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Understanding In Vitro Embryo Development through Classical Germination Measurements: A Case Study of Dragon's Blood (*Croton lechleri* Müll Arg.)

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Abstract: Sample size fluctuation and the restriction of measurements that demonstrate kinetics (typical of physiological processes) are two of the largest inferential constraints in studies on embryonic development in vitro. Thus, we hypothesize that a practical and robust way of aggregating knowledge on aspects of embryonic development in vitro is to use measurements based on the binary counting component. These are typically used to measure the germination process (intraeminal embryonal development). Our biological model was Dragon's blood (*Croton lechleri* Müll Arg.), a species native to the Amazon with great socioeconomic impact. Matrices originating from two populations (one native and another cultivated) were the source of biological material. From this material, we studied five sampling densities (5, 25, 50, and 100 embryos), forming a 2 × 4 factorial ANOVA. Among the measurements studied, the coefficient of variation of time, uncertainty, and the synchronization index were the most sensitive to sample-size fluctuation. The synchronization index, however, also proved to be an interesting measurement to detect the parental effect related to the place of occurrence of the matrices. The embryonic development ability, mean development time, and mean development rate were not affected by fluctuations in the sample size or the origin of the material, demonstrating highly conserved traits of the species. Finally, in general, the measurements based on binary counting demonstrated robustness for modeling embryonic growth.

Keywords: embryo development; functional traits; growth and development; modeling and measurements; sample size

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1. Introduction

Plant tissue culture is a science that is based on the determination of hormonal balance (mainly auxin-cytokinin interactions) in species-specific protocols [1,2] to facilitate the management of germplasm banks and to clarify hormonal functions [2–4]. However, few algebraic aspects have been determined to explore the patterns of in vitro development. Only absolute measurements, as the percentage value of the number of events, were popularized among plant scientists [5,6]. These measurements are practical, but they do not allow a clear analysis of the kinetics of physiological processes. The kinetic aspects play a key role in understanding patterns of development in an organism, organ, cell, or tissue [7,8]. Thus, we ask: How can we improve our understanding of in vitro development aspects? An answer could come from growth/development measurements made for various aspects of the seed germination process.

The development aspects as a whole can be studied by means of continuous (in which any real value can be attributed to the event) or discrete characteristics (in which the event happens or not, i.e., a binomial phenomenon), which ends up defining inferential aspects associated with data distribution [8–11]. In general, germination is the most studied discrete physiological process [7], whereas mass accumulation (fresh or dry) is the most commonly studied continuous event [11]. In the case of in vitro cultivation, embryonic development is only quantified through the total percentage of positive events and, in some cases, through the dry mass [12]. Thus, to make inferences about in vitro embryonic development, the researcher either only assigns absolute values, working with small samples, or substantially increases the sample size due to the destruction of individuals at each time of analysis, for example, the measurement of growth dynamics, such as biomass curves (destructive analysis) [8]. What is not considered in this type of analysis is the fact that embryonic development presents the potential to be considered a binomial event, in which development occurs or not. For this, it is possible to make an analogy with seed germination, an intraseminal amphibolic process where the embryo grows/develops through the seed wraps (reserve material + teguments) [13]. Germination is measured at each time interval using embryonic protrusion as a criterion; if the primary root is the protruded embryo structure, the geotropic curve is used to quantify a germination event in the sample; if it is the shoot, the chlorophyll pigmentation on the seedling or young plant sensu stricto is used to identify the germination process [7,14,15]. This is done so that only individuals with proven vitality are recorded. This criterion can be used in any experimental design that investigates embryonic growth/development, including in vitro. The problem, in this case, is that the classic measurements of seed germination may be sensitive to sample-size fluctuation [16], which is common in in vitro experiments. Now, the question arises: would the seed germination measurements be valid to assess embryonic development in vitro since the sample size is much smaller than that used for experimental designs for seed germination? We expected the answer to be yes.

To date, there have been almost no reports on aspects of sample size for in vitro cultivation of plants. The few reports we found tried to develop a model to promote robust and standardized inferences about the sample size [17,18]. For example, some authors used one (e.g., *Buthia Eriospatha* Mart. by [19]), others four (e.g., *Jatropha* sp. L. by [20]), and others eight embryos (e.g., *Phoenix dactylifera* L. by [21]) as samples for experiments on embryonic development. This becomes even more significant in the case of native species, which have a low degree of genetic improvement and therefore great intraspecific variability. This variability may affect inferences on early development patterns and, therefore, may reveal physiological traits affected by sample-size fluctuation. This makes it difficult to manage biological material (e.g., germplasm banks) or compare results, whether for technical or academic purposes [16,22].

Croton lechleri Müll Arg is a monotypic arboreal species originating in the Amazon and known for its medicinal properties [23]. Morphologically, there are records of individuals measuring up to 20 m in height and with a diameter at breast height (dbh) of 30 cm, even in commercial fields [24]. The cordiform leaves and the minute clustered unisexual cream-yellow flowers (terminal inflorescence bisexual) are peculiarities used to identify the species in the field [25]. Other important biological reports on the botany of the species are (i) the anemochory, hydrochory, and zoochory dispersion of the fruits (capsule type), which contain small seeds (5.37 mm × 5.82 mm × 5.80 mm) according to [26], and (ii) the cyclic phenological behavior, with flowering (in the dry season) and the dissemination of well-defined seeds (in the rainy season) [27]. From an ethnopharmacological point of view, the sap of the species is used in the treatment of ulcers, gastritis, and diarrhea; to increase immunity in patients with AIDS [27]; and as an antitumor treatment [28,29]. The species has been conquering important markets, including commercialization in Western countries [30]. Nevertheless, there are no studies related to embryonic development in vitro for the genus Croton. This fact is worrying, considering the predatory practices that occur with most of the native medicinal species of the Amazon [31]. Considering this and the

fact that the lack of standardization of the experimental sample size tends to be greater for native species that have a lower economic impact and a higher ecological impact than cultivated species [32], *C. Lechleri* was used as the biological model for our study.

Given the above, using development measurements determined for binomial events, we have two expectations: *i.* to obtain new insights into classical biotechnology; and *ii.* to establish the effects of sample-size fluctuation on physiological inferences about in vitro embryonic development. This second expectation is very important because the sample effort (i.e., a functional limit between variability and increment of sample size, where increasing the sample size does not entail statistical improvement) excessively restricts the sample size studied. For this, the hypothesis is that classical measurements of the seed-germination process may be relevant to the understanding of the in vitro embryonic development process, with the most appropriate measurements being those that are robust and not sensitive to sample-size fluctuation. The objective, therefore, is to provide a new way to understand the physiology of development processes in vitro from measurements based on binary counting.

2. Materials and Methods

2.1. Fruit Collection and Processing

We used fruits from mother plants established at two sites: a native environment and a cultivation environment (Table S1). The mother plants are separated by approximately nine kilometers (Table S1). The number of populations supplying biological material was restricted according to the fertility of the individuals (mother plants) within the perimeter of 20 km of the collection area (a naturally occurring forest). In any case, the establishment of populations in such distant places ensures adequate variability among plant populations for sample size studies. The collection was conducted before the dehiscence of the fruits, and the removal of the seeds was conducted in the laboratory with the aid of orthodontic pliers, scissors, and tweezers.

2.2. Seed Disinfestation and Embryo Development In Vitro Protocols

The disinfestation process occurred in a laminar flow chamber. The seeds were immersed in ethanol at 70% purity for one minute and then immersed in a sodium hypochlorite solution at 1% of the commercial compound (NaOCl), supplemented with a drop of Tween-80 (for each 50 mL) for 15 min. The process was terminated after three rinses in reverse-osmosis water.

To extract the embryos from the seeds, a stereomicroscope was used, and the extracted embryos were immediately placed in test tubes (150 mm × 25 mm) with 10 mL of QL culture medium [33]. Each test tube contained one embryo. The choice of this type of medium was attributable to positive results obtained in pretests, which were probably due to the high concentration of total nitrogen (39.0 mM) [34]. The medium was supplemented with 30 g L^{-1} sucrose and solidified with 7 g L^{-1} agar. The pH was adjusted to 5.8.

As there is a possible effect of mutual stimulation related to embryonic development processes in native species [16,35], we chose to study the robustness of development measurements through the effect of sample-size fluctuation with different numbers of test tubes to simulate the sample sizes under study. This experimental design followed that recommended by [16], in which the experimental units are randomized according to the precepts of the casualization assumption [36], but the number of individuals per experimental unit fluctuates according to the sample size factor. Here, the sample factor had four levels, i.e., 5, 25, 50 and 100 embryos. The samples were divided into five subsamples (=five replicates), which had 1, 5, 10, and 20 embryos as the experimental units. Notably, the extraction of embryos is extremely laborious because the structure of the seed is highly sensitive to handling. Therefore, the largest experimental unit was made up of 20 embryos.

The embryos conditioned to the nutritive medium were kept in the in vitro cultivation room at 25 °C with a photoperiod of 16 h (16 L: 9 D, mean photosynthetic photon flux density (PPFD) equal to 22.5 µmol m^{-2} s^{-1}). Assessments of embryonic development were

performed every 12 h at the same times until the number of embryos developed reached stabilization, which occurred seven days after conditioning. The criterion established for development was the geotropic curvature of the root and/or the pigmentation of the cotyledon (Figure 1), which confers autotrophic ability for the early establishment of the plant individual.

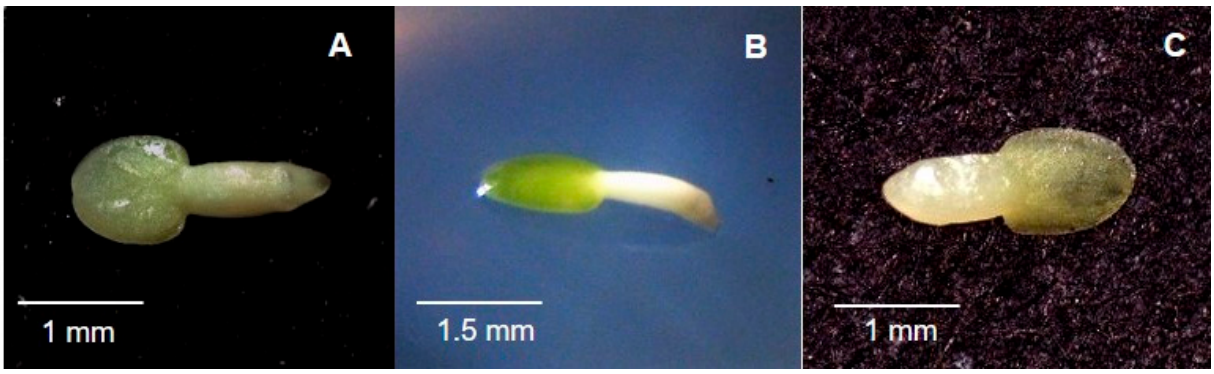

Figure 1. Embryo of Dragon's blood (*Croton lechleri* Müll Arg.). (**A**) Freshly collected/extracted from seed; (**B**) embryo developed in vitro after 48 h, with green pigmentation of the chlorophyll cotyledon and geotropic curvature of the root; (**C**) embryo not developed after 192 h of experimental data recording.

The other factor in the study was the origin of the population of embryo donor seeds. The mother plants were selected from two populations since there is a known maternal effect (due to the maternal environment) on the quality of the embryo produced [37–39]. The experimental design followed a double factorial scheme with fixed factors: 2 (mother plant population) × 4 (sample size). The null hypothesis of this interaction was that the development measurement was robust enough to study embryonic development, regardless of the associative effect between the sample size and the population of origin of the biological material. When there was no interaction between the factors, the study of the main effects was carried out, in which (i) the results of the studied character are not affected by the sample size, regardless of the effect of the plant population; (ii) the results of the studied character are not affected by the plant population, regardless of the sample size. The term "percentage of developed embryos" was used because, according to [7,15], germination is a process of embryonic development through the wraps (reserve material + teguments) of a seed, starting with the imbibition per se of the seed/diaspore and ending with the embryo protrusion. In the present study, the embryo was removed from the mechanical wraps, and therefore, the nomenclature 'germination process' or 'germination' is not appropriate.

2.3. Embryo Development Measurements

The number of developed embryos [(DE (%)] is the number of functional embryos in the sample. Therefore, DE is the percentage of developed embryos with geotropic curvature and/or pigmentation under experimental conditions. This indicates the autotrophic capacity of young plants sensu stricto [7,14]. Other measurements used here are the mean development time of the embryo [$\bar{t}$(hours)], calculated by the expression proposed by [40], in which the number of embryos developed in the time intervals established for data collection is used for weighting [7]. The coefficient of variation of the development time [*CVt* (%)], according to [7], measures the degree of dispersion of embryonic development around the mean development time, allowing the evaluation of the uniformity of embryonic development across the experiment time. The mean development rate of the embryo [$\overline{\overline{v}}$ (hours^{-1})] was calculated according to [41] as a frequency that allows the observation of embryonic development across time [7]. According to [16], this is a measurement with similar criteria to that used by chemical kinetics to measure the velocity of reactions. The

uncertainty of embryonic development [U (bits)], proposed by [42], associated with the distribution of relative frequency highlights the variation of embryonic development across time. The lower the uncertainty value, the more predictable the development will be. The synchronization index of embryonic development (Z), calculated according to [7], evaluates the overlap of events, being more synchronous to the sample that represents greater overlap and, therefore, a value closer to 1 [7,43]. In addition, the frequentist measurement, here called the relative frequency of embryonic development (fi), calculated according to [42], was plotted. Through frequency, it is possible to observe how embryonic development occurs across time [14]. In the text, we also describe the development time range, i.e., the time between first and last stages of embryonic development. This development time range can be observed from graphs of the relative frequency of embryonic development. All embryo-development measurements (calculus and nomenclature) were contextualized from seed science, and further information can be found in [7,44].

2.4. Experimental Design and Statistical Analysis

The treatments were arranged in a randomized complete block design (RCBD) with a 2 × 4 (mother plant population × sample size or number of embryos) factorial scheme in five replicates. The level of the sample-size factor equal to 5 embryos was excluded for dataset processing when the embryo-development measurements had an algebraic limitation to calculus, i.e., 0.5, when the embryo number in a subsample or experimental unit was equal to one (see details in Results and Discussion). Consequently, in this case, the factorial scheme was 2 × 3 (mother plant population × sample size). This experimental design is according to [16,22]. For the statistical analyses, the Shapiro–Wilk test was used to test the normality of the residual; Levene's test was used to test the homogeneity between the variances; and Tukey's test was used to test the additivity between the blocks, all at the 0.01 level of significance ($\alpha = 0.01$; Table 1). Once these assumptions were accepted, the Snedecor test (ANOVA) was applied to the dataset, and then the Tukey's test for comparison between means was used, all at the 0.05 level of significance ($\alpha = 0.05$). We opted to perform parametric analysis of the percentage of developed embryos (%) that, even when transformed, did not meet all assumptions but reduced the F value of the Levene test and/or increased the W value of the Shapiro–Wilk test, as suggested by [45].

Table 1. Statistics and probability (*P*) of tests used for processing the dataset of the development pattern of embryos of Dragon's blood (*Croton lechleri* Müll Arg.) in vitro.

Statistics			Characters											
			$\bar{t}$ (Day)		*CVt* (%)		*U* (Bit)		*Z*		*ED* (%)		$\bar{v}$ (Day^{-1})	
ANOVA Assumptions		*W*	0.924		0.951		0.983		0.951		0.962		0.927	
		(P)	(0.010)		(0.084)		(0.787)		(0.080)		(0.193)		(0.012)	
		^{1}F	2.688		2.059		3.200		1.635		4.721		2.323	
		(P)	(0.26)		(0.78)		(0.011)		(0.161)		(0.001)		(0.049)	
		^{2}F	3.666		3.592		0.648		0.565		20.848		1.790	
		(P)	(0.066)		(0.069)		(0.428)		(0.459)		(0.000)		(0.192)	
Model	**Source of Variation**	**DF**	**MS**	**^{3}F (*P*)**	**MS**	**^{3}F (*P*)**	**MS**		**MS**	**^{3}F (*P*)**	**DF**	**MS**	**^{3}F (*P*)**	**MS**	**^{3}F (*P*)**
Factorial ANOVA	Sample Size	2	0.022	0.740 (0.5372)	0.619	28.427 (0.000)	3.257	419.583 (0.0000)	0.943	19.882 (0.0000)	3	0.150	1.261 (0.3068)	0.0002	0.273 (0.8442)
	Mother Plant Population	1	0.002	0.051 (0.8222)	0.046	2.117 (0.1568)	0.022	2.846 (0.1027)	0.240	5064 (0.0325)	1	0.486	4.085 (0.0529)	0.00004	0.066 (0.7997)
	Sample Size × Mother Plant Population	2	0.005	0.152 (0.9276)	0.032	1.1492 (0.2383)	0.010	1.300 (0.2940)	0.072	1.521 (0.2307)	3	0.171	1.440 (0.2520)	0.0001	0.142 (0.9340)
	Block	4	0.048	-	0.039	-	0.011	-	0.038	-	4	0.003	-	0.008	-
	Error	20	0.030	-	0.022	-	0.008	-	0.047	-	28	0.019		0.006	
		***CV* (%)**	7.79			41.14	10.40		49.28	-	-	1.89		18.16	

Note: *W*: statistic of Shapiro–Wilk test for residual normality ($p \geq 0.01$); ^{1}F: statistic of Levene test for homogeneity of variances ($p \geq 0.01$); ^{2}F: statistic of Tukey test for additivity ($p \geq 0.01$); DF: Degrees of Freedom; MS: Mean Square; ^{3}F: statistic of Snedecor test (Factorial-ANOVA; $p < 0.05$). $\bar{t}$: mean development time for the embryo; *ED*: percentage of embryos developed; $\bar{v}$: mean development rate for the embryo; *CVt*: coefficient of variation of the development time for the embryo; *Z*: synchronization index for the embryo. The statistical analyses for characters were based on transformed data by $\sqrt{x}$ or, in case of percentage data, by arcsine $\sqrt{x/100}$. *CV*: Coefficient of Variation.

3. Results and Discussion

The embryo-development measurements were not influenced by the associative effect between the sample size and the population of mother plants (Table 1, Figure 2). Thus, these measurements can determine patterns of in vitro development without compromising statistical inferences. In the inferential scope, the standard practice for the area is to only quantify the percentage of total embryos developed during the experimental time [6]. In the case of our model plant, the embryos had a high development capacity (ED $\geq$ 90%) (Figure 2), and therefore, the plausible conclusion would be that the maternal effect was not strong enough to alter the pattern of embryonic development of the species. This makes much more sense when taking into consideration that the core of the maternal effect was associated with wraps [46], which were discarded for our in vitro embryo cultivation. Those wraps had most or all of the genetic material of maternal origin [37,39]. On the other hand, the question is: what is the benefit of using the other measurements? The answer is in the inference about the kinetics and uniformity of the physiological process.

When considering only the absolute value related to the total development events, one neglects how this process proceeded across time [47]. Thus, little is known about metabolic aspects, for example. Many classical physiologists have used the calculations of robust measurements to infer the time, velocity, and uniformity of binomial physiological processes, i.e., whether they occur or not [7]. These calculations have been made easier with review articles, interactive spreadsheets (e.g., [44]), and biological computational advances (e.g., those achieved with R). Currently, these measurements are used not only with the objective of demonstrating the physiological process per se but also as a guide for molecular measurements, which are more sophisticated and have the ability to deepen knowledge about specific aspects of biological processes (e.g., [48]). Here, our biological model demonstrated that time measurements (especially mean development time and development time range—descriptive measurements from graphs of the relative frequency of embryo development; Figures 2 and 3) can be used to classify development performance without losing statistical robustness due to sample-size fluctuation. This, however, does not mean that these measurements do not suffer from the size of the experimental unit. One of the reasons for the mean development time not distinguishing between the plant populations was the large amount of variability among the values found for the different blocks, even though they had lower experimental variability (see CV, Mean Square for sample size and of the block in contrast to the Error Mean Square; Table 1). When the plot unit was only one embryo, the mean development time represented the pattern of one individual, and therefore, the population effect was mischaracterized by the weight of the individual physiology (see Figure 3). That means the subsample becomes non-representative of the sample and, consequently, of the species (or any other treatment being studied). It is also important to emphasize that the mean development time for the embryo, when presented with residuals adhering to a normal distribution, demonstrates the moment when it will be possible to observe the largest number of embryos developed in the sample (see [7]). Therefore, the measurement may be useful for the production of protocols aimed only at punctual observations of the occurrence of total sample events.

The mean development rate of the embryo measures the kinetics. This measurement was similar between the treatments because the embryos demonstrated low variability according to the population and/or the sample size fluctuation (see Mean Square values in Table 1; Figure 2). This corroborates the theory that, by measuring the kinetics of events in a nonlinear way [7,16], the measurement is a robust inference for the metabolic performance of a binomial event distributed across time, such as germination [7,16,49–51]. The mean development rate has also been considered a fingerprint for the species [16,52]. Thus, the embryonic development of *Croton Lecheleri* presents a mean metabolic rate of 0.019 h^{-1} (Figure 2). It is important to note that, for experimental designs that use measurement as a way to measure development, the experimental unit should be standardized, as is done with seed testing (e.g., [53]), or the individual should be isolated in exclusive containers, as was done in the present work. These measurements prevent the mutual stimulus attributable to

volatile signals, hormonal or not, finely regulated by some species and demonstrated as a possible fragility of the measurement [16], from being a non-controlled source of variation for laboratory tests.

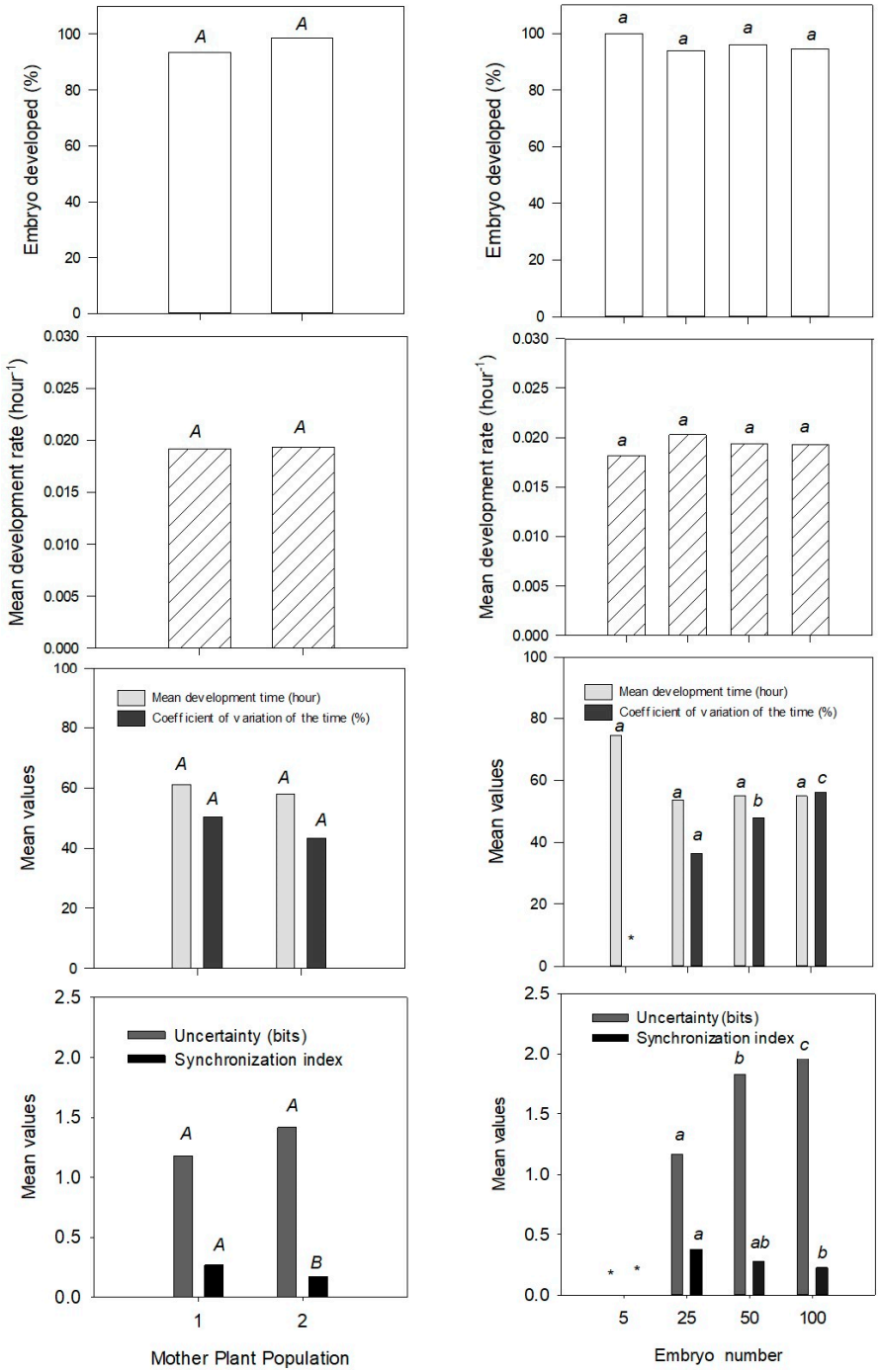

Figure 2. The development measurements calculated from embryo development in vitro of Dragon's blood (*Croton lechleri* Müll Arg.) in which developmental pattern was defined by different sample size (embryo number) and/or mother plant population (1: mother plant population established in natural environment area; 2: mother plant population established in cultivated environment area). The capital letter represents the Tukey test statistic ($\alpha = 0.05$) for the mean comparison of the sample size factor, while the lower case represents the Tukey test statistic for the mean comparison of the mother plant population factor. *: It is a highlight for the exclusion of lower sample size for some characters due to its algebraic limitation for replicates without variability (i.e., only one embryo per experimental unit).

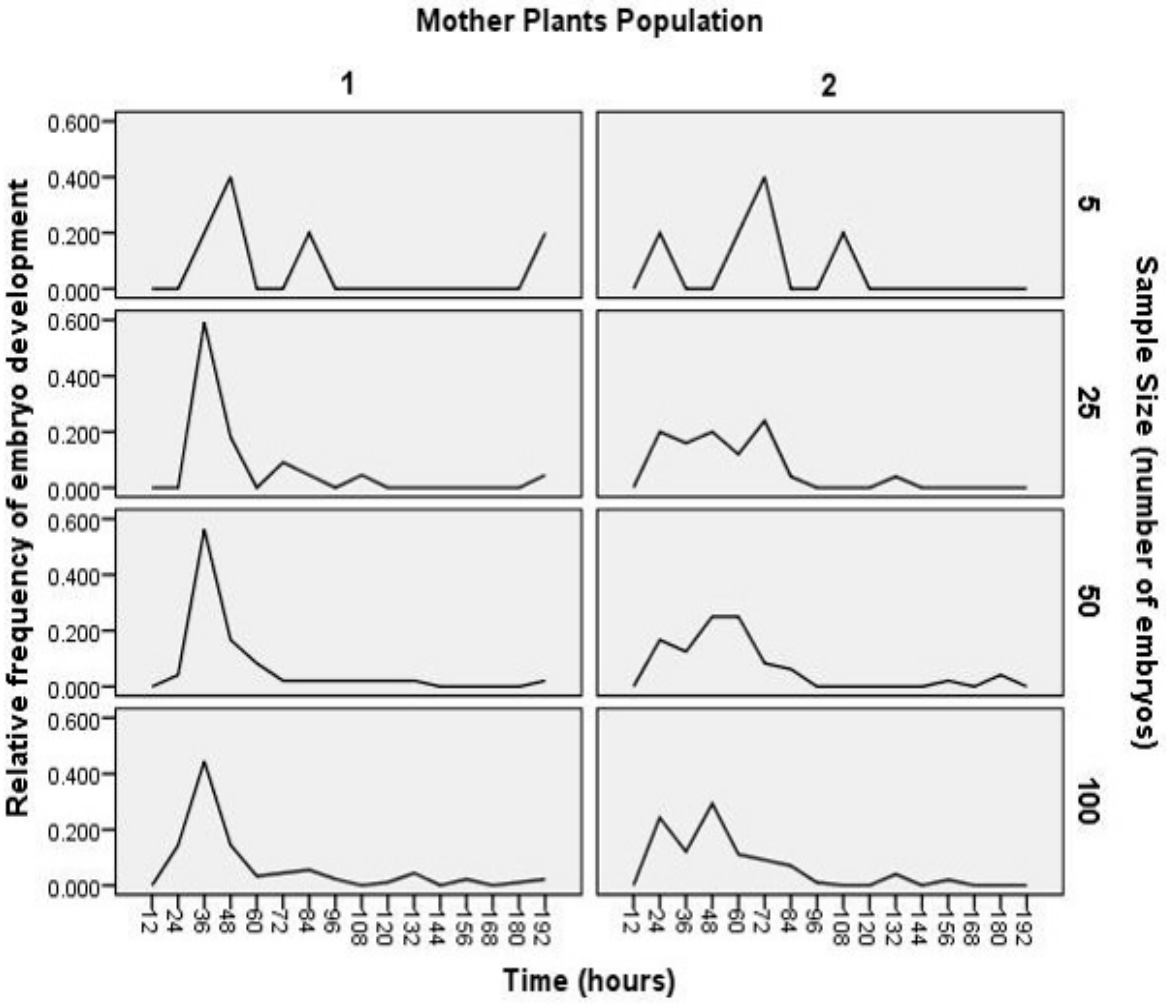

Figure 3. Relative frequency of embryonic development based on in vitro assays of Dragon's blood (*Croton lechleri* Müll Arg.) in which developmental pattern was defined by different sample sizes (embryo number) and/or mother plant populations (1: mother plant population established in natural environment area; 2: mother plant population established in cultivated environment area).

In the case of classical biotechnological aspects of plants, such as tissue culture, one of the most interesting precepts is the uniformity of the seedlings produced [12]. In this way, the study of the coefficient of variation of the development time, uncertainty, and synchronization index of the embryo can be quite interesting for the stage of multiplication and/or establishment of the protocols, whether routine or experimental. This is clear in the results presented here (Figure 2); even when the percentage of developed embryos, time, and velocity measurements do not show significant differences, the uniformity measurements manage to demonstrate these differences. The problem here, however, is robustness against the sample-size variation. All measurements of the uniformity of the embryonic development process in *Croton Lecheleri* were sensitive to sample-size variation. This had been mentioned for Z and *CVt* when the object of study was seed germination of native species [16], but this is the first time that sensitivity to the sample size is confirmed for measuring uncertainty. This characteristic is derived from the Shannon index, which is considered by ecologists to be sensitive to sample-size fluctuations [54]. Before this, however, it is possible to highlight some important points. (i) These measurements should naturally be sensitive to sample-size variation because they measure the sample disturbance related to the occurrence of a random event. Thus, the greater the number of individuals in the sample, the greater the probability of an event occurring and, therefore, the greater the disturbance in the sample. This can be visually noticed through the relative frequency graphs

for embryonic development, in which the amplitude of developing events (development time range) increases with the increase in the sample size (see fi in Figure 3), as well as the values of these measurements. However, even when there were a greater number of explants per sample and/or subsample, the inference obtained from the measurements did not change. This was the opposite of what was noted for seed germination of non-dormant and/or native species sensitive to the phenomenon of mutual stimulation [16]. This is because, from a sufficient sample size point of view, the development measurements in fact measure uniformity (see Table 1 and Figure 3). (ii) Here, it is worth remembering that there are models, such as the modified maximum curvature method [55], that have been successfully adapted to calculate the sufficient sample size for physiological characteristics with binomial occurrence, such as seed germination [22,56], in an easy and safe way. Therefore, sample size standardization can be made feasible not only for practical purposes, such as the use of universal protocols, but also for academic purposes, in which the use of the measurements would become a framework for the collection of inferential information about the physiological uniformity of the treatments under study. (iii) Still on the nature of the measurements, it is important to point out that the uncertainty, the coefficient of variation of time, and the synchronization index cannot be calculated when only one individual composes the experimental unit [7]. This is because an individual does not allow the calculation of variance and therefore cannot infer sample or population processes. Thus, whether for measurement calculation or statistical inference, one should avoid using only one explant as an experimental unit based on the fact that the death of this explant inflates the statistics and/or hinders the analyses when it is considered a lost experimental unit.

Some investigators may understand that the increment of the coefficient of variation of the development time is a problem that should not occur since there is widespread thinking that the coefficient of experimental variation is a marker of 'experimental precision' (e.g., [57]). This thinking is unsound [45] because it disregards practical issues, such as the biological material under study [14]. In general, native species, such as *Croton lecheleri*, have high intraspecific genetic variability, which increases this coefficient of variation. This is not related to experimental precision. In this sense, it is also possible that the coefficient of variation of the development time is not synonymous with the coefficient of variation from the experimental conditions; therefore, the interpretation should be more biological and focused on precepts once defined in the crosstalk between physiology and experimental statistics (see [8,16,58]). Similarly, it is worth noting that the synchronization index, being the only measurement capable of validating differences between the plant populations, is quite interesting because it demonstrates that overlapping events are a phenomenon associated with the ability of the mother plant to provide seedling recruitment in the face of the environment. This would make inferences about the measurement sensitive if the nature of the measurement and the fact that it is drastically affected by the size of the experimental unit were not considered and therefore usable in universal protocols.

We are unaware that some other report has considered binomial development measurements to define patterns established for plant explants in in vitro studies or discussed how the sample-size fluctuation can affect the physiological inferences of these laboratory techniques. This, by itself, would make the present work a guide to be improved by other authors, in which aspects such as the stage of fruit development could be studied as a cause of variation in the ability of an embryo of a native species to develop evenly and quickly. However, this work also sets a precedent that not only the sample size be studied as a variation factor in in vitro cultivation designs but also the size of the experimental unit. For example, it is necessary to study other types of explants by extrapolating the sample size used in this research, which had a sample size limit of 100. It is also noteworthy that this value was established since the manipulation of embryos is a delicate and exhaustive work, which would not be the case, for example, for seeds or explants of leaf, root, stalk, or meristem. For all these types of explants, development can be considered a physiological phenomenon with a binomial pattern. The production of roots with a size greater than

2 mm, also usual for some seed physiologists (e.g., [59]), or the production of calli are two examples of this binomial pattern (in this case, seeds and vegetative parts, respectively).

In a restrictive way, despite the satisfactory embryo development rate of Dragon's blood, the process did not occur in a uniform way, regardless of the population and quantity of embryos that comprised the sample ($32.47\% \leq CVt \leq 56.14\%$) (Figure 2). This heterogeneity was also observed by [60] for the germination of *Anadenanthera Colubrina* (Vell.) Brenan. The authors of the aforementioned work attribute such a pattern to native species that are subject to selective pressures from the environment. This environmental pressure may have caused heterogeneity in the development of the embryos of the species we studied. We standardized the embryo size during the experimental implantation, but it was not possible to measure its early capacity for development and/or maturation in the seed. This reinforces that the measurements capture even the nuances of the development process and therefore have the capacity to detail patterns when using different hormonal treatments or culture media. The contribution is that these measurements can extract more information from the same test when considering what is currently used in the area of plant tissue culture.

4. Conclusions

From this study, we conclude that (i) among the measurements presented, the least robust measurements to sample-size fluctuation for inferences on embryonic development are those that measure uniformity (*CVt*, U, and Z). (ii) In addition to the total percentage of embryonic development, it is possible to infer with statistical robustness aspects of developmental kinetics when using mean development time and mean development rate. (iii) Frequentist measurements, such as the frequency of embryonic development and the development time range, complement the absolute measurements by promoting visual insights into the process. (iv) Small samples, with only one individual as a replicate, should be avoided so that physiological aspects may be representative of the sample and/or biological population under study. Thus, it is recommended that studies on the sufficient sample size of in vitro cultivation be encouraged, especially when the target biological material is a native species that has high intraspecific variability.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13081618/s1, Table S1: Geographic coordinates of the population of the mother plant population of Dragon's blood (*Croton lechleri* Müll Arg.). UTM: Universal Transverse Mercator.

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