In vitro propagation and seedling acclimatization of *Caesalpinia ferrea* Mart., a valuable medicinal plant in the Amazon (Fabaceae)

Propagação *in vitro* e aclimatação de plântulas de *Caesalpinia ferrea* Mart., uma valiosa planta medicinal na Amazônia (Fabaceae)

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Abstract: The objective of this work was to establish a protocol for the rapid *in vitro* multiplication of *Caesalpinia ferrea* Mart., aimed at genetic selection and *in vitro* germplasm conservation. Seeds treated in different sodium hypochlorite solution concentrations were inoculated in Murashige and Skoog (MS) culture medium and maintained in a growth room. After 30 days, sodium hypochlorite at 0.25% concentration resulted in 96.67% survival and 90% germination of the seeds. In the explants multiplication phase three types of medium culture were evaluated supplemented with cytokinins and auxins in different concentrations and combinations. The addiction of 0.1 mg·L^{-1} Benzylaminopurine (BAP) and 0.1 mg·L^{-1} Indole butyric acid (IBA) induced 100% of explants with shoots and 32% with root formation, while a complete absence of callus was found. It was observed that 90% of explants derived from the buds developed shoots with 2.73 cm and greater multiplication rate (10.06). The seedlings obtained from *in vitro* multiplication were acclimated without the presence of roots in different types of substrate. After 60 days, soil substrate was most indicated for acclimatization, showing 63.33% of healthy and uniform plants. *In vitro* propagation of *C. ferrea* allows the establishment and multiplication of *in vitro* seedlings from nodal segments and axillary buds.

Keywords: Biotechnology. Conservation. Cell regeneration. Plant multiplication.

Resumo: O objetivo deste trabalho foi estabelecer um protocolo para a rápida multiplicação *in vitro* de *Caesalpinia ferrea* Mart., visando o melhoramento genético e a conservação *in vitro* de germoplasma. As sementes tratadas com diferentes concentrações de hipoclorito de sódio foram inoculadas em meio de cultura Murashige e Skoog (MS) e mantidas em sala de crescimento. Após 30 dias, a concentração de 0,25% de hipoclorito de sódio resultou em 96,67% de sobrevivência e em 90% de germinação das sementes. Na fase de multiplicação dos explantes, foram avaliados três tipos de meios de cultura, suplementados com diferentes concentrações e combinações de citocininas e auxinas. A adição de 0,1 mg·L^{-1} de 6-benzilaminopurina (BAP) e 0,1 mg·L^{-1} de ácido indol-3-butírico (AIB) ao meio MS induziu 100% de explantes com brotações e 32% com formação de raízes, sendo constatada ausência total de calos. Foi observado que 90% dos explantes originados das gemas desenvolveram brotos com 2,73 cm de altura e maior taxa de multiplicação (10,06). As plântulas provenientes da multiplicação *in vitro* foram aclimatadas sem a presença de raízes em diferentes tipos de substratos. Após 60 dias, o substrato terra foi o mais indicado para a aclimatação, apresentando 63,33% de plantas sadias e uniformes. A propagação *in vitro* de *C. ferrea* permite o estabelecimento e a multiplicação de plântulas a partir de segmentos nodais e de gemas axilares.

Palavras-chave: Biotecnologia. Conservação. Regeneração celular. Multiplicação vegetal.

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INTRODUCTION

The Amazon region presents a rich flora with an immense variety of therapeutic plant species. Among the numerous families of plants found in the Amazon the family Fabaceae stands out with *Caesalpinia ferrea* Mart., a species commonly known as ironwood (Lorenzi & Matos, 2002). It is a medicinal plant used by the population of the Brazilian north and northeast, as a tea made from the powdered bark that is used to treat wounds, fever, relieve chronic cough, and lung conditions. It acts against anemia and diabetes with good results, which stimulates interest in biotechnological and pharmacological studies of this species (Roque *et al*., 2010).

Studies with *C. ferrea* performed by Nozaki *et al*. (2007) and Ohira *et al*. (2013) identified anticancer activity of the active substance 'Pau ferrol' against human topoiosomerase II, which inhibited cell growth by induction of apoptosis, and it can be used as an important tool in the treatment of human HL60 leukemia.

The extraction activities practiced by people in natural populations of *C. ferrea* contributed to the genetic erosion and loss of valuable genotypes of this species (Benedito *et al*., 2012). Therefore, it is necessary to improve the propagation methods aimed at selection or commercial planting programs. The micropropagation technique allows the multiplication of high quality and physiologically rejuvenated propagules in short time with phytosanitary guarantee and genetic stability of plantlets (Gao *et al*., 2010; Morais *et al*., 2012; Carvalho *et al*., 2013).

Thus, this study aimed to establish a methodology for *in vitro* propagation of *C. ferrea*, as well as for its conservation and rapid multiplication, making it available in the future for the development of phytotherapics by the sustainable use of this species.

MATERIAL AND METHODS

C. ferrea seeds used in the experiments provided by Dr. Luiz Augusto Gomes de Souza were stored about six years at the Seed Bank of the Microbiology and Soil Fertility Laboratory of the National Institute of Amazonian Research (INPA), and the plant exsiccate is deposited in the INPA Herbarium under number 228.022.

The explants were kept in a growth room with temperature of 25 ± 2 °C and 16 h of photoperiod with light intensity of 52 $\mu mol \cdot m^{-2}s^{-1}$, under two cool white fluorescent lamps (GE.85W). The culture media basal salts, Murashige and Skoog (MS) (Murashige & Skoog, 1962), B5 Gamborg (B5) (Gamborg *et al*., 1968), and Wood Plant Medium (WPM) (Lloyd & McCown, 1981), were supplemented with vitamins and 30 $g \cdot L^{-1}$ sucrose, solidified with 7 $g \cdot L^{-1}$ agar, except the WPM supplemented with 20 $g \cdot L^{-1}$ sucrose, and all were supplemented with specified plant growth regulators in each experiment. The pH was adjusted to 6.0.

The seeds of *C. ferrea* were scarified at the opposite side of the hilum using an emery stone in order to break the dormancy and to allow seed water imbibition. After scarification, the seeds were sterilized by washing with neutral detergent and rinsing in tap water for one minute. They were immersed in Carbendazim (2.0% v/v) fungicide solution for one hour under constant stirring at 100 rpm. Subsequently, the seeds were immersed in 70% ethanol for one minute and then dipped respectively in different concentrations of sodium hypochlorite (NaOCl): 0.0%, 0.10%, 0.25%, 0.50%, or 1.0% (v/v) for 30 minutes, also with the same stirring. Subsequently, the seeds were rinsed four times with sterile distilled water. After sterilization, seeds were inoculated in test tubes containing 10 ml of MS medium. The explants were kept in the growth room and were evaluated after 30 days for the presence or absence of microorganisms, survival rate, and percentage of germination.

Three experiments were performed to develop *in vitro* multiplication protocols of this species. In the first experiment, nodal segments were taken from seedlings of *C. ferrea* germinated *in vitro* and then these were inoculated in MS medium supplemented

with 6-Benzylaminopurine (BAP), Kinetin (KIN), and Thidiazuron (TDZ) at concentrations of 0.0; 0.1; 1.0; 3.0 and 5.0 mg· L^{-1}, respectively. In the second experiment, buds from different positions on the stem were cut and inoculated in MS medium supplemented with 0.1 mg· L^{-1} BAP. In the third experiment, nodal segments obtained from seedlings grown *in vitro* in MS medium were inoculated in culture medium Woord Plant Mediun (WPm), Murashige and Skoog (MS), and Gamborg B5 (B5) in the original concentrations, as well as in their concentrations diluted by half and the quarter part, all supplemented with 0.1 mg· L^{-1} BAP to determine the best culture medium for the multiplication of *C. ferrea*. After 30 days of growth in these culture media, the explants were evaluated in relation to the percentage of explants with shoot proliferation, the number of shoots per bud, number of buds per stem, the multiplication rate (the number of shoots per bud multiplied by the number of buds per stem), the height of the shoots and the formation of calluses and roots.

The nodal segments excised from seedlings grown *in vitro* were inoculated in MS medium in the original concentration plus 0.1 mg· L^{-1} BAP and supplemented with Naphthaleneacetic Acid (NAA), Indole-3-Acetic Acid (IAA), and 3-Indole Butyric Acid (AIB) at concentrations of 0.0; 0.1; 1.0; 3.0 and 5.0 mg· L^{-1}. After 30 days of growth in culture media, the explants were evaluated in relation to the percentage of explants with shoot proliferation, the number of shoots per bud and number of buds per stem, the multiplication rate, the height of the shoots, and the formation of calluses and roots.

For this experiment, 150 plantlets multiplied *in vitro* were used without roots and with shoots of approximately 7 cm height and were washed with distilled water until complete removal of residues of culture medium. After that, they were planted in polyethylene germination trays 41 x 27 x 8 cm (length, width and height), using the following substrates: earthworm humus, vermiculite, soil, sand, and 1:1:1 soil + sand + vermiculite. The substrates were autoclaved for 1 hour at 121 °C. These seedlings were kept covered with clear glass flasks 10 x 7 cm (height and diameter) for 30 days in the Seedling Nursery of the Plant Tissue Culture Laboratory and after this period, the glass flasks were removed. The plantlets evaluation was performed after 30 and 60 days in relation to the survival or not in *ex vitro* environment.

The experimental design was randomized in all experiments and used three replicates of ten explants per treatment. Data were submitted to Analysis of Variance (ANOVA) and comparisons of treatment means was done by Tukey test ($p < 0.05$) using the ASSISTAT program version 7.7.

RESULTS AND DISCUSSION

Aseptic treatment with 0.25% Sodium Hypochlorite (NaOCl) resulted in 96.67% survival and 90% germination in seeds. Furthermore, this provided the lowest percentage of contamination, 6.67% by fungi and total absence of bacteria, compared with other treatments, indicating that low concentrations are effective for disinfection of the seeds.

After scarification and asepsis, the seeds of *C. ferrea* inoculated *in vitro*, showed high germination rates when compared with other medicinal species such as *Croton antisyphiliticus* Mart. (Oliveira *et al*., 2011) and *Copaifera langsdorffii* Desf. (Noleto & Silveira, 2004) that did not show good and rapid germination *in vitro.* Considering the number of shoots per bud multiplied by the number of buds per stem (Santos *et al*., 2006), this species has high and rapid rate of multiplication after one month of *in vitro* inoculation. Therefore, one single seedling of *C. ferrea* is able to give ten nodal segments on average, making it possible to develop a protocol of micropropagation of this species from the *in vitro* establishment (Figure 1).

The effect of different concentrations and types of cytokinins showed statistically significant differences at the 5% level for all variables (Table 1). The most appropriate treatment for multiplication was MS supplemented with

0.1 mg·L^{-1} BAP, which provided the best rate of explants with shoots (100%), large number of shoots per bud (3.37), buds per stem (2.70), and shoot height (1.52 cm), and moreover it promoted a greater multiplication rate (9.01) and did not induce callus formation (Figure 2A). According to Santos *et al*. (2006), the multiplication rate is an important parameter that allows checking on the speed of the *in vitro* propagation process. In conformity with Grattapaglia & Machado (1998), BAP is the most efficient cytokinin to promote shoot proliferation, and it presents the lowest economic cost.

A high percentage of callus formation was promoted by high concentrations of BAP, KIN, and TDZ (5.0 mg· L^{-1}). Furthermore, it was observed that callus formation interfered in the development of explants, and it gave rise to the smallest multiplication rates and shoot heights (Table 1). In relation to root formation, it was found that none of the treatments with cytokinins induced rooting.

Figure 1. Establishment of *Caesalpinia ferrea* seeds *in vitro* medium after 30 days of culture. Photo: Daniel da Silva.

In relation to the percentage of explants with shoots, there was no statistical difference between bud position treatments. However, in relation to number of buds, bud number, and height of the shoot, bud positions

Table 1. Effect of different concentrations and types of cytokinins on the *in vitro* development of *C. ferrea* explants. Means followed by the same letter in the same column do not differ statistically among themselves by Tukey test ($p < 0.05$).

Growth regulator (mg·L^{-1})	Shoot proliferation (%)	Number of shoots/bud	Number of buds/stem	Multiplication rate	Shoot height (cm)	Callus formation (%)
Control	76.67 a	1.13 cd	2.06 ab	2.34 bcd	0.37c	0.00 d
BAP						
0.1	100.00 a	3.37 a	2.70 a	9.01 a	1.52 a	0.00 d
1.0	93.00 a	2.66 a	1.76 ab	4.85 bc	1.20 ab	0.00 d
3.0	93.00 a	2.56 ab	2.15 ab	5.23 b	1.29 ab	0.00 d
5.0	20.00 bc	1.00 cd	1.20 bc	2.15 bcd	0.15 c	90.00 a
KIN						
0.1	90.00 a	1.43 c	1.73 ab	1.76 cd	1.21 ab	0.00 d
1.0	86.00 a	1.40 c	2.23 ab	2.38 bcd	0.70 bc	0.00 d
3.0	83.33 a	1.36 c	2.06 ab	3.15 bcd	0.80 abc	0.00 d
5.0	56.67 ab	1.46 bc	1.07 bc	3.12 bcd	0.79 abc	58.89 b
TDZ						
0.1	86.67 a	1.28 c	1.61 ab	1.38 d	0.63 bc	0.00 d
1.0	86.67 a	1.23 cd	1.56 ab	1.95 cd	0.73 bc	6.67 cd
3.0	83.33 a	1.03 cd	1.30 bc	1.60 d	0.65 bc	20.00 c
5.0	3.33 c	0.13 d	0.10 bc	0.20 d	0.13 c	100.00 a

showed statistically significant difference at the 5% level (Table 2), and the intermediate bud at position 5 (Figure 2B) showed the best performance when compared to other bud positions. Similar responses were found in the micropropagation of *Acmella oleracea* (L.) R.K. Jansen (electric daisy, Asteraceae), whose intermediate buds induced the greatest percentage of explants with shoots (Malosso *et al*., 2008). This shows that not all bud positions on the stem are potentially viable and should not be used indeterminately in the subculture process because they do not provide uniform explants for *in vitro* development.

According to Table 3, a statistically significant difference was found at the 5% level for all parameters analyzed at different dilutions and kinds of culture medium. B5 culture medium at the original concentration, as well as MS/2, MS/4, and WP/4, showed the lowest multiplication rates, shoot height, and consequently callus formation at the base of the seedlings, representing an inconvenient characteristic for the subsequent rooting stage.

Therefore, MS culture medium at the original concentration is best for *in vitro* multiplication of this species, because it produced the greatest percentage of explants with shoots (90%), showed total absence of callus formation, and promoted the greatest number of shoots (3.36), number of buds (2.90) and the greatest multiplication rate (10.29) (Table 3, Figure 2C). Similar results were found in the micropropagation of Amazonian plants like *Cissus sicyoide* L. (Abreu *et al*., 2003) and *Ananas comosus* (L.) Merr. (Moreira *et al*., 2011).

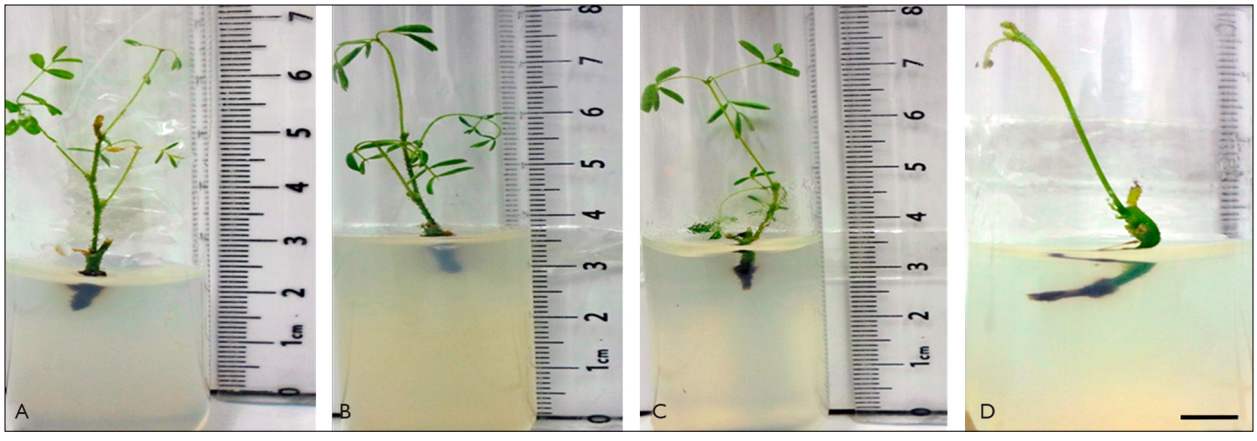

Figure 2. Aspects of seedlings provided from different experiments of in vitro multiplication of *Caesalpinia ferrea* after 30 days of culture: (A) shoot proliferation on MS; (B) shoot position on the stem in MS medium; (C) dilution of MS medium at original concentration with 0.1 mg L^{-1} BAP; and (D) shoots with root formation cultivated in MS medium with 0.1 mg L^{-1} BAP and 0.1 mg L^{-1} de AIB. Photos: Daniel da Silva.

Table 2. Influence of different bud positions on the stem on the *in vitro* development of *C. ferrea* explants. Means followed by the same letter in the same column do not differ statistically among themselves by Tukey test ($p < 0.05$).

Bud position on the stem	Shoot proliferation (%)	Number of shoots/ bud	Number of buds/ stem	Multiplication rate	Shoot height (cm)
1	76.67 a	2.70 b	1.93 ab	5.23 b	1.65 b
3	83.00 a	2.66 b	1.63 b	4.32 b	2.25 ab
5	90.00 a	4.66 a	2.33 a	10.06 a	2.73 a
7	90.00 a	3.10 ab	2.00 ab	6.34 ab	2.18 ab
9	80.00 a	3.03 ab	1.76 ab	5.36 ab	2.49 ab

Table 3. Effect of different dilutions of culture media (MS, B5, and WP) on the *in vitro* development of *C. ferrea* explants. Means followed by the same letter in the same column do not differ statistically among themselves by Tukey test ($p < 0.05$).

Basal culture medium and dilutions	Shoot proliferation (%)	Number of shoots/bud	Number of buds/stem	Multiplication rate	Shoot height (cm)	Callus formation (%)
MS	90.00 a	3.36 a	2.90 a	10.29 a	2.00 a	0.00 b
MS/2	66.67 ab	1.56 bc	1.36 bc	2.14 b	1.02 bc	20.00 a
MS/4	86.67 a	1.83 abc	1.56 bc	3.09 b	0.89 bc	6.67 ab
B5	70.00 ab	1.50 bc	1.46 bc	2.23 b	1.05 bc	6.6 ab
B5/2	73.33 ab	1.50 bc	1.60 bc	2.46 b	1.09 bc	0.00 b
B5/4	70.00 ab	1.56 bc	1.63 abc	2.55 b	1.03 bc	0.00 b
WP	96.67 a	2.96 ab	2.43 ab	7.27 ab	1.42 ab	0.00 b
WP/2	66.67 ab	1.60 bc	1.26 bc	2.43 b	0.77 bc	0.00 b
WP/4	40.00 b	0.56 c	0.50 c	0.31 b	0.32 c	23.00 a

The effect of different kinds of auxins added to MS medium resulted in the formation of adventitious roots *in vitro* and callus formation in nodal segments of *C. ferrea* (Table 4). The treatment containing AIB at the concentration of 0.1 mg· L^{-1} was the most effective and promoted the highest percentage of rooting formation and explants with shoots, buds and shoots number, multiplication rate and shoot height (Figure 2D). The best results for rooting *in vitro* using AIB have been reported in several species of Fabaceae (Kielse *et al*., 2009; Costa *et al*., 2010; Gutiérrez *et al*., 2011; Fermino Junior & Scherwinski-Pereira, 2012).

During the rooting phase, treatment with NAA, IAA, and AIB at concentration of 3.0 and 5.0 mg· L^{-1} promoted callus formation at the base of the shoots and decreased the percentage of adventitious root formation. On the other hand, callus formation was not observed at concentrations under 0.1 and 1.0 mg· L^{-1} of those auxins, thus being financially advantageous because the use of low amounts of plant growth regulators makes the protocol economically low cost, and lowest concentrations induce lower rates of somaclonal variation. Similar results were obtained by Ghimire *et al*. (2016), who found a great number of roots per explant in *Melastoma malabatricum* Linn. in MS medium supplemented with AIB, also indicating that this auxin was more effective to induce the rooting than IAA.

Considering the low rates of root formation in *C. ferrea* (Table 4), it is suggested that following studies might be performed at different combinations of these two growth regulators, auxin and cytokinin, with the goal to increase the rooting formation, because the cytokinins are active in the division process, cell elongation, and differentiation, especially in interaction with auxins (Taiz & Zeiger, 2009).

In vitro rooting conditions depend on several factors, among which are the levels of endogenous auxin, the conditions of the mother plant such as genotype or youthfulness, the culture medium, the presence of growth regulators and carbohydrates, the mineral nutrition, the presence of polyamines and substances such as activated carbon and phenolic compounds, and the environmental growth conditions of *in vitro* seedlings, among others (Souza & Pereira, 2007).

In general, the development of the *in vitro* root system of the Amazon species represents a major challenge to be achieved in the scope of efficient protocols with the use of micropropagation techniques in different species such as *C. ferrea.* Therefore, *in vitro* rhizogenesis presents a complexity of various physical and biochemical factors that are directly related to the formation of roots.

In treatments with different substrates there were significant differences at the 5% level of probability in

Table 4. Effect of different concentrations and types of auxins on the *in vitro* development and rooting of *C. ferrea* explants. Means followed by the same letter in the same column do not differ statistically among themselves by Tukey test ($p < 0.05$).

Growth regulator ($mg \cdot L^{-1}$)	Shoot proliferation (%)	Number of shoots/bud	Number of buds/stem	Multiplication rate	Shoot height (cm)	Callus formation (%)	Root formation (%)
Control	66.67 a	3.10 a	1.90 a	5.90 ab	1.66 ab	0.00 e	3.33 ab
NAA							
0.1	56.67 ab	2.36 ab	1.86 a	4.42 ab	1.45 ab	0.00 e	16.67 ab
1.0	53.33 abc	1.56 abc	1.10 abc	1.74 ab	0.70 bcd	0.00 e	0.00 b
3.0	13.33 cd	0.30 cd	0.30 bc	0.17 ab	0.25 cde	40.00 cd	3.33 ab
5.0	16.67 bcd	0.33 cd	0.33 bc	0.12 ab	0.11de	40.00 cd	6.67 ab
IAA							
0.1	66.67 a	2.03 abc	1.70 a	3.53 ab	1.28 abc	0.00 e	6.67 ab
1.0	63.33 a	2.23 ab	1.46 ab	3.27 ab	0.98 abc	0.00 e	20.00 ab
3.0	16.67 bcd	0.53 bcd	0.40 bc	0.32 ab	0.21 cde	66.67 bc	3.33 ab
5.0	3.33 d	0.06 d	0.67 c	0.01 b	0.05 b	73.33 ab	10.00 ab
AIB							
0.1	80.30 a	3.20 a	2.03 a	7.37 a	1.90 a	0.00 e	32.21 a
1.0	66.67 a	3.13 a	2.06 a	6.63 ab	1.27 abc	0.00 e	20.00 ab
3.0	53.33 abc	3.16 a	1.93 a	6.64 ab	1.09 abc	20.00 de	0.00 b
5.0	0.00 d	0.00 d	0.00 c	0.00 b	0.00 e	100.00 a	0.00 b

relation to the percentage of seedling survival acclimated at 30 and 60 days. The soil substrate provided the greatest percentage of survival (63.33%) in seedling nursery conditions, where seedlings remained green and vigorous (Figure 3A). However, the same substrate did not differ significantly from sand (53.37%) and soil + sand + vermiculite (50%) substrates. It was found that the lowest percentage of survival occurred when the seedlings were transplanted to an earthworm humus substrate (10%).

Therefore, this species grew and developed rapidly on soil substrate (Figure 3B) when compared to other treatments, and soil was the most suitable substrate for the supply of mineral nutrients and most like the natural environment. Thus, it can be verified that the large differences in the index of survival in the different substrates occurred due to the capacity of water retention, aeration, and nutrients supply to plants (Malvestiti, 2011). This result shows the possibility of acclimatization on substrate of low cost, corroborating to satisfactory results found in the seedling formation of *Vernonia condensata* Baker (Vicente *et al*., 2009) and *Morinda citrifolia* L. (Silva *et al*., 2014).

Figure 3. Acclimation of *Caesalpinia ferrea* seedlings on germinating trays with soil substrate: (A) covered by transparent glass flask at 30 days, and (B) after removal of the glass flask at 60 days. Photos: Daniel da Silva.

According to Girardi & Pescador (2010), another limiting factor in acclimatization is the adaptation of the plants from *in vitro* conditions, after transferring to environmental conditions *ex vitro*. This method sometimes entails low survival rates of the seedlings as a consequence of low photosynthetic rates of plants that are not completely autotrophic.

Several studies report that the success of the acclimatization phase is directly related to seedlings which exhibit well developed roots *in vitro* (Lédo *et al*., 2007; Silva *et al*., 2008; Pelizza *et al*., 2013). However, propagules of *C. ferrea* cultured *in vitro* even without the presence of roots survived transplanting, and subsequently initiated the formation of the root system during the acclimatization period in the nursery, allowing for growth viability and production of suitable seedlings for planting (Figure 3B).

CONCLUSION

The micropropagation of *Caesalpinia ferrea* was possible from nodal segments and intermediate buds, allowing the establishment and multiplication of *in vitro* seedlings. However, further studies are necessary to fully understand the rooting and acclimatization processes of the multiplied *in vitro* seedlings in the greenhouse.

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