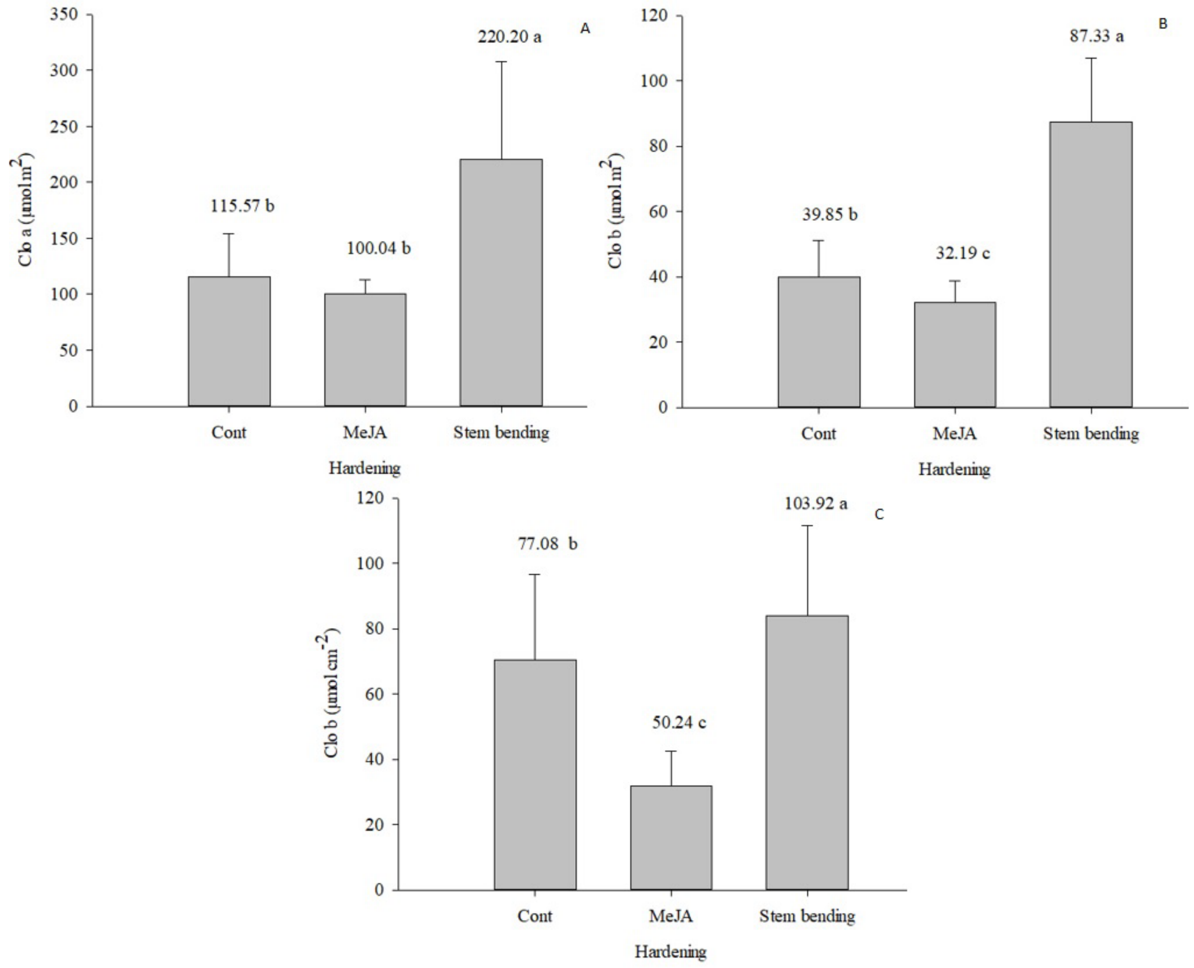
Figure 3: Electrolyte (EL) loss in *Hymenaea courbaril* seedlings as a function of hardening methods and growth stages I (A) and II (B). The means followed by the same letter do not differ statistically from each other by the Tukey test at 1 and 5% probability.

chemically and mechanically hardened, respectively. With stage II seedlings, SPAD indices were 20.63; 28.83; 25.14 µg cm^{-2} while with stage III the indices were 25.10; 27.10; and 29.53 µg cm^{-2}.

The increase in chlorophyll content coincided with the reduction of leaf area in stage I seedlings hardened with stem bending. To compensate for that reduction, there was an increase in the synthesis of both chlorophyll *a* and chlorophyll *b* (Figures 4). This compensation prevents the production of photoassimilates, energy acquisition, and primary metabolism of plants from being interrupted and harming development. In contrast to our findings, Cadorin *et al.* (2021) observed that after the quantification of chlorophyll *a* and *b* in *Tabebuia roseo-alba* seedlings, there was a reduction in those parameters as a function of the application of stem bending for 4 and 8 weeks.

The reduction in chlorophyll concentration as a function of stem bending was also observed by Coutand (2010) who associated the decrease in pigments with a reduction in aboveground biomass, as well as a reduction in photosynthetic rate. The above author emphasized the fact that each species will respond in a specific and differentiated way to stressful stimuli. Furthermore, in the same species and stage, the application of MeJA resulted in a reduction in the levels of chlorophyll a (Figure 4A).

The exogenous application of jasmonates may be associated with the process of leaf senescence and, consequently, the degradation of chlorophyll pigments. These pigments are linked to the capture of light energy, through the conversion and consumption of ATP and NADPH, and in this case chlorophylls and their reduction will influence the rate of net assimilation of CO$_2$ (Vieira *et al.*, 2010).



Source: Rocha (2022).

Figure 4: Chlorophyll a concentration in growth stage I (A) and b in stages I (B) and III (C) in *Hymenaea courbaril* seedlings as a function of hardening methods. The means followed by the same letter do not differ statistically from each other by the Tukey test at 1 and 5% probability.

Control seedlings from stage I (Figure 7A) and from stage III (Figure 4C) resulted in greater variation in chlorophyll *b* compared to mechanical hardening. Seedlings treated with stem bending showed an increase ($P < 0.05$) compared to control seedlings at stage I with an increase of 119.15%. While, seedlings treated with methyl jasmonate reduced their levels in both growth stages compared to the control seedlings (Figures 4B and 4C).

Previous research with MeJA has resulted in leaf abscission and reduction of chlorophyll pigments externalized through chlorosis (Sembdner & Parthier, 1993) and this may explain the reduction that occurred in the levels of chlorophyll pigments in seedlings treated with MeJA, when compared to other treatments.

Jatoba seedlings of stages I (50 DAE), II (80 DAE), and III (110 DAE) showed 100% survival regardless of treatments 180 days after planting. Matheus *et al.* (2011) highlighted that *Hymenaea courbaril* is very tolerant to environmental adversities, in addition to presenting strategies to better survive under those conditions, such as leaf loss (Costa *et al.*, 2011).

Seedlings from stage I 30 days after outplanting started the process of drastic defoliation, resulting from thermal stress (temperatures below 13 °C) and therefore were not morphologically evaluated at 90 days. The regrowth of leaves of those seedlings at this stage only started 150 days after planting when temperatures were higher. Seedlings of stages II and III at 90 days after planting showed no difference ($P > 0.05$) for the morphometric parameters as a function of hardening treatments. In stage II seedlings, the increments were 2.93; 2.13; 2.28 cm in height and 0.30 cm; 0.36; 0.30 mm in stem diameter for control and those submitted to MeJA and stem bending, respectively. Defoliation was also evidenced in seedlings from stage II, but only in the evaluations carried out after 90 days of outplanting and without drastic defoliation.

At 90 days after outplanting, jatoba seedlings of stage III presented height increments of 7.74; 6.74; 6.66 cm and stem diameter increments of 1.21; 0.94; and 0.88 mm in control and those submitted to MeJA and stem bending, respectively. The increment values of stage III seedlings submitted to MeJA and stem bending were similar ($P > 0.05$).

The low values of morphometric increments of jatoba seedlings after 90 days of outplanting is a characteristic of the species, classified as climax and therefore has slow initial growth as its main characteristic (Lorenzi, 2002; Carvalho, 2003). The difference observed between the increments in seedlings of stages II and III is related to the period in which they were outplanted; stage II seedlings were planted close to the winter (Figure 2).

Cadorin *et al.* (2015) evaluated the growth of *Cordia trichotoma* (Vell.) Arrab. ex Steud and did not detect a statistically significant difference between the hardening treatments 90 days after planting. However, at 180 days after planting, the authors mentioned above reported that seedlings with the highest growth were those that received 20 daily stem bending and MeJA for 8 weeks.

Mechanical and chemical hardening can be recommended to promote acclimatation of seedlings from the nursery to the outplanting environment. Hardening processes result in distinct and interesting responses and morphometric and physiological changes when evaluating the quality parameters of seedlings. However, the mechanical method is more laborious while the application of plant regulators appears as a possibility to shape quality parameters, as well as reduce the activities carried out in the management of forest seedlings. Despite the advantages, the chemical method can be limiting in terms of its acquisition, since its price is not accessible to all consumers, despite that, the concentrations used and recommended are very low.

Still, unveiling quality standards and their correlations with seedling survival field remains a major challenge. Therefore, furthermore research of hardening processes need to executed in order to satisfy the “target seedling” principle.

CONCLUSIONS

Seedling physiological characteristics are altered at all stages due to hardening. However, for the chlorophyll pigment in the leaves, the treatment with the highest average is the mechanical hardening resulting in an intensification of the green color, as a way of compensating photosynthetic activity. In relation to phenolic compounds, there was also an increase in their concentration in seedling of stages I and II in leaves and roots, as this is a metabolite related to plant defense. However, for older seedlings (stage III) the opposite occurred in the leaves, resulting in a reduction as hardening was applied, which reinforces the idea that younger seedlings are more susceptible to stress. Therefore, primary and secondary routes of the defensive system are activated in a more intense and therefore noticeable way in initial seedling growth stage.

Therefore, under the conditions and with the specie studied stem bending would be the recommended, as it managed to modulate the physiological parameters. Furthermore, young seedlings from stage II onwards can be subjected to hardening without major harm to their development.

Hardened and control seedlings did not differ statistically for outplanted survival, height and stem diameter increments evaluated 180 days after planting.

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