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Germination Behaviour Evaluated Using The Identity Test For Nonlinear Models

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Abstract: When considered continuously over time, the germination behaviour is better described by regression models then by speed germination index and/or germination percentage. However, very few studies have evaluated the differences between models and their respective parameters using statistical criteria to explain the effects of treatments. The objective of this study was to apply the identity test for nonlinear models to evaluate germination behaviour continuously over time and compare the results with single-value indices. Data from an experiment on Jatobá were used in which the seeds were subjected to five different freezing time periods at - 20°C (0, 3, 6, 9 and 12 days). The identity test for nonlinear models was applied to these data, and the germination index and the final germination percentage were calculated. Unlike the single-value indices, for which there were no statistically differences among treatments, the identity test for nonlinear models discriminated accurately the adjusted equations for each treatment. The identity test proved to be effective in elucidating the behaviour of cumulative germination curves.

Key words: logistic function, seeds, *Hymenaea courbaril*.

INTRODUCTION

Several indices have been proposed to describe seed germination patterns in the laboratory and under different field conditions. However, the usefulness of these indices has been questioned because they differ in the extent to which they summarize the germination patterns (Torres and Frutos, 1989). Indices such as the germination index (GI) (Maguire, 1962) and the final germination percentage are still widely used, however, they do not reflect germination behaviour continuously over time (Brown and Mayer, 1988a). The most appropriated is the method that evaluates the evolution of the germination rate over time and the behaviour of each phase of this process (Brown and Mayer, 1986).

The GI evaluates the germination vigor of seeds and has been used in several studies by researchers in the field of agricultural sciences (Grus et al., 1984; Albuquerque et al., 1998; Costa et al., 2007; Zhang et al., 2010). Theoretically, the GI is influenced by the mean time and the speed of seed germination, i.e., if germination occurs soon after seeding, the value of the index will be higher than if it occurs later. In addition, the GI is suitable only for comparisons between samples or treatments in experiments that utilize the same number of germinated seeds (Santana and Ranal, 2000). Therefore, in studies in which the main objective is to test hypotheses regarding the effects of different factors on germination, this index would only be appropriate if these hypotheses are not rejected. The GI also becomes limited by not considering the seeds that do not germinate.

To overcome this limitation, several researchers present the results of their studies using germination regressions models with or without fitting them using mathematical models. The studies performed by Brown and Mayer (1988b), Torres and Frutos (1989 and 1990), Timmermans et al. (2007) and Joosen et al. (2010) involved the use of equations from which parameters, such as the speed of germination and the highest germination rate, are estimated. Based on the sigmoidal nature of the germination process, the models that best fit this phenomenon are nonlinear. Such models are also logistic since the independent variable (time) is continuous and the dependent variable (germination) is categorical. Meanwhile, studies assessing the differences between these models, which are characteristic of the germination process generated by these equations are rare.

The identity test for nonlinear models proposed by Regazzi (2003) and Regazzi and Silva (2004) has great potential for use in analysing germination processes because it allows researchers to assess whether the fit equations of a given data set are identical in any nonlinear regression model, i.e., to assess whether the phenomenon under study can be represented by a single equation that can discriminate between treatment conditions.

Within this context, the present study aimed to apply the identity test for nonlinear models to continuously evaluate germination behaviour over time and compare the results with single-value indices.

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MATERIALS AND METHODS

The seeds of *Hymenaea courbaril* L. (Jatobá) used in this study were obtained from fruits collected in August 2010 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).

After collection, seeds samples were submitted to five subzero treatments (zero, 3, 6, 9 or 12 days in a -20°C freezer). 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.

For each treatment, the cumulative germination over time was fit to the model that best represented it, using as a selection criterion the coefficient of determination and the graphical analysis of residuals (Torres and Frutos, 1989). A logistic model was chosen, as in the studies by Torres and Frutos (1989, 1990).

For each treatment, the following equations were fit to the logistic model $y_{ij} = a_i/(1 + b_i e^{-c_i x_{ij}}) + \varepsilon_{ij}$, 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, b_i and c_i are the parameters of the model; and ε_{ij} is the random error not observable in the model (Torres and Frutos, 1989).

The equivalence between the equations was determined by comparing their coefficients using the identity test for nonlinear models according to the methodologies proposed by Regazzi (2003) and Regazzi and Silva (2004). The main steps of the proposed test are described in Table 1.

Table 1: Sequence and criteria used in the identity test for nonlinear models, adapted from Regazzi (2003) and Regazzi and Silva (2004).

Step	Sequence	Criterion
1	Fit the data to a single model.	Choose the best growth model according to the coefficient of determination (R^2) and the graphical analysis of the residuals.
2	Formulate hypotheses (H_i)	$H_0^{(1)}$: a_1=...=a_g (=a) *vs*. $H_a^{(1)}$ = not all a_i values are equal; $H_0^{(2)}$: b_1 =...= b_g (= b) *vs*. $H_a^{(2)}$ = not all b_i values are equal; $H_0^{(3)}$: c_1 =...= c_g (= c) *vs*. $H_a^{(3)}$ = not all c_i values are equal; $H_0^{(4)}$: a_1 =...= a_g (= a), b_1 =...= b_g (= b) and c_1 =...= c_g (= c) *vs*. $H_a^{(4)}$; at least one equality is an inequality.
3	Estimate the parameters	$\hat{a}_i = \frac{y_B(y_A y_B + y_B y_C - 2 y_A y_C)}{y_B^2 - y_A y_C}$ For $\hat{b}_i$ and $\hat{c}_i$, linearization of the fitted equation should be performed. Evaluate the appropriateness of the method by adding a constant one (1) to y_i to estimate all parameters because, in the early days of counting, the germination is zero.
4	Calculate the sum of squares of the regression residuals	Use the parameters estimated in step 3 for each equation generated for each tested hypothesis and for the complete model (without restriction). Use dummy variables to compare pairs of equations for i = 1,..., g and the variables "dummy D_i", such that D_i = 1 if the observation y_{ij} belongs to group i and 0 otherwise. $y = \sum_{i=0}^{n} D_u [a_u/(1 + b_u e^{-c_u x_{ij}})] + \varepsilon_{ij}$, where j=1, ..., n_i, i=1, ..., g.
5	Compare the equations using the F-test or chi-square test.	For $N \geq 120$, use the F-test or chi-square test; for $N < 120$, use the F-test.
a, b, c = model parameters; y_A, y_B and y_C = cumulative germination at equidistant points A, B and C; N = sample size.		

The GI (Maguire, 1962) and the final germination percentage (G%) were also calculated, the latter of which was calculated as G% = (NGS_n/TNS) x 100, where NGS is the total number of germinated seeds on the last day of treatment and TNS is the total number of seeds.

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

Results:

The results for the four hypotheses formulated for each combination of two treatments (-20°C for different freezing times) are presented in Table 2. The hypothesis of complete identity of the model ($H_0^{(4)}$) was not rejected for comparisons between six and nine and between nine and twelve days of freezing. Therefore, the identity test for nonlinear models was performed on these three equations. The results of this test confirmed the equality among these three treatments (Table 2). Thus, these three treatments were represented by a single model (Figure 1).

Table 2: 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, 3, 6, 9 and 12 days) of freezing (-20°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)
$H_0^{(1)}$: $a_0=a_{12}$	103.28*	1	0.000	$H_0^{(2)}$: $b_6=b_{12}$	102.55*	1	0.000
$H_0^{(1)}$: $a_6=a_{12}$	101.79*	1	0.000	$H_0^{(2)}$: $b_0=b_{12}$	30.86*	1	0.000
$H_0^{(1)}$: $a_3=a_6$	40.08*	1	0.000	$H_0^{(2)}$: $b_6=b_9$	3.09^{ns}	1	0.086
$H_0^{(1)}$: $a_0=a_3$	24.72*	1	0.000	$H_0^{(2)}$: $b_6=b_9=b_{12}$	2.94^{ns}	1	0.094
$H_0^{(1)}$: $a_3=a_9$	24.32*	1	0.000	$H_0^{(2)}$: $b_0=b_9$	1.44^{ns}	1	0.237
$H_0^{(1)}$: $a_3=a_{12}$	24.09*	1	0.000	$H_0^{(2)}$: $b_3=b_6$	0.55^{ns}	1	0.463
$H_0^{(1)}$: $a_0=a_9$	19.04*	1	0.000	$H_0^{(2)}$: $b_9=b_{12}$	0.35^{ns}	1	0.557
$H_0^{(1)}$: $a_0=a_6$	16.82*	1	0.000	$H_0^{(2)}$: $b_0=b_6$	-0.34^{ns}	1	0.563
$H_0^{(1)}$: $a_6= a_9=a_{12}$	1.02^{ns}	1	0.319	$H_0^{(2)}$: $b_3=b_9$	-1.52^{ns}	1	0.225
$H_0^{(1)}$: $a_6=a_9$	0.44^{ns}	1	0.511	$H_0^{(2)}$: $b_3=b_{12}$	-2.56^{ns}	1	0.117
$H_0^{(1)}$: $a_9=a_{12}$	0.06^{ns}	1	0.808	$H_0^{(2)}$: $b_0=b_3$	-2.79^{ns}	1	0.102
Identity for the parameter c				Identity for the parameters a, b and c			
Hypotheses	Fc	GL	P(F)	Hypotheses	Fc	GL	P(F)
$H_0^{(3)}$: $c_6=c_{12}$	104.10*	1	0.000	$H_0^{(4)}$: $a_6=a_{12}$; $b_6=b_{12}$; $c_6=c_{12}$	44.14*	3	0.000
$H_0^{(3)}$: $c_6=c_9$	3.85^{ns}	1	0.057	$H_0^{(4)}$: $a_0=a_3$; $b_0=b_3$; $c_0=c_3$	35.75*	3	0.000
$H_0^{(3)}$: $c_6=c_9=c_{12}$	3.56^{ns}	1	0.066	$H_0^{(4)}$: $a_3=a_6$; $b_3=b_6$; $c_3=c_6$	12.12*	3	0.000
$H_0^{(3)}$: $c_0=c_9$	1.62^{ns}	1	0.210	$H_0^{(4)}$: $a_3=a_{12}$; $b_3=b_{12}$; $c_3=c_{12}$	11.32*	3	0.000
$H_0^{(3)}$: $c_3=c_6$	1.46^{ns}	1	0.234	$H_0^{(4)}$: $a_3=a_9$; $b_3=b_9$; $c_3=c_9$	10.74*	3	0.000
$H_0^{(3)}$: $c_0=c_{12}$	0.89^{ns}	1	0.351	$H_0^{(4)}$: $a_0=a_{12}$; $b_0=b_{12}$; $c_0=c_{12}$	8.42*	3	0.000
$H_0^{(3)}$: $c_9=c_{12}$	-0.01^{ns}	1	0.921	$H_0^{(4)}$: $a_0=a_6$; $b_0=b_6$; $c_0=c_6$	7.79*	3	0.000
$H_0^{(3)}$: $c_0=c_6$	-0.08^{ns}	1	0.779	$H_0^{(4)}$: $a_0=a_9$; $b_0=b_9$; $c_0=c_9$	6.23*	3	0.001
$H_0^{(3)}$: $c_3=c_9$	-0.59^{ns}	1	0.447	$H_0^{(4)}$: $a_6=a_9=a_{12}$; $b_6=b_9=b_{12}$; $c_6=c_9=c_{12}$	2.12^{ns}	6	0.075
$H_0^{(3)}$: $c_0=c_3$	-1.58^{ns}	1	0.216	$H_0^{(4)}$: $a_6=a_9$; $b_6=b_9$; $c_6=c_9$	1.98^{ns}	3	0.133
$H_0^{(3)}$: $c_3=c_{12}$	-2.88^{ns}	1	0.097	$H_0^{(4)}$: $a_9=a_{12}$; $b_9=b_{12}$; $c_9=c_{12}$	0.50^{ns}	3	0.684

Estimates of the parameters a, b and c for different freezing times at -20°C						
Parameters	0 days	3 days	6 days	9 days	12 days	6, 9, and 12 days
a	75.90887	97.42955	84.47049	85.83696	87.10428	85.81032
b	115.83390	219.34957	267.17679	68.59787	90.61642	98.57347
c	0.47319	0.51940	0.57233	0.40420	0.40496	0.43937
R^2	0.98214 0.98	0.99129 0.99	0.98360 0.98	0.98143 0,98	0.98360 0,98	0.98612 0,98
"*x*" Max	10.04281	10.37864	9.76344	10.46082	11.12859	10.44860

a_0, b_0 and c_0: parameters of the equation corresponding to 0 days of freezing; a_3, b_3 and c_3: parameters of the equation for 3 days of freezing; a_6, b_6 and c_6: parameters of the equation for 6 days of freezing; a_9, b_9 and c_9: parameters of the equation for 9 days of freezing; a_{12}, b_{12} and c_{12}: parameters of the equation for 12 days of freezing. ^{ns}: not significant and *: significant at 5% probability. "*x*" Max is the time (in days) at which the germination rate reached its maximum value, given by the expression Ln_b/c.

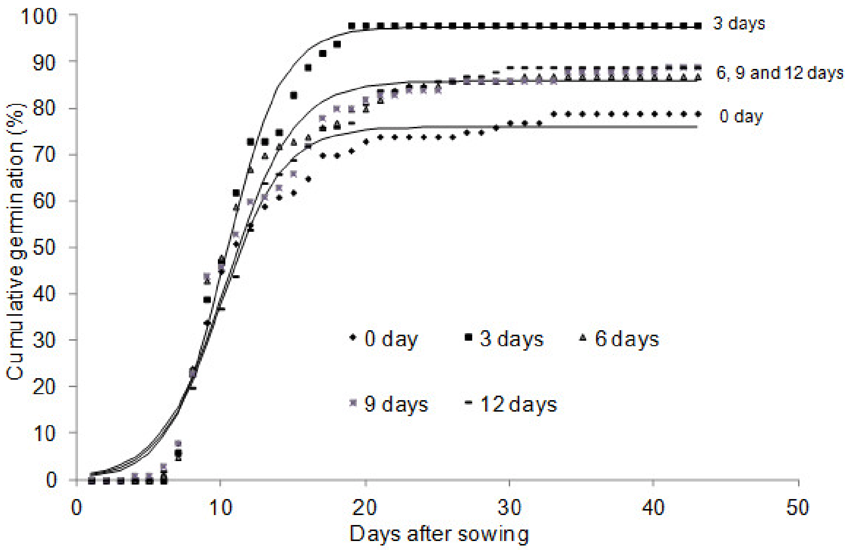

Fig. 1: Cumulative germination curves for Hymenaea courbaril seeds submiyyed to freezing for one of five time periods, accords to the model $y=a\ (1+be^{-cx})$ for each equation are presentd in Table 1.

The equations for the treatments of zero and three days of freezing were significantly different from each other and from the others, implying a separate equation for each of these treatments (Table 2, Figure 1). Despite these differences, Figure 1 shows that, for each model, the deviations between the observed and expected data were negligible, an observation confirmed by high coefficients of determination (Table 2).

The hypothesis of the identity between the parameters a_0 and a_3 in all combinations involving zero and three days of freezing, both for separate treatments and treatment combinations, was rejected. The hypothesis of the identity between a_6 and a_{12} was also rejected. However, the hypothesis of the identity between a_6 and a_9 and between a_9 and a_{12} was not rejected (Table 2).

Regarding comparisons of the estimates of the parameters b_i, the estimate of b_{12} differed significantly from the estimates of b_0 and b_6. None of the other comparisons were rejected (Table 2). The parameter *c* differed significantly only for the freezing times of six and twelve days. However, when the hypotheses of equality among the parameter *c* of the equations for six, nine and twelve days of freezing were assessed together, they were not significantly different.

A summary of the analysis of variance for the variables GI and final germination percentage for five treatments (five freezing times at -20°C) is presented in Table 3. For the variables GI and final germination percentage, there were no significant differences among the different freezing times despite the differences observed in the dispersion of the original data (Figure 1).

The means of the GI and final germination percentage for each freezing time are shown in Table 4.

Table 3: Summary of ANOVA results for the GI and final germination percentage of *Hymenaea courbaril* seeds submitted to freezing for one of five time periods (0, 3, 6, 9 or 12 days) at -20°C.

		GI		Germination percentage	
FV	GL	QM	P-value	QM	P-value
Treatments	4	0.274	0.755	0.061	0.422
Residuals	15	0.579		0.059	
Total	19	0.853		0.119	
Coefficient of variance		25.29%		18.73%	

Table 4: Mean GI and final germination percentage of *Hymenaea courbaril* seeds submitted to freezing for one of five time periods (0, 3, 6, 9 or 12 days) at -20°C.

	Duration of freezing (-20ºC) in days				
Variables	0	3	6	9	12
Mean GI	1.72	2.35	2.13	2.12	2.02
Final germination percentage	79%	98%	87%	88%	89%

Discussion:

To support the discussion of the parameters *a*, *b* and *c* and their biological implications, it is important to consider the function under study when $x = 0$ and when $x => \infty$, where $y = a/(1+b)$ and $y => a$, respectively.

For the parameters *b* and *c*, it is also important to consider the expression Ln_b/c, which represents the value of *x* where the function has its maximum slope (Table 2), obtained by equating the second derivative of the function under study $\{-abc^2(e^{cx}-b^2e^{-cx})/(e^{cx}+2b+b^2e^{-cx})^2\}$ to zero.

The most important biological implication of the parameter *a* is obtained when considering $x => \infty$ because this case represents the maximum percentage of germinated seeds and is asymptotically approached over time. Observing the estimates for this parameter (Table 2, Figure 1), it is evident that the seeds under submitted to three days of freezing yielded the highest percentage of germination. Because the estimate of the parameter a_3 was significantly different from the estimates of the other a_i parameters, we conclude that the three days of freezing at -20ºC promoted a final percentage of germination that was significantly higher than that obtained from the other treatments. However, the final germination percentage and the GI were not significantly different among the five treatments according to the F-test in the ANOVA (Table 3). Therefore, the identity test among the parameters a_i of the model in question was more effective in detecting the final differences in the germination rate.

The biological implications of the parameter *b* are also more important for values of *x* above zero because, along with *c*, this parameter determines the point (Ln_b/c) of maximum slope of the curve. For the same value of *c*, the higher the value of *b*, the greater the interval over which the curve remains asymptotically near the *x* axis. In the present study, this relationship is important because the *x* axis refers to time; therefore, high values of *b* imply a greater delay in the beginning of germination and/or a lower speed (rate) of germination. Considering these statements and the identity tests for the *b* estimates (Table 2), we can conclude that, in the present study, the time at which germination began, as well as its rate, was not significantly different in any combination involving zero and nine days of freezing. This is another finding not elucidated by the GI or the final germination percentage, again demonstrating the effectiveness of the identity test for nonlinear models in germination studies.

The parameter *c* is also involved in determining the maximum slope (Ln_b/c) of the curve. For a given value of *b*, the lower the value of *c*, the greater the value of *x* which corresponds to the maximum curvature.

For the data under analysis, because the time values are on the *x* axis, the lower the estimate of *c*, the slower germination speed (Figure 1). From the results of the analysis, it can be inferred that the germination resulting from twelve days of freezing was slower than that resulting from six days of freezing (Table 2). The estimates of *c* for the other freezing time periods did not differ from each other.

One important observation to note is that the identity between the estimates of *b* was rejected for six and twelve days of freezing but not for six and nine days of freezing (Table 2). The difference between these *b* estimates for the first case (i.e., six days of freezing) was greater than that between the estimates for the second case (i.e., twelve days of freezing) (Table 2). This apparent contradiction may be due to the joint behaviour of the parameters *b* and *c*.

The estimated time to reach the highest germination rate (Ln_b/c) ranged from 9.8 to 11.1 days for the treatments considered, including the treatment combination involving six, nine and twelve days of freezing (Table 2). Although there is no statistical test to verify the identity between these estimates, their small degree of variation implies that the time to reach the highest germination are essentially the same for all freezing times.

For the full model identity tests, we found that the identity between six and nine days of freezing and between nine and twelve days of freezing was not rejected. Although the identity was rejected when considering six and twelve days of freezing, it was not rejected when considering a single model for a freezing time of six or more days (Table 2). Therefore, it can be concluded that a single model can represent the seed germination behaviour of the species for six or more days of freezing (Figure 1).

The use of equations to demonstrate the behaviour of seed germination relative to single-value indices was emphasized in some studies (Brown and Mayer, 1988b; Torres and Frutos, 1989, 1990; Timmermans *et al*., 2007; Joosen *et al*., 2010). Although more elucidative than the traditional method, the approach taken in such studies is not ideal because, in experiments in which there is more than one treatment under test, the approach is limited to estimating parameters that determine the best model for each treatment. The identity test for nonlinear models allows for a comparison between the estimated parameters, thereby enabling a detailed analysis of the biological phenomenon under investigation.

In the literature, we found several studies that used the identity test for linear and nonlinear models (Sarmento *et al*., 2006; Casali *et al*., 2008; Tironi *et al.*, 2009; Pompelli *et al*., 2012). These tests are commonly used in other research fields, demonstrating their usefulness beyond investigations of seed germination. However, their use is still rare, and further studies in the area of seed germination are required.

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