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Storage of *Hymenaea Courbaril* Seeds In Subzero Temperature Up To Six Months

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ABSTRACT

The *Hymenaea courbaril* is a specie with great economic and social interest, been important to know the influence of their seeds storage on germination process. The objective of this study was to evaluate de germinative behavior of *Hymenaea courbaril* seeds as a function of freezer storage at -18ºC for 0, 2, 4 and 6 months using the completely randomized design. All the dates were submitted to variance analyses and to the dates about percentage of germination was applied the identity test of nonlinear models. The percentage of germination. It was observed by identity test for nonlinear models that there was no difference in germination rate between different storage times and the seeds stored for four and two months have the same germination behavior. *Hymenaea courbaril* seeds can be stored up to six months in freezer at -18ºC without significant losses in the final percentage of germination and despite the increase of moisture in seeds stored, this does not affect the seedlings emergency and the germination.

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INTRODUCTION

The specie *Hymenaea courbaril* L. known as jatobá da mata in Brazil, belongs to the Fabaceae/Caesalpinaceae family. This specie produces high commercial value wood with adequate characteristics to the furniture chain, phytochemistry, medicinal and pharmochemical sector (Vargas-Rechia *et al*., 1998; Busato, *et al*., 2001; Nogueira *et al*., 2001; Silva *et al*., 2001; Sano and Fonseca, 2003; Santos and Buckeridge, 2004; Fernandes, *et al*., 2005; Gonçalves *et al*., 2005; Imai *et al*., 2007). This specie is basically explored in native forest with restricted studies about their silviculture. The domestication process of any specie, starts with their propagation capability, consequently, knowledges about the storage and the germination of seeds, are essentials.

The specie *Hymenaea courbaril* L. has seeds with hard coat, orthodox with irregular germination (Lorenzi, 2002). These characteristics becomes onerous the seedling production in commercial scale.

The orthodox seeds tolerates the drying and storage at low temperatures (Baskin e Baskin, 2001). By presenting these characteristics, one of the strategies to increase the storage time of *Hymenaea courbaril* L. seeds, is the reduction of their storage temperature (Farias *et al*., 2006). The maintenance at low temperatures can reduce the metabolic rates (Walters *et al*., 1998; Bonner and Karrfalt, 2008) ensuring thus, the viability of seeds through time.

The conventional system of germoplasm bank consists in the storage of seeds in small units controlled at temperature of 10°C and 40% of moisture (Farias *et al*., 2006). Another form to storage seeds, is the cryopreservation method, which consists in the storage of seeds in cryogenic containers, where these containers are immersed in liquid nitrogen at temperature of –196 °C or just in contact with nitrogen vapor (without immersion) at temperature of -170 °C. However, those two techniques require the use of specific and impracticable equipments when used to stored seed in a small scale.

Within this context, the present study aimed to evaluate the viability of storage of *Hymenaea courbaril* L. seeds at freezer at -18°C up to six months, without submit seeds to the drying process.

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MATERIAL AND METHODS

The fruits of *Hymenaea courbaril* L. were collected from 16 seed tree after natural fall in August, 2010, in remnant of 170 hetares of Semideciduous Seasonal Forest on the Experimental Farm of Moura, Curvelo-MG, which belongs to the Federal University of the Vales do Jequitinhonha e Mucuri-UFVJM (18º49'19.25"S / 44º24'19.82"W). These fruits were kept in plastic closed bags for 30 minutes at Integrated Center of Research and Forest Studies (CIPEF) of UFVJM, Diamantina-MG. After this period, the seeds were removed from the fruits and the coat was broken with hammer help. With a knife, the endocarp was separated of the seeds. Were descarted all the seeds which presented damages and/or bad formation. The rest of seeds were separated in nine lots, each one containing 115 seeds.

The nine lots were stored in cold chamber at 6°C with relative humidity of 25%, at CIPEF in UFVJM until the beginning of experiments.

In order to evaluate the effect of freezing, was realized a pre test of germination with five of nine lots storage in cold chamber by 90 days. The seeds were submitted to five treatments (0, 3, 6, 9 and 12 days of freezing at 18°C). When removed from the freezer, the seeds remained in a cold chamber for 24 hours (6°C and 25% relative humidity). After this period, they were disinfested with a 2,5% active chlorine solution for five minutes, laterally scarified with number 80 sandpaper and kept submerged in distilled water for 24 hours to soak. Next, they were placed in a germination chamber to germinate at 30°C under previously washed, medium-textured sand and sterilized in the oven at 200°C for 2 hours (Brazil, 2009). The experiment featured a completely randomized design, with five treatments and four replicates containing 25 seeds per plot. Germination was evaluated daily.

The results of pre test demonstrated the viability of storage of *Hymenaea courbaril* seeds up to 12 days in cold chamber freezer (-18 °C), because no difference was identified ($p>0,05$) between the treatments to the variables, final germination percentage, germination speed index (GI) and moisture content, being the final germination percentual remained high in all the treatments, 92% of average.

With the positive results of pre test, proceeded with the storage of four resulting lots of Hymenaea courbaril seeds up to six months in freezer (-18 °C).

Were tested four periods of storage (treatments): T0- the seeds remained only in cold chamber; T2- seven months in cold chamber followed by two months in freezer; T4- five months in cold chamber followed by four months in freezer and T6- three months in cold chamber followed by six months in freezer.

After their respective periods of freezing, the seeds were placed off freezer and placed by two and four hours in cold chamber (6°C and 25% relative humidity), with the objective of defrosting slowly, avoiding with this, any type of damage caused by big variation of temperature.

Part of these seeds were used to determine the moisture content by oven method at 105°C ± 3°C for 24 hours (Brazil, 2009). Were used 15 seeds divided in 3 replicates, totalizing 5 seeds per replicate. The seeds were broken in four parts (Andrade *et al*., 2010), with hammer help before the realization of moisture content test. With the dates, the moisture content (%) was calculated and the dates were submitted to variance analyses and Tukey test at 5% of probability.

The seeds used in the germination test were disinfested with a 2,5% active chlorine solution for five minutes, laterally scarified with number 80 sandpaper and kept submerged in distilled water for 24 hours to soak.

After the pre germinative tests, the seeds were placed to germinate at Silviculture and Seedling Production Laboratory, from UFVJM, in three cold chambers, type B.O.D. - NI 1718 (New Instruments) at medium and constant temperature of 30°C, with constant light, using like substrate medium-textured sand washed and sterilized in the oven at 200°C for 2 hours (Brazil, 2009).

Daily, was realized counts of germinated seeds and was realized the irrigation of the experiment, considering germinated, seeds with root protrusion superior than 2 mm of length (BRASIL, 2009).

The experiment featured a completely randomized design, with four previously mentioned treatments and four replicates containing 25 seeds per plot.

The germination was evaluated by germination speed index (GI) (Maguire, 1962), by cumulative germination curves and final percentage of germination. At the end of experiment were accounted the quantity of produced seedling per treatment, considering like seedlings, those plants which presented emitted the first leaves. The dates were submitted to descriptive analyses and analysis of variance with Tukey test with 5% of probability.

Due the germinative process presents a characteristic sigmoidal, logistical models were adjusted in attempting to try describe this process, once that the independent variable (time) is continuous and the dependent variable (germination) is categorical.

In this way, were adjusted to germination equations under the logistical model, in which: $y_{ij} = a_i/(1 + b_i e^{-c_i x_{ij}}) + \varepsilon_{ij}$, where y_{ij} is the observed value (cumulative germination) for the i^{th} treatment (i=1,...,g) on the j^{th} day (j=1,...,n); x_{ij} is the value of the independent variable (j^{th} day for the i^{th} treatment) ; a_i

(maximum expected value - final germination percentage), b_i (in this case, as higher b to the same c value, higher is the time that the germination needs to start) and c_i (medium rate of curve growth - germination speed).are the parameters of the model; and ε_{ij} is the random error not observable in the model (Souza, *et al*., 2013).

With the equations adjusted, were apply identity test for nonlinear models, following the methodologies proposed by Souza *et al*. (2013). The equations were considered equals when all the hypotheses tested were not significant.

All analyses were performed using the software program Statistica 10.0 (STATSOFT INC., 2010).

Results:

The treatments effects under the moisture content of seeds were significant (Table 1).

Table 1: Medium moisture content in percentage and coefficient of variance of treatments of *Hymenaea courbaril* seeds submitted to four times of storage 0, 2, 4 and 6 months) at -18°C.

	----------Treatments----------			
	T0	T2	T4	T6
Moisture content (%)	11,90 a	13,07 b	13,23 b	14,79 c
Coefficient of variance (%)	1,58	1,82	1,44	1,68

Means followed by the same letters in column do not differ significantly by the Tukey test ($p < 0.05$).

The germination started in the sixth day of evaluation, lasting until 47, 44, 45, 46 days after the beginning of experiment, with final percentage of 82, 87, 87 and 73%, respectively to the treatments T0, T2, T4 and T6. In all treatments was verified higher speed germination index between the sixth and the fourteenth day (Figure 1).

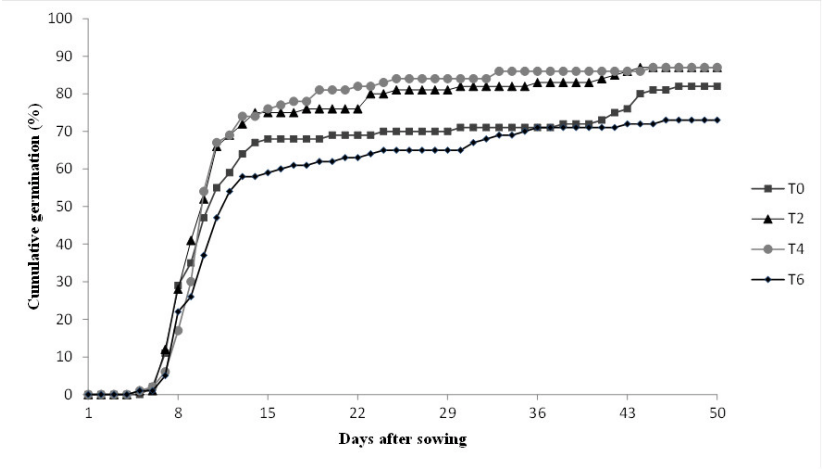

Fig. 1: Cumulative germination for *Hymenaea courbaril* seeds submited to storage (- 18 ºC) for one of four time periods.

The treatments effects were significant to the final percentage of germination and GI and no significant to the quantity of seedlings produced per treatment (Table 2).

Table 2: Resume of variance analyses to the variables percentage of germination, GI and quantity of seedlings obtained in the experiment with *H. courbaril* seeds submitted to 4 times of storage (0, 2, 4 and 6 months) at -18°C.

		-% Germination-		---------IVG-------		Seedlings Quantity	
FV	GL	QM	P-Valor	QM	P-Valor	QM	P-Valor
Treatments	3	174,33	0,025	0,182	0,007	15,729	0,246
Residuals	12	39,00		0,028		9,979	
Total	15	213,33		0,210		25,708	
Coefficient of variance		8,03		10,81		12,44	

In the table 3 are found the comparisons between averages to the three variables by Tukey test at 5% of probability.

Table 3: Estimated averages to percentage of germination, GI, and final number of seedlings obtained in the experiment with *H. courbaril* seeds submitted to 4 times of storage (0, 2, 4 and 6 months) at - 18°C.

Treatments	% Germination	GI	Seedlings Quantity Obtained
T2	87 a	2,165 a	66 a
T4	87 a	2,122 a	72 a
T0	82 a b	1,921 a b	64 a
T6	73 b	1,699 b	53 a

Means followed by the same letters in column do not differ significantly by the Tukey test ($p < 0.05$).

By the identity test for nonlinear models, was observed no significant difference to the treatments T4 and T2. Thus, these two treatments were represented by a single model. Already among these and the others treatments this identity was not verified (Table 4 and Figure 3).

It is observed yet, that the final percentage of germination, represented by coefficient *a* in the equation, was higher and equal in the treatments T2 and T4, followed by treatments T0 and T6 (Table 4 and Figure 3). Already the germination speed, represented by coefficient *c* in the equation, was equal in all treatments.

The coefficient *b* in the equation was different only between the treatments T6 and T4 (Table 4).

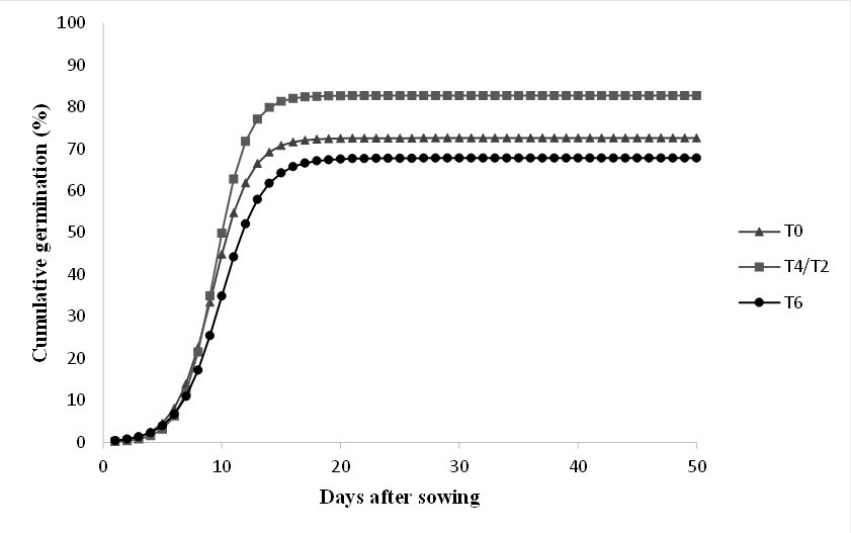

Fig. 2: Cumulative germination curves for *Hymenaea courbaril* seeds submited to storage (- 18 ºC) for one of four time periods, according to the model $y = a/(1+be^{-cx})$. The estimated parameters for each equation are presented in table 4.

Table 4: Identity tests for the equations derived in this study, where the dependent variable is the seed germination rate of *Hymenaea courbaril* and the independent variable is the duration (0, 2, 4 and 6 months) of storage (- 18°C) to which seeds were subjected, along with the degrees of freedom, estimated F and respective level of significance.

Identity for the parameter a				Identity for the parameter b			
Hypotheses	Fc	GL	P(F)	Hypotheses	Fc	GL	P(F)
$H_0^{(1)}$: $a_6=a_4$	206,29*	1	0,0000	$H_0^{(2)}$: $b_6=b_4$	-4,89*	1	0,0294
$H_0^{(1)}$: $a_6=a_2$	126,63*	1	0,0000	$H_0^{(2)}$: $b_6=b_2$	$-3,06^{ns}$	1	0,0835
$H_0^{(1)}$: $a_4=a_0$	77,93*	1	0,0000	$H_0^{(2)}$: $b_4=b_2$	$2,63^{ns}$	1	0,1082
$H_0^{(1)}$: $a_2=a_0$	42,36*	1	0,0000	$H_0^{(2)}$: $b_4=b_0$	$0,75^{ns}$	1	0,3888
$H_0^{(1)}$: $a_6=a_0$	16,94*	1	0,0001	$H_0^{(2)}$: $b_2=b_0$	$0,32^{ns}$	1	0,5756
$H_0^{(1)}$: $a_4=a_2$	$2,89^{ns}$	1	0,0927	$H_0^{(2)}$: $b_6=b_0$	$-0,80^{ns}$	1	0,3740
Identity for the parameter c				Identity for the parameters a, b and c			
Hypotheses	Fc	GL	P(F)	Hypotheses	Fc	GL	P(F)
$H_0^{(3)}$: $c_6=c_2$	-4,00*	1	0,0485	$H_0^{(5)}$:$a_6=a_4;b_6=b_4;c_6=c_4$	66,88*	3	0,0000
$H_0^{(3)}$: $c_6=c_4$	$-3,50^{ns}$	1	0,0645	$H_0^{(5)}$:$a_6=a_2;b_6=b_2;c_6=c_2$	41,70*	3	0,0000
$H_0^{(3)}$: $c_4=c_2$	$2,26^{ns}$	1	0,1365	$H_0^{(5)}$:$a_4=a_0;b_4=b_0;c_4=c_0$	28,32*	3	0,0000
$H_0^{(3)}$: $c_6=c_0$	$-1,76^{ns}$	1	0,1877	$H_0^{(5)}$:$a_2=a_0;b_2=b_0;c_2=c_0$	15,06*	3	0,0000
$H_0^{(3)}$: $c_4=c_0$	$1,50^{ns}$	1	0,2242	$H_0^{(5)}$:$a_6=a_0;b_6=b_0;c_6=c_0$	5,84*	3	0,0011
$H_0^{(3)}$: $c_2=c_0$	$0,07^{ns}$	1	0,7875	$H_0^{(5)}$:$a_4=a_2;b_4=b_2;c_4=c_2$	$2,27^{ns}$	3	0,0849
Estimates of the parameters a, b and c for different freezing times at - 18°C							
Parameters	0 months (T0)	2 months (T2)	4 months (T4)	6 months (T6)	2 and 4 months (T2/T4)		
a	72,5991	81,6075	83,8310	67,7899	82,7448		
b	364,0380	602,4059	1997,9862	279,9200	978,2219		
c	0,6380	0,6950	0,7900	0,5695	0,7302		
R^2	0,9720	0,9840	0,9898	0,9756			

a_0, b_0 and c_0: parameters of the equation corresponding to 0 months of storage; a_2, b_2 and c_2: parameters of the equation for 2 months of storage; a_4, b_4 and c_4: parameters of the equation for 4 months of storage; a_6, b_6 and c_6: parameters of the equation for 6 months of storage. ^{ns}: not significant and *: significant at 5% probability.

Discussion:

The most part of orthodox species seeds remains viable after desiccation up to a moisture content low than 10% and can be stored in subzero temperature for a long time (Bonner and Karrfalt, 2008; Santos 2000). The *H. courbaril* seeds presented moisture content higher than 10% (Table 1). This value is closest to 15%, which is established like limit of moisture for storage of seeds at temperatures under to -15°C (Bonner e Karrfalt, 2008).

When the moisture content overcomes this tolerance limit can occurs increase in the respiratory rate during the storage with consequences like, loss of germinative vigor. This effect was not observed in seeds used in this study, which presented elevated germinative capacity and production of normal seedlings when frozen up to six months in medium temperature of -18 °C. This results demonstrates yet, that *H. courbaril* seeds can be stored up to six months in freezer without pass through the dry process.

During the storage, the moisture content increases according with the time of storage (Table 1). The moisture increase of *Psidium guineense* seeds, was observed when these seeds were storage in same conditions by 180 days, however, when was used a glass recipient, the increase of moisture seeds was not significant (Cisneiros *et al*., 2003). This behavior was also observed when sunflowers (*Helianthus annuus*) was storage in paper bags for three months at - 20°C (José *et al*., 2010).

When seeds are stored with elevated moisture in subzero temperatures, can occurs ice crystal formation from the free water presents into cells causing injuries and loss of germinative capacity (Santos, 2000; Fonseca and Freire, 2003). In this present study, this was not a problem because the treatments presented high germination and besides this, the treatments T2 and T4 were superiors in germination than the treatment T0, which was not submitted to the storage in freezer (Table 3, Figure 1).

Another problem which influences the quality of seeds during the storage and it is directly related with the moisture, is the presence and proliferation of fungi (Rupollo, 2006). However, it was not verified big attacks during the conduction of experiment.

There was a gain in the germination when seeds were frozen for two and four months, however, by Tukey test, at 5% of probability, this gain was not significant in relation to the treatments where the seeds were not frozen (Table 3). Gains with the storage of *H. courbaril* seeds, stored at temperatures around - 20°C and - 30°C are described in the literature (Farias *et al*., 2006; Lima *et al*., 2008).

Another result that call attention is, despite of no apparent significant differences in the final number of seedlings production, the treatment with seeds, frozen for four months it was the one which promoted the higher production (Table 3). This value must be considered, viewed the big difference between the treatment which presents the higher production (T4 = 72) with that treatment which produces less (T6 = 53) (Figure 3).

Low temperatures can result in low establishment of seedlings and in the reduction of biomass, mainly between tropical or subtropical species (Bedi and Basra, 1993; Bewley and Black, 1994). In this case, just not the low temperature (-18°C), also the moisture and the time that the seeds stayed frozen, influenced in the establishment of seedlings, being more prejudicial to the seeds the freeze for six months (Figure 3).

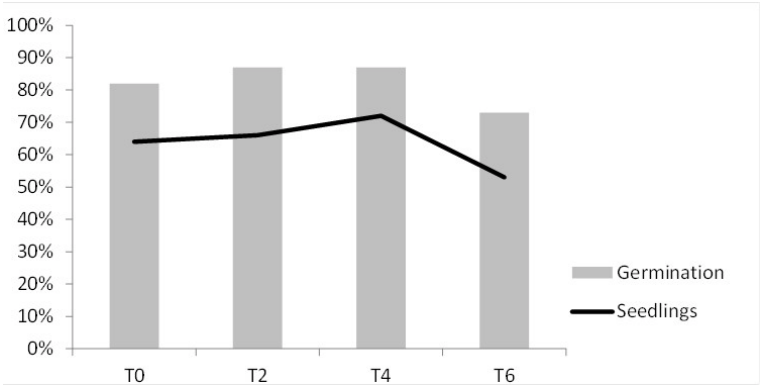

Fig. 3: Germination percentage and quantity of seedlings obtained per treatment in the experiment with *H. courbaril* seeds submitted to four times of storage (0, 2, 4 and 6 months) at -18°C.

The identity models test shows that seeds stored for four and two months presented the same germinative behavior (Tables 3, 4 and Figure 2). And just as the GI, shows also that the storage didn´t influence in the speed germination of seeds.

Another important observation about the identity models and the parameters of adjust equation is the time that the germination takes to start. A higher b to the same c, is a higher time that the germination takes to start. (Souza *et al*., 2013). In this study, despite b, be different only in the treatment T4 and T6, the time for the beginning of germination was higher in the treatments T4/T2, T0 and T6 (Table 4 and Figure 2). In this way the treatments T4/T2 despite presents higher percentage of germination took more time to start the germination process.

Conclusion:

It is viable the storage of *Hymenaea courbaril* in freezer (18°C) up to six months. Up to four months there is a gain in final percentage of germination and at six months the percentage of germination is less than the storage, however the germination keeps higher.

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