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DRYING TOLERANCE IN COCOA SEEDS AND USE OF THE SUPEROXIDE DISMUTASE AS A BIOCHEMICAL MARKER

Isadora de Andrade Tronco[1*], Vitória de Andrade Tronco[1], Victor Augusto Forti[2]

[1]Federal University of São Carlos, Campus of Agricultural Sciences, Araras, São Paulo, Brazil.
E-mails: isadora.tronco@gmail.com; vitoria.tronco@gmail.com;
[2]Federal University of São Carlos, Department of Agroindustrial Technology and Rural Socioeconomics, Campus of Agrarian Sciences, Araras, São Paulo, Brazil. E-mail: viaugu@ufscar.br

*Corresponding author: isadora.tronco@gmail.com

Cocoa is a recalcitrant species, sensitive to low temperatures and reduced water levels. This may be related to the inefficiency of its defense mechanisms against stressful conditions. The aim of this study was to determine the activity of the enzyme SOD (superoxide dismutase) as a biochemical marker of drying sensitivity in cocoa seeds, as this enzyme is responsible for removing free radicals that can led to cell death. To achieve this, two lots of cocoa seeds, extracted from ripe fruits, were subjected to continuous drying in an oven with forced air circulation at 34 ± 2°C to obtain treatments with varying water contents. The seeds from each treatment were evaluated for water content, germination, viability, and biochemical analysis to evaluate the activity of the SOD enzyme. It is concluded that the quality of the lots can interfere with the response of SOD enzyme activity. However, a tendency for a reduction in SOD activity was observed with the reduction of water content in both lots, which adversely impacts the viability of the seeds. Therefore, the assessment of the activity of this enzyme can be used as a reliable marker of drying sensitivity.

Keywords: Recalcitrant seed, seed quality, *Theobroma cacao* L.

Tolerância à secagem em sementes de cacau e uso da superóxido dismutase como marcador bioquímico. O cacau é uma espécie recalcitrante, sendo sensível a baixas temperaturas e a baixos teores de água. Isso pode estar relacionado à ineficiência dos mecanismos de defesa em condições de estresse. O trabalho teve como objetivo determinar a atividade da enzima SOD (superóxido dismutase) como um marcador bioquímico de sensibilidade à secagem em sementes de cacau, uma vez que essa enzima é responsável por remover os radicais livres que provocam a morte celular. Para isso, dois lotes de sementes de cacau, extraídas de frutos maduros, foram submetidos a secagem contínua em estufa com circulação de ar forçada a 34 ± 2 °C para obter tratamentos com diferentes teores de água. As sementes de cada tratamento foram avaliadas quanto ao teor de água, germinação, a viabilidade, e a análise bioquímica para a avaliação da atividade da enzima SOD. Conclui-se que a qualidade dos lotes pode interferir na resposta da atividade da enzima SOD. Entretanto, observou-se uma tendência de redução da atividade da SOD com a redução do teor de água em ambos os lotes, o que impacta a viabilidade das sementes. Dessa forma, a avaliação da atividade dessa enzima pode ser utilizada como marcador de sensibilidade à dessecação.

Palavras-chave: Semente recalcitrante, qualidade de semente, *Theobroma cacao* L.

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Introduction

In 2022, the largest cocoa production was on the African continent, representing 69.85% of world production. The second largest producer was the American continent, which accounts for 17.26% of world production (FAOSTAT, 2024). This crop production, especially in agroforestry systems, has a high economic, social, and environmental value for the population, as it can promote biodiversity, contribute to the economic income of producers, and generate employment (Gama-Rodrigues et al., 2021). Cocoa beans are used as raw material for food and for producing seedlings for grafting, especially for rootstock production (Sodré, 2013; Di Mattia et al., 2017).

Cocoa seeds are considered recalcitrant (Li and Sun, 1999; Salles et al., 2019) due to their inability to survive prolonged periods of storage, low temperatures, and low water contents. This is in contrast to orthodox seeds, which are more resilient (Gasparin et al., 2019). The analysis of critical and lethal water contents in seeds is a parameter used to identify recalcitrance. Nair et al. (2020) and Viana et al. (2020) demonstrated that for the storage of recalcitrant seeds, they should be kept with water contents higher than the critical level, as lower values result in a drastic loss of seed viability. Upon reaching the lethal level, germination did not occur (Viana et al., 2020).

This drying sensitivity is related to the cellular protection mechanisms present in seeds. The enzyme superoxide dismutase (SOD) serves as a defense mechanism against stresses such as water stress and indicates vigor in aging conditions (Francini et al., 2006; Liu et al., 2020). During the process of eliminating free radicals, the enzyme functions by reducing the oxygen ion (O_2^-) into hydrogen peroxide (H_2O_2) and oxidizing another O_2^- to form oxygen (O_2) (Marcos-Filho, 2015). Consequently, SOD plays a pivotal role in regulating the level of free radicals and ensuring the retention of proteins within living tissues. Its activity is subject to change as seeds age, which consequently affects their viability (Ardebili et al., 2019; Brar et al., 2019).

In recalcitrant seeds of *Quercus robur* L., the decline in SOD activity in the embryonic axis was observed to occur concomitantly with the loss of water in the seed during drying, as well as the accumulation of free radicals and increased lipid peroxidation in the axes (Hendry et al., 1992). This increase in free radicals was also observed by Chandra et al. (2021) in seeds of *Madhuca latifolia* Roxb., which exhibited a decrease in water content. In cocoa seeds, SOD declined with drying, and there was an increase in lipid peroxidation products in the cotyledons, as reported by Li and Sun (1999).

In light of the aforementioned considerations, biochemical markers (such as SOD) represent crucial mechanisms for identifying seed quality, as they allow for the elucidation of protection mechanisms against oxidative stress. The objective of this study is to ascertain the activity level of the enzyme superoxide dismutase (SOD) as a biochemical marker of drying sensitivity in recalcitrant cocoa seeds (*Theobroma cacao* L.).

Material and Methods

The present study involved the analysis of two batches of cocoa beans procured from ripe fruit purchased at the Campinas Supply Center (CEASA de Campinas). Subsequently, the seeds were extracted and processed to remove the pulp by manual rubbing through a fine sand sieve. The seeds were then homogenized and washed in running water, after which they were dried on the surface using paper towels. For this work, batches were not used for comparative purposes but rather for repetition.

Following surface drying, the seeds were subjected to continuous drying in an oven with forced air circulation at 36 °C to obtain 11 different treatments, which considered different drying times, spaced at three-hour intervals, for a total of 30 hours. The drying times were as follows: 0 h, 3 h, 6 h, 9 h, 12 h, 15 h, 18 h, 21 h, 24 h, 27 h, and 30 h.

The seeds from each treatment were evaluated for water content (four replicates of five seeds) and germination (four replicates of 25 seeds) and viability using the tetrazolium test (four replicates of 10 seeds). To determine the water content, the oven method was employed at 105 ± 3 °C for 24 hours (Brasil, 2009). For the germination test, the seeds that germinated in vermiculite within a closed germination chamber at 25°C, following the methodology of Voigt et al. (1995) with adaptations. The substrate was moistened to 60% of its water retention capacity in order to ensure that sufficient water would be available for germination. The seeds in the germination test were not treated with sodium hypochlorite because the presence of fungi is important in the assessment of germination (Brasil, 2009).

An evaluation of normal seedlings was conducted, with particular attention to those that exhibited the growth of primary and secondary roots. In the first batch, germination was evaluated on four occasions, with the final assessment occurring on the 26th day following the initiation of the experiment. In Batch 2, three evaluations were conducted concluding on the 27th day. The final assessment was determined by considering the time at which the formation of normal plants stabilized.

The tetrazolium test was carried out to assess the viability of cocoa seeds. A preliminary test was conducted to determine the optimal concentration of 0.075% and the optimal immersion time in the tetrazolium solution of 7 hours. The concentrations tested in the preliminary experiment were 0.075%, 0.1%, and 0.5%, while the times analyzed were 2, 3, 4, 5, 6, and 7 hours.

Before immersion of the seeds in the tetrazolium solution, they were wrapped in germination paper that had been moistened to a weight ratio of 2.5 times the dry weight of the paper. The seeds were then stored until the tegument was removed. The seed coat was removed using a razor blade with the objective of creating a superficial lateral cut opposite the embryonic axis. Subsequently, the seeds from each treatment were placed in a 0.075% tetrazolium solution for a period of seven hours. Afterward, the seeds were rinsed in running water and maintained in water at a temperature of 25 °C in a BOD until analysis. For the purposes of this analysis, the viability of the seeds from each treatment was classified according to the following criteria (Table 1 and Figure 1).

Furthermore, a portion of the seeds from each treatment was separated and frozen in a freezer set to -10 °C for subsequent assessment of SOD enzyme activity. The methodology employed was that of Sekita (2012), with adaptations, using only the whole embryonic axes for extraction.

Table 1. Cocoa seed viability class for the tetrazolium test.

Class	Critéria
Class 1	Viable. Pink embryonic axis.
Class 2	Viable. Pink embryonic axis. Presence of small spots with a more intense pink color, less than 50% of the total area.
Class 3	Viable. Embryonic axis with an intense pink color that represents more than 50% of the total area.
Class 4	Not viable. Embryonic axis with more than 50% white color, mixed with intense pink tones.
Class 5	Dead. Embryonic axis completely white.

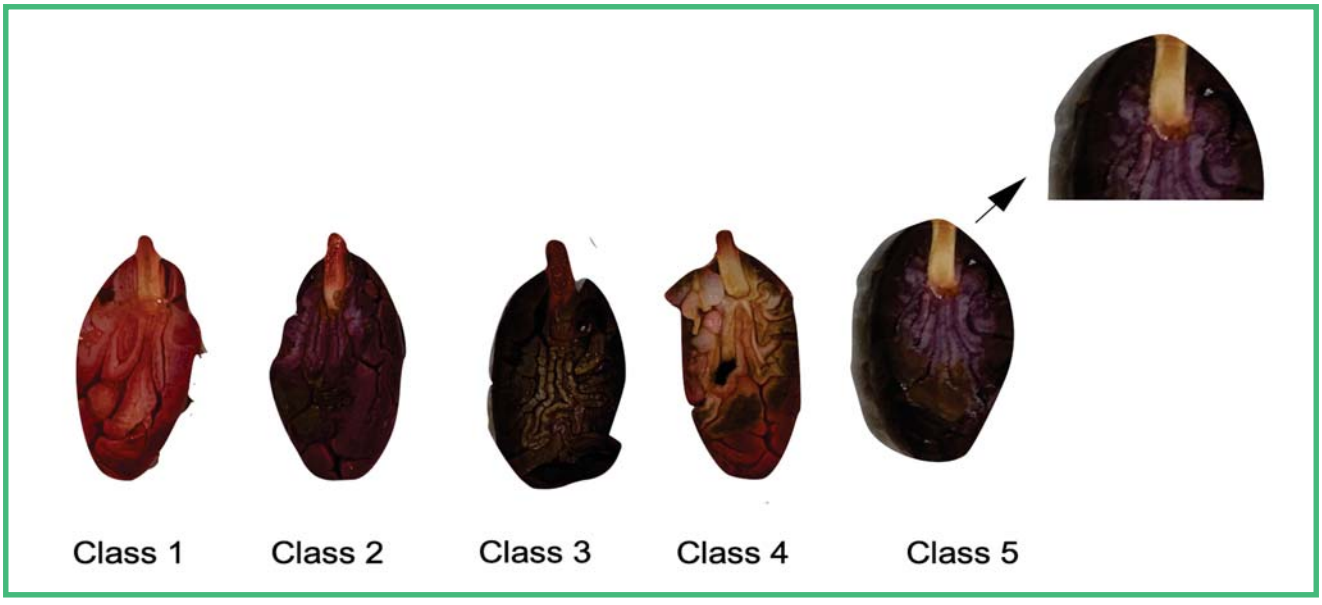

Figure 1. Viability analysis of cocoa seeds using the tetrazolium test and a zoomed image of the dead embryonic axis in class 5. Class 1: Pink embryonic axis. Class 2: Embryonic axis with small patches of more intense pink color. Class 3: Embryonic axis with intense pink color in more than 50% of the total area. Class 4: Embryonic axis colored white over 50% of the total area. Class 5: Embryonic axis completely white.

The results of the germination and viability tests were subjected to analysis of variance (ANOVA) to identify any significant effects. In the event of a significant effect the means were analyzed using the Tukey test at a 5% probability. The R software was used for statistical analysis.

Results

A reduction in the water content of the cocoa seeds was observed with exposure to the different drying times for both batches (Figure 2). For Batch 1, at time zero, the water content was 57.24%, reaching a water content of 26.75% after 30 hours of drying For Batch 2, the initial water content was 67.58%, reaching 23.91% after 30 hours of drying.

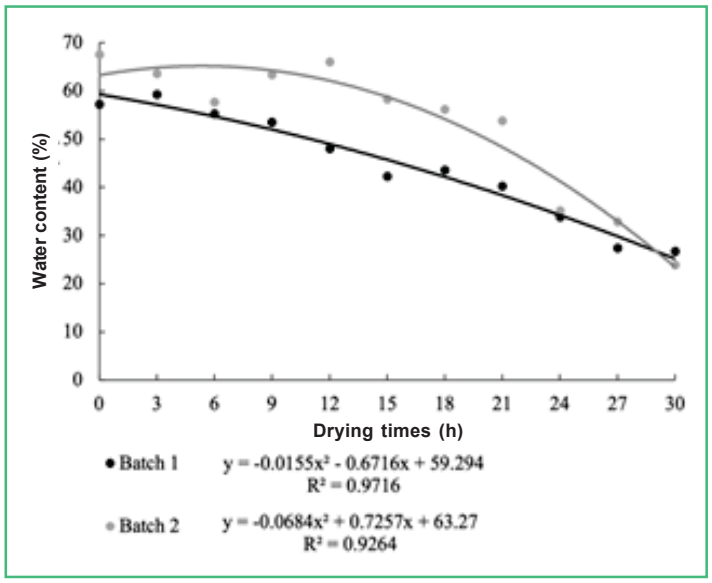

Figure 2. Water content (in percentage) of *Theobroma cacao* L. seeds in batch 1 and batch 2 at different drying times (in hours) in a forced circulation oven at 36º C.

The data pertaining to the drying rate (percentage of water loss in relation to the initial water content), germination, and viability, as determined by the tetrazolium test, are presented in Table 2. It can be observed that following the drying process, the viability of the seeds decreased for both batches, as cocoa seeds are recalcitrant and cannot tolerate losses in water content. For Batch 2, there was a loss of data during the collection process at the 24-hour drying time, which is not reflected in the table. For Batch 1, at a water content of 26.75%, only 15% of the seeds germinated. For Batch 2, germination was 51% when the seeds reached a water content of 23.91%.

Table 2. Water content (WC), drying rate (DR), germination (G) and viability by tetrazolium (TZ), in percentage, in *Theobroma cacao* L. seeds with different drying times in a forced circulation oven at 36 ºC for Batchs 1 and 2.

Drying times (h)	Batch 1				Batch 2			
	WC (%)	DR (%)	G (%)	TZ (%)	WC (%)	DR (%)	G (%)	TZ (%)
0	57.24	0.00	68 a*	93.33 a	67.58	0.00	72.00 a	82.50 a
3	59.29	3.58	51 ab	87.50 ab	63.63	-5.84	83.00 a	82.50 a
6	55.32	-3.35	54 ab	95.00 a	57.69	-14.63	73.00 a	85.00 a
9	53.53	-6.48	61 a	95.00 a	63.38	-6.21	73.00 a	87.50 a
12	48.07	-16.02	40 abcd	82.50 abc	66.04	-2.28	51.00 b	90.00 a
15	42.33	-26.05	46 abc	62.50 abcd	58.35	-13.66	54.42 b	87.50 a
18	43.61	-23.81	26 bcd	65.00 abcd	56.20	-16.84	58.39 b	82.50 a
21	40.23	-29.72	12 d	53.61 bcd	53.84	-20.33	60.00 b	92.50 a
24	33.86	-40.85	20 cd	46.11 cd	-	-	-	-
27	27.45	-52.04	13 d	37.50 d	32.91	-51.30	51.30 b	30.00 b
30	26.75	-53.27	15 d	30.00 d	23.91	-64.62	51.00 b	40.00 b
CV (%)			31.34	16.89			24.42	20.60

*Means followed by the same letter in the columns do not differ by the Tukey test at 5% probability

The critical water content, or the point at which a significant decline in germination was observed, was determined to be 43.61% (drying time of 18 h) for Batch 1 and 66.04% (time of 12 h) for Batch 2. This critical content is defined by the point at which there is a notable decline in germination due to the drying of the seeds.

In general, viability, as determined by the tetrazolium test exhibited a similar trend to that observed for germination (Table 2). However, a difference was observed between the viability of the seeds assessed by these two methods.

Regarding the drying rate it was observed that this was lower in Batch 1 (-53.27%) than in Batch 2 (-64.62%) in comparison to the initial period up to 30 h (Table 2).

As for SOD activity it can be observed that in Batch 1, no clear trend in activity was evident (Table 3). However, for Batch 2, a correlation was observed between the reduction in SOD activity and the drying process.

The observed difference in SOD activity between the two batches may be attributed to differences in seed quality. Batch 1 (Figure 3A) was found to be of lower quality than Batch 2 (Figure 3B), which may explain some of the observed variations in the results.

Table 3. Drying time and percentage of photoreduction by the superoxide dismutase (SOD) sample in *Theobroma cacao* L. seeds with different drying times in a forced circulation oven at 36 ºC.

Drying times (h)	% of photoreduction by sample (SOD)	
	Batch 1	Batch 2
0	81.52	231.35
3	88.40	170.64
6	114.58	156.67
9	82.70	173.91
12	121.03	189.98
15	75.02	137.49
18	98.65	134.30
21	83.75	125.65
24	104.40	89.60
27	76.03	127.48
30	111.10	132.64

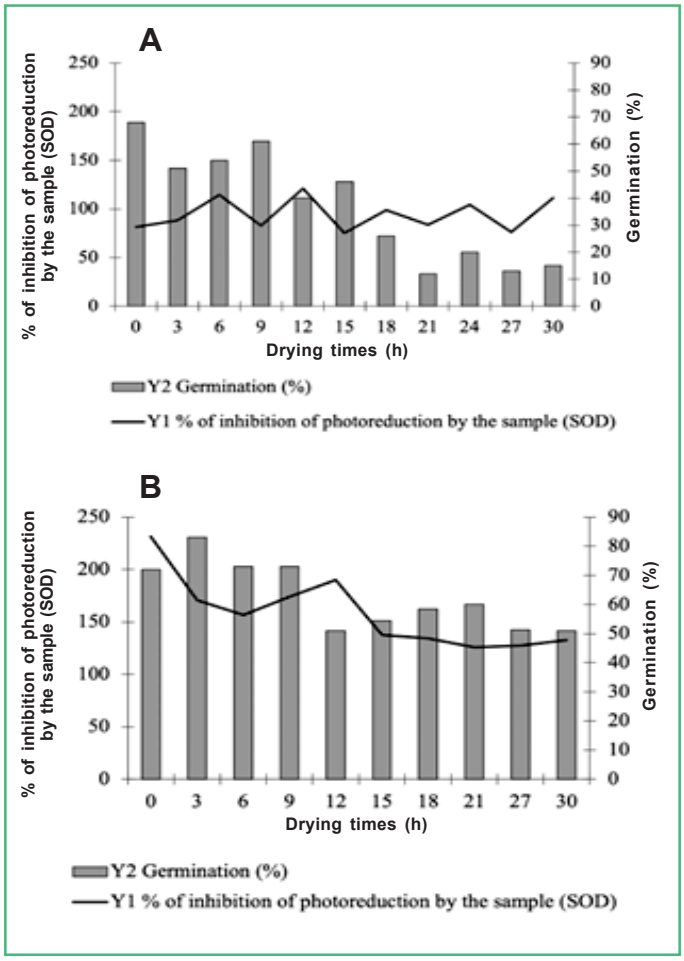

Figure 3. Effect of drying on batch 1 (A) and 2 (B) in relation to germination percentage and the percentage of inhibition of photoreduction by the sample (SOD).

Discussion

Drying, which causes a reduction in water content, negatively impacts germination of recalcitrant species, as seen in this study for cacao species. Seiffert et al. (2006), Negi and Rawal (2019), and Viana et al. (2020) observed that the viability of recalcitrant seeds decreased when the water content in the seed was reduced. Also, in Viana et al. (2020), seeds showed a significant decline in germination after reaching the critical water content, similar to that observed in cacao seeds.

The observed difference in drying rate between Batch 1 and Batch 2, despite their exposure to the same drying time, may be attributed to the initial water content of the seed, which was higher in Batch 2. Additionally, there was a discrepancy in germination rates between the two batches,

suggesting that Batch 2 exhibited superior physiological potential due to its higher germination rates at 30 hours.

This discrepancy in germination, between the batches, may also be attributed to the drying rate. As elucidated by Marcos-Filho (2015), when drying occurs at a slower pace, there is a heightened susceptibility to damage to the seed, which can culminate in damage to biochemical processes and cell structure. Some observed damages caused by drying include changes in protein structure, changes in the concentration of sugars in the embryonic axes and cotyledons (Connor and Sowa, 2003), and even in the activity of enzymes such as SOD (Li and Sun, 1999; Ardebili et al., 2019; Brar et al., 2019; Anusha et al., 2022; Walt et al., 2022).

One hypothesis for the decline in germination when the water content of the seeds is reduced is related to the failure of some biochemical mechanisms that did not preserve the viability of the seed (Connor and Sowa, 2003). Some of the observed damage is electrolyte leakage, which increases with drying time and negatively affects seed viability (Vieira et al., 2022). It was observed in recalcitrant *Camellia sinensis* seeds that drying resulted in changes in the responses of stress proteins, such as catalase and ascorbate peroxidase, and proteins related to metabolic activity. Prolonged drying resulted in changes in the activity of proteins related to metabolic activity due to the use of energy to overcome the stress caused by drying. The authors also observed that the use of ROS scavengers helped to increase the germination of the seeds studied (Chen et al., 2011). As stated by Sharanya et al. (2023), the exposure of seeds to drying conditions has been shown to compromise germination and the cell membrane, thereby interfering with their physiological quality.

The water content of cocoa seeds interferes with the biochemical activity of SOD. The reduction of SOD was also observed by Feng et al. (2017) in their study of *Ginkgo biloba* seeds, the activity of superoxide dismutase increased when the water content was 25% in the seed and subsequently decreased along with the drying of the seeds. In Peng et al. (2023), SOD was also found to be affected by drying in recalcitrant seeds of *Sassafras tzumu* (Hemsl.) Hemsl.

It is important to note that SOD is one of the initial response mechanisms against stress. Additionally, other enzymes, such as catalase and peroxidase, play a role in the capture of free radicals and contribute to the protection against seed deterioration (Marcos-Filho, 2015). Consequently, the behavior (increase or decrease in activity) of these enzymes during drying varies with the presence of these radicals and the efficiency of these mechanisms (Varghese and Naithani, 2002; Viana et al., 2020).

According to Liu et al. (2020), SOD activity can act as an indicator of batch vigor, as the inefficiency of the enzyme system has been shown to indicate loss of vigor in *Metasequoia glyptostroboides* seeds. Another study found that in recalcitrant *Syzygium maire* seeds, the water content of the seed influenced SOD activity, which decreased with rapid drying (Walt et al., 2022). Furthermore, the authors observed that SOD exhibited no activity when the seed was not viable. This indicates that SOD activity can be a parameter of viability, as observed in this study. Batch 1 was found to have lower physiological quality than Batch 2, as it exhibited lower levels of SOD activity, which coincided with low viability (both in the germination test and in the tetrazolium test at 30 hours).

In light of these observations, it can be postulated that the oscillation in SOD activity observed in Batch 1 may be attributed to the presence of the superoxide anion. This suggests that the enzyme is still functioning to protect the seed, albeit with the seed's physiological potential being affected. This hypothesis can be corroborated by the findings of Varghese and Naithani (2002), who demonstrated that SOD activity can increase due to the growth of superoxide anion in seeds, even after the seed has reached critical water content. This indicates that the defense mechanism is still active. In this same study, it was observed that the enzymes catalase and peroxidase exhibited a reduction in their activity following the critical content, likely due to a failure in the underlying mechanism, even with the observed increase in lipid peroxidation.

It can be observed that in Batch 2, the decline in SOD activity occurred subsequent to the seeds reaching the critical content (66.04%). Similarly, in the study by Viana et al. (2020), it was observed that in *Garcinia gardneriana* seeds, SOD activity decreased following the attainment of the critical level. In contrast, catalase exhibited an increase. This correlation between the decline in SOD activity and the point at which the seed reaches the critical level suggests the potential use of biochemical analysis of SOD enzyme activity as a marker

of drying sensitivity. This is because the decline in SOD activity after reaching the critical level has also been observed in intermediate seeds (Vieira et al., 2022), not only in recalcitrant seeds.

In general, viability using the tetrazolium test followed the same trend as the germination test (decline). But the difference between the germination test and tetrazolium viability may have been due to the presence of fungi in the seeds, which may have affected root growth during germination in the germination test. Cocoa seeds with root blackening and no adventitious growth were considered abnormal and were not included in the germination percentage. Consequently, the germination percentage was found to be lower than that indicated by the tetrazolium test. This discrepancy may be due to the fact that the tetrazolium test is not influenced by the action of certain fungi, which can result in high viability values in this test (Neto and West, 1989). In Brasil (2009), seedlings infected with microorganisms are considered normal at germination if the seedlings are intact. Therefore, seedlings were abnormal if their full structure did not develop. This indicates that the germination test is more sensitive than the tetrazolium test.

Conclusion

The physiological quality of the batches may influence the response of the superoxide dismutase enzyme. Furthermore, it was determined that the analysis of SOD activity can be utilized as a marker of drying sensitivity, but not as a sole indicator of recalcitrance.

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