Special Supplement: *Cerrado* **[Brazilian Savanna]**

Seed viability test of orchids native to the Brazilian Savanna[1]

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ABSTRACT

An essential factor for the formation of a native orchid seed bank is the identification of the viability of stored seeds. This study aimed to determine a methodology for optimizing the tetrazolium test, in the identification of the viability of stored seeds of two Orchidaceae medicinal species native to the Brazilian Savanna. Seeds of *Miltonia flavescens* Lindl. and *Schomburgkia crispa* Lindl. were submitted to three pre-conditioning conditions: no soaking (control), sucrose solution or distilled water. The seeds were then submitted to a tetrazolium solution, with three types of conditioning: oven, water bath or ambient temperature. The *M. flavescens* seeds showed a germination of 86.91 %, with a higher percentage of viable seeds (62.32 %) when submitted to pre-conditioning with sucrose + oven conditioning. For *S. crispa*, the germination was 97.78 %, with a higher percentage of viable seeds for the control treatment + ambient temperature (89.49 %). These results suggest that specific protocols should be used to conduct the tetrazolium test in Orchidaceae. Moreover, when performed on a sample basis, the tetrazolium test should only be used to indicate the seed viability.

KEYWORDS: *Miltonia flavescens* Lindl., *Schomburgkia crispa* Lindl., Orchidaceae.

RESUMO

Teste de viabilidade de sementes de orquídeas nativas do Cerrado brasileiro

Um fator essencial para a formação de um banco de sementes de orquídeas nativas é a identificação da viabilidade das sementes armazenadas. Objetivou-se determinar uma metodologia para a otimização do teste de tetrazólio, na identificação da viabilidade de sementes armazenadas de duas espécies medicinais de Orchidaceae nativas do Cerrado. Sementes de *Miltonia flavescens* Lindl. e *Schomburgkia crispa* Lindl. foram submetidas a três pré-condicionamentos: sem embebição (controle), solução de sacarose ou água destilada. Em seguida foram submetidas a solução de tetrazólio, com três tipos de condicionamento: estufa, banho maria ou temperatura ambiente. As sementes de *M. flavescens* apresentaram germinação de 86,91 % e, quando submetidas ao pré-condicionamento com sacarose + condicionamento em estufa, mostraram maior porcentagem de sementes viáveis (62,32 %). Já para *S. crispa*, a germinação foi de 97,78 % e observou-se maior porcentagem de sementes viáveis no tratamento controle + temperatura ambiente (89,49 %). Esses resultados sugerem que, em Orchidaceae, protocolos específicos devem ser utilizados na condução do teste de tetrazólio e, ainda, a realização do teste de maneira amostral deve ser utilizada apenas para a indicação da viabilidade das sementes.

PALAVRAS-CHAVE: *Miltonia flavescens* Lindl., *Schomburgkia crispa* Lindl., Orchidaceae.

INTRODUCTION

Orchidaceae is one of the largest and most diverse families among angiosperms, second only to Compositae (Chase et al. 2015, The Plant List 2020). A total of 2,692 native orchid species are listed in the Flora do Brasil Project (Flora do Brasil 2021). These species are distributed in 251 genera, and 1,490 are endemic to the country. The Orchidaceae family is the third most representative of the plant biodiversity in the Brazilian Savanna biome (Batista et al. 2005, Mendonça et al. 2008), showing species with little explored economic and ornamental potential. According to Flora do Brasil (2021), this biome accounts for 701 species, distributed in 126 genera.

Some orchid species are considered medicinal. According to Diazgranados et al. (2020), in the world checklist of useful plant species, 636 Orchidaceae species have medicinal use. Belloto et al. (2017) reported the isolation of a new natural product, called crispoic acid, from the epiphyte orchid *Schomburgkia crispa* Lindl., in addition to six other

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chemical compounds already known, which are effective against cancer in cells. For the epiphyte *Miltonia flavescens* Lindl., the presence of bioactive compounds with pharmacological properties was already reported, including antifungal and anticancer agents (Porte et al. 2014).

Thus, storing seeds of native species is an important tool to prevent the loss of genetic resources, ensuring the preservation of biodiversity of this highly threatened plant family. *Ex situ* plant conservation - mainly with the formation of seed banks, aiming at the maintenance of genetic variability - may contribute to the restoration of natural populations (Yang et al. 2017, Gale et al. 2018, Seaton et al. 2018).

An essential factor for the formation of a native orchid seed bank is the identification of the viability of stored seeds which can be used for propagation and conservation of the species, since this viability may be affected even under favorable conditions (Doria 2010, Hosomi et al. 2017, Mercado et al. 2020). Therefore, the use of fast tests such as tetrazolium is important, mainly to decision-making regarding the management of seed banks (Marcos Filho 2015).

The tetrazolium test is based on the detection of the activity of dehydrogenase enzymes, particularly the malic acid dehydrogenase. This dehydrogenase reduces the 2,3,5 triphenyl tetrazolium chloride salt in the seed living tissues which transfer hydrogen ions to the salt (Marcos Filho 2015). A reduction reaction occurs in living cells when the seed is immersed in a tetrazolium solution, resulting in the formation of a non-diffusible red compound known as triphenylformazan. Such color indicates the occurrence of respiratory activity in the mitochondria and, consequently, the viability of the tissue. Dead (non-viable) tissues do not react with the solution, preserving their natural color (França Neto et al. 1998).

Methodologies for the tetrazolium test in Orchidaceae seeds have been proposed in the literature (Hosomi et al. 2011, Hosomi et al. 2012, Hosomi et al. 2017, Macedo et al. 2014, Soares et al. 2014). However, changes in the seed pre-conditioning and conditioning procedures for the test may increase the seed efficiency, since these changes aim to improve the visualization of viable seeds (Salazar & Gélvez 2015, Custódio et al. 2016, Seaton et al. 2018, Mercado et al. 2020). For *Trichocentrum jonesianum* Rchb. f., soaking in distilled water for 24 h provides better results for visualizing the seed viability (Lallana & Garcia 2013). Hosomi et al. (2017), evaluating the tetrazolium test on *Cattleya labiata* and *C. tigrina* seeds, report that pre-conditioning in a 10 % glucose or 10 % sucrose solution is more effective for visualizing viable seeds. Mercado et al. (2020) found that the use of deionized water improves the efficiency of the tetrazolium test on *Epidendrum fimbriatum*, *E. microtum* and *E. elongatum* seeds.

Thus, this study aimed to determine a methodology for optimizing the tetrazolium test in the identification of the viability of stored seeds of the Orchidaceae species *M. flavescens* and *S. crispa*, native to the Brazilian Savanna.

MATERIAL AND METHODS

The experiment was conducted at the Universidade Federal da Grande Dourados, in Dourados (Mato Grosso do Sul state, Brazil). *Miltonia flavescens* Lindl. and *Schomburgkia crispa* Lindl. seeds were obtained from manual pollination and mother plants with more than ten years old and were grown in a nursery covered by the overlap of two 50 % shading screens (Figure 1). The average irradiance, temperature and relative humidity were, respectively, 235 μmol m^{-2} s^{-1}, 22.6 ± 5 ºC and 73.9 ± 10 %. Closed capsules were collected eight months after the pollination. Their seeds were removed, homogenized, weighed and kept in a desiccator with silica gel for 15 days. After this period, the seeds of each species were separately packed in aluminum foil, stored in opaque polypropylene screw-capped vials containing silica gel, and refrigerated at 4 ± 2 ºC for up to 180 days.

A total of thirty-six 0.001 g samples of stored seeds of each species were weighed and placed in test tubes. Sets of twelve tubes (10 mL) were pre-conditioned with three soaking pre-treatments: no soaking (control); 3 mL of sucrose solution (10 %) for 24 h; and 3 mL of distilled water for 24 h.

After 24 hours of pre-conditioning, the seeds (excluding those from the control treatment) were washed with distilled water three times and submitted to a 3 mL aqueous solution of 2,3,5 triphenyl tetrazolium chloride (0.5 %) (Soares et al. 2014). They were then placed in the darkness for 24 h and each set of twelve tubes coming from the pre-conditioning treatments were divided into three conditioning treatments: oven (40 ºC); water bath

Figure 1. Mother plants of the orchid species *Miltonia flavescens* Lindl. (A) and *Schomburgkia crispa* Lindl. (B).

(40 ºC); and ambient temperature (25 ± 2 ºC), with eight replicates of one tube each.

After 24 hours, 7 mL of distilled water were added to the 3 mL of tetrazolium solution in the tube and stirred to dilute the solution. Then, 1 mL was pipetted in a Peters chamber for identification and counting of viable seeds under a binocular stereoscopic zoom microscope. Seeds with totally carmine embryos, partially colored embryos and colorless embryos, as well as embryo-free seeds, were counted, the last three ones being considered non-viable seeds. From this counting, the percentage of viable seeds was determined using the following formula modified by Soares et al. (2014): (number of seeds with totally carmine embryos x 100)/total number observed seeds.

The treatments were photographed with a digital camera coupled to a stereoscopic microscope, using the AxionVision version 3.1 (Zeiss™) software. To confirm the results, immediately after the test, a sample of 0.005 g of seeds of each studied species (not subjected to tetrazolium) was taken to an aseptic environment and disinfected with 15 mL of a 0.8 % sodium hypochlorite solution, remaining immersed for 5 min. Subsequently, the seed suspension was diluted to 50 mL and then washed three times with sterile distilled water (121 ºC and 1 atm pressure, for 20 min). Then, the volume of the suspension was made up to 50 mL with sterile distilled water. For *in vitro* sowing, 1,000 µL of seed suspension were inoculated per vial, with four culture vials for each species.

An amount of 60 mL of Murashige & Skoog (1962) culture medium at half the normal salt concentration (½ MS) was used per 600 mL vial (previously sterilized in an autoclave under 121 ºC and 1 atm pressure, for 20 min). Subsequently, the cultures were placed in a growth room with controlled temperature and photoperiod (25 ± 2 ºC; 16 h), and 22 µmol m^{-2} s^{-1} of irradiance were provided by white fluorescent lamps.

The germination percentage was evaluated after 45 days of cultivation. For this purpose, 10 mL of distilled water were added to each culture vial. After manual shaking, the propagules suspension was poured into a Petri dish and the total number of seeds and the number of germinated seedlings were counted using a binocular stereoscopic zoom microscope. The germination percentage was calculated using the following formula (Rosa et al. 2013): (number of geminated seedlings x 100)/total number of observed seeds.

A completely randomized design was used for each studied species, with the treatments arranged in a 3 x 3 factorial scheme (three pre-conditionings and three conditionings), with four replicates of one tube each. The experiment was performed in triplicate at the same time. The data were subjected to analysis of variance. When significant, the means of the treatments were compared by the Tukey test ($p < 0.05$). The statistical analyses were conducted using the Sisvar v.5.3. software (Ferreira 2011).

RESULTS AND DISCUSSION

There was a combined effect ($p < 0.05$) of pre-conditioning and conditioning for the percentage

of viable seeds in both species. For *M. flavescens*, the highest percentage of viable seeds was observed when the seeds were soaked in 3 mL of the sucrose solution (10 %) for 24 h, and, subsequently, conditioned in an oven (40 ºC) (62.32 %), although without statistical difference from those conditioned in ambient temperature (25 ± 2 ºC) and water bath (40 ºC) (44.67 and 38.90 %, respectively) and pre-conditioned in 3 mL of distilled water for 24 h (47.58 %) (Table 1).

For *S. crispa*, the highest percentage of viable seeds was observed for the control (no soaking) and, subsequently, for conditioning in ambient temperature (25 ± 2 ºC) (89.49 %), but there were no statistical differences between the pre-conditioning and conditioning in oven (40 ºC) (74.62 %) (Table 2).

The results allowed to infer that the pre-conditioning of seeds before the test is a determinant factor for the increased visualization of *M. flavescens* and *S. crispa* viable seeds identified by the tetrazolium test.

The use of a 10 % sucrose solution as pre-conditioning may increase the accuracy of the tetrazolium test (Hosomi et al. 2011, Hosomi et al. 2012, Hosomi et al. 2017, Custódio et al. 2016, Seaton et al. 2018), since the soaking of seeds in this solution for 24 h favors the activation of the respiratory chain of enzymes in the embryo prior to the exposure to the tetrazolium solution (Seaton et al. 2018). When studying the pre-conditioning in *Cattleya*, Hosomi et al. (2012) observed favorable responses with the use of sucrose, which increased by 10 % the number of viable seeds visualized in the tetrazolium test, when compared to the control.

Regardless of the pre-conditioning, there is an increase in the visualization of viable seeds of *S. crispa* when conditioned in ambient temperature (25 ± 2 ºC). Hosomi et al. (2011 and 2012) reported that the sucrose pre-conditioning provides good viability results for *Cattleya*, while, for this species, the conventional tetrazolium test can be used (without prior seed soaking and with incubation at ambient temperature).

The difference in the viability results for both species (Figures 2 and 3) suggests that specific protocols should be used when conducting the tetrazolium test for a better visualization of viable orchid seeds (germination of 100 %).

The highest viability values found in the tetrazolium test were lower than those for germination

Table 1. Percentage of viable seeds of *Miltonia flavescens* Lindl., as a function of pre-conditioning and conditioning in the tetrazolium test.

Pre-conditioning	Viable seeds (%)		
	Conditioning		
	Oven (40 ºC)	Water bath (40 ºC)	Ambient temperature (25 ± 2 ºC)
Control	13.11 bC*	41.54 aA	45.91 aA
Sucrose (3 mL; 24 h)	62.32 aA	38.90 bA	44.67 bA
Distilled water (3 mL; 24 h)	47.58 aB	43.82 aA	46.84 aA
Average	41.00	41.42	45.81
CV (%)	4.89		

* Means followed by the same lowercase letter in the row and uppercase letter in the column do not differ by the Tukey test ($p < 0.05$).

Table 2. Percentage of viable seeds of *Schomburgkia crispa* Lindl., as a function of pre-conditioning and conditioning in the tetrazolium test.

Pre-conditioning	Viable seeds (%)		
	Conditioning		
	Oven (40 ºC)	Water bath (40 ºC)	Ambient temperature (25 ± 2 ºC)
Control	2.94 bB*	3.83 bC	83.93 aA
Sucrose (3 mL; 24 h)	74.89 bA	86.43 aA	88.46 aA
Distilled water (3 mL; 24 h)	74.62 bA	67.12 bB	89.49 aA
Average	50.82	52.46	87.29
CV (%)	3.51		

* Means followed by the same lowercase letter in the row and uppercase letter in the column do not differ by the Tukey test ($p < 0.05$).

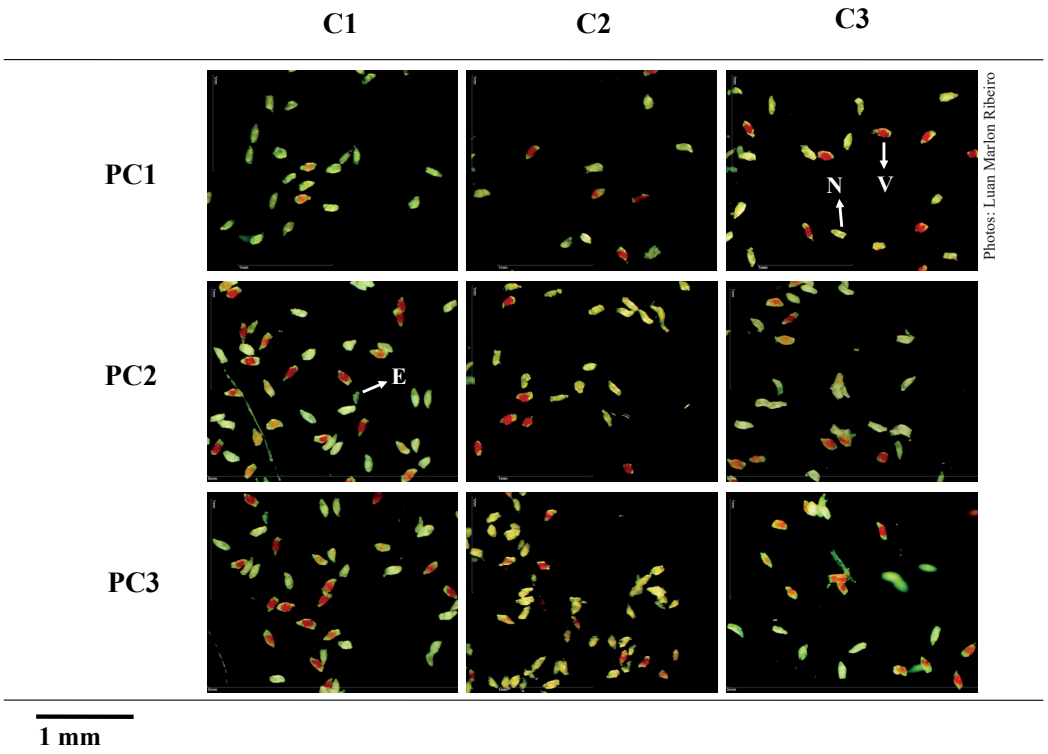

Figure 2. *Miltonia flavescens* Lindl. seeds, as a function of pre-conditioning [PC1: no soaking (control); PC2: 3 mL of a 10 % sucrose solution, for 24 h; PC3: 3 mL of distilled water, for 24 h] and conditioning [C1: oven (40 ºC); C2: water bath (40 ºC); C3: ambient temperature (25 ± 2 ºC)] in the tetrazolium test. V, N and E: viable, non-viable and empty seed, respectively.

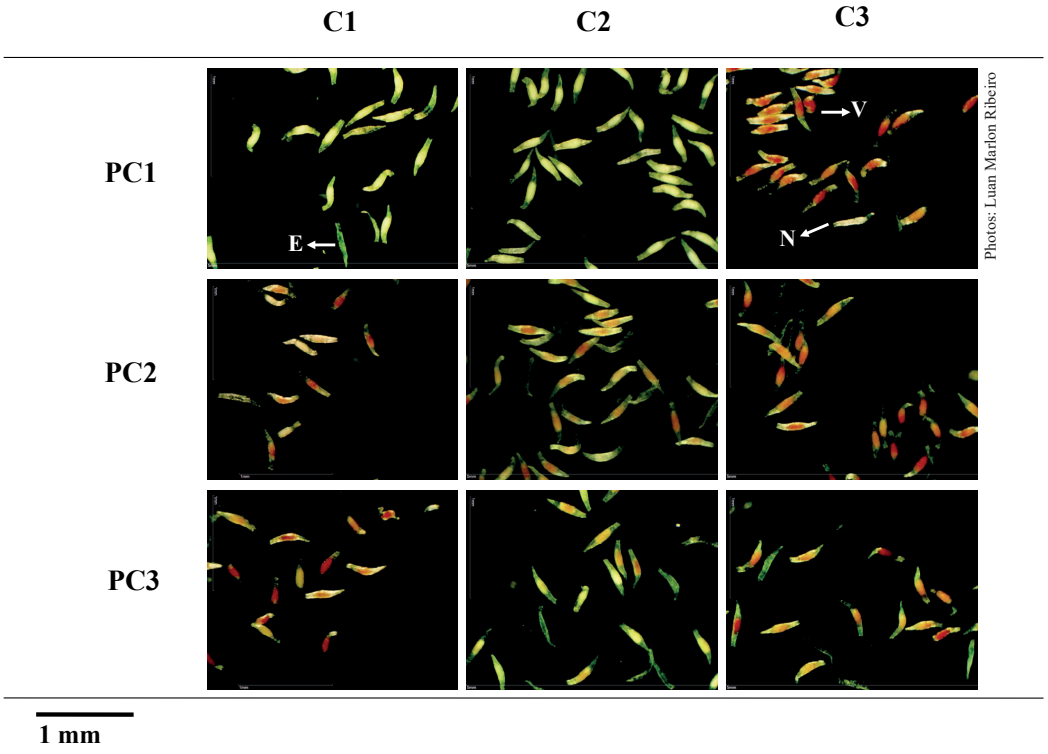

Figure 3. *Schomburgkia crispa* Lindl. seeds, as a function of pre-conditioning [PC1: no soaking (control); PC2: 3 mL of a 10 % sucrose solution, for 24 h; PC3: 3 mL of distilled water, for 24 h] and conditioning [C1: oven (40 ºC); C2: water bath (40 ºC); C3: ambient temperature (25 ± 2 ºC)] in the tetrazolium test. V, N and E: viable, non-viable and empty seed, respectively.

in the two studied species. *M. flavescens* showed a percentage of viable seeds of 62.32 % and germination of 86.91 %, while *S. crispa* presented a percentage of viable seeds of 89.49 % and germination of 97.78 %, at 45 days after sowing.

The number of potentially viable seeds observed in the tetrazolium test often may not correspond to the number of germinated seeds. When studying the viability of *Brassavola tuberculata* Hook. seeds, Rosa et al. (2013) reported a low correlation of the tetrazolium test results with the germination percentage, inferring that the sample method of this test can only be used to predict seed viability and not the likely germination percentage. These results refer to the mode of action of the test, which reflects the activity of the dehydrogenase enzymes involved in the respiration process, making it possible to distinguish the living parts of the seeds from the dead ones, which do not change color (Oliveira et al. 2005). The rules for seed analysis (Brasil 2009) recommend that partially colored embryos may or may not be viable, since the position and size of necrotic areas is what determines the seed viability.

Furthermore, orchid seeds are small, around 0.05 mm (Arditti & Ghani 2000), what makes it difficult to perform cuts allowing a full visualization of the interior of the embryo for the proper assessment of damage to its essential tissues. Thus, seeds that were considered unfeasible in the tetrazolium test may have germinated under appropriate conditions, and seeds that were considered viable may not have germinated.

CONCLUSIONS

1. The difference in the results of percentage of viable seeds observed in *Miltonia flavescens* and *Schomburgkia crispa* suggests that specific methodologies should be used when conducting the tetrazolium test for a better visualization of viable orchid seeds;
2. Performed on a sample basis, the tetrazolium test may only be used to indicate the viability of stored seeds, and not the species germination potential.

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