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The influence of pre-treatment on germination of three species of *Catasetum* (Orchidaceae)

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ABSTRACT: Asymbiotic germination techniques are successful in species of the *Catasetum* genus. The diverse composition of the culture media can be critical; however, viable seeds are required for asymbiotic germination. The reliable method for viability assessment is the tetrazolium test. A decisive step in the efficiency of the tetrazolium test is the seeds preconditioning with sucrose solution, which has the potential to improve germination. Therefore, this work aimed to evaluate the seed germination of three species of the genus *Catasetum* in culture media, preconditioned or not with sucrose solution. Seeds of the species *Catasetum osculatum*, *Ctsm. galeritum* and *Ctsm. complanatum* were assessed by tetrazolium test and germinated in two different media, pretreated or not with 10% sucrose solution. The statistical analysis showed that the use of sucrose pretreatment significantly increased the germination rates of the species. We conclude that the pretreatment with 10% sucrose for 24 hours, regardless of the species and the culture medium, significantly increases the germination of *Ctsm. complanatum*, *Ctsm. galeritum* and *Ctsm. osculatum* seeds.

Index terms: orchid, plant culture, seeds, sucrose solution, tetrazolium.

RESUMO: Técnicas de germinação assimbiótica são bem sucedidas em espécies do gênero *Catasetum*. A composição diversificada dos meios de cultura pode ser crítica. No entanto, sementes viáveis são necessárias para a germinação assimbiótica. O método confiável para avaliação de viabilidade é o teste de tetrazólio. Uma etapa decisiva na eficiência do teste de tetrazólio é o pré-condicionamento das sementes com solução de sacarose, o que tem potencial para melhorar a germinação. Portanto, este trabalho teve como objetivo avaliar a germinação de sementes de três espécies do gênero *Catasetum* em meios de cultura, pré-condicionados ou não com solução de sacarose. Sementes da espécie *Catasetum osculatum*, *Ctsm*. *galeritum* e *Ctsm*. *complanatum* foram avaliadas pelo teste de tetrazólio e germinadas em dois meios diferentes, pré-tratados ou não com solução de sacarose a 10%. A análise estatística mostrou que o uso do pré-tratamento com sacarose aumentou significativamente as taxas de germinação das espécies. Concluímos que o pré-tratamento com sacarose 10% por 24 horas, independente da espécie e do meio de cultura, aumenta significativamente a germinação de sementes de *Ctsm. complanatum*, *Ctsm. galeritum* e *Ctsm. osculatum*.

Termos para indexação: orquídea, cultura vegetal, sementes, solução de sacarose, tetrazólio.

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INTRODUCTION

The species within the Orchidaceae family have low occurrence in populations and are restricted to small forest fragments (Daviña et al., 2007). The disturbance of these habitats and the indiscriminate collection placed many species at risk (Hirano et al., 2005). The genus *Catasetum* comprises 121 species, of which 92 are endemic to Brazil (Petini-Benelli, 2022), all suffering from continuous habitat destruction, unbalancing the flowering and fruiting percentages along with other genera (Cardoso et al., 2016; Petini-Benelli, 2016). Most of the *Catasetum* species have no information on the threat status, making a real assessment of its situation impossible (Petini-Benelli, 2022).

To avoid the over collection of plants from natural populations, since the germination rate in nature is low (< 5%), one solution is to produce high numbers of plants through asymbiotic germination techniques (Arditti and Ernst, 1993; Utami and Hariyanto, 2020) and turn them easily available to the consumers (Seaton and Pritchard, 2011). With this methodology, in vitro germination, it is possible to supply carbon to the embryo to fulfill the energy needs that would be provided in nature by mycorrhizal fungi (Zhang et al., 2013).

Germination occurs differentially among orchid species. For epiphytic species such as *Cattleya* (Hengling et al., 2020; Fileti et al., 2021), the embryo swells, breaks up the testae, and turns green. For terrestrials, the embryo swells, fulfils the testae before breaking it up and stay white for long, emitting rhizoids and leaf primordia before becoming green (Seaton and Ramsay, 2005). *Catasetum* species, even an epiphytic genus, follows the germination pattern of the terrestrials. Although many authors show prominent results for asymbiotic germination in the *Catasetum* genus (Ferreira et al., 2018; Zakizadeh et al., 2019), for the species *Ctsm. galeritum*, *Ctsm*. *osculatum* e *Ctsm*. *complanatum*, Brazilian endemics (Petini-Benelli, 2022), which exist in environments subjected to high anthropic pressure, has no data available until now. Some authors point out that the composition of the medium can be critical to achieving high germination rates, as the formulation can be specific to the genus or even the species (Suzuki et al., 2012; Diantina et al., 2020).

The different formulations and compound quantities, as well as the nutrients available (organic are crucial for germination in different species. Determining an ideal medium can improve large-scale production and contribute to the conservation of threatened species (Suzuki et al., 2012).

The culture media are diverse, and the MS medium is the most common (Murashige and Skoog, 1962). Another important medium, recommended especially for terrestrial species, is the MM, Malmgren medium (Malmgren, 1996), which effectiveness was not yet evaluated for the germination of the *Catasetum* genus. The main difference is the presence of nitric nitrogen in the high concentrations in the MS medium, which has an inhibitory effect in the germination of some terrestrials (Ponert et al., 2013; Figura et al., 2020), in the Malmgren medium nitric nitrogen is absent.

Nevertheless, viable seeds are necessary for asymbiotic germination. The most common, practical, and reliable method is the tetrazolium test, which has the potential to assess seed viability and quality (Hosomi et al., 2011, 2012; Vudala and Ribas, 2017). A decisive step in the efficiency of the tetrazolium test is the seeds preconditioning with sucrose solution. This stage aims to hydrate evenly and activate the embryo's metabolism (George et al., 2008). To increase seed germination, this step would be of good improvement for the germination rates.

Therefore, this work aimed to evaluate if the pretreatment step with sucrose before seed germination of three species of the genus *Catasetum* in culture media can increase germination rates.

MATERIAL AND METHODS

Seed Collection and storage

Seeds of *Ctsm. osculatum* K.G.Lacerda and V.P.Castro, *Ctsm*. *galeritum* Rchb.f. and *Ctsm*. *complanatum* F.E.L.Miranda and K.G.Lacerda (Table 1) were produced by crossings, as this genus exhibits sexual dimorphism in Bela Vista Orchids

(Assis – SP, -22.67299 -50.41754). At dehiscence, seeds were removed from capsules, put in paper envelopes and these were kept over silica gel for three days at 25 °C. They were then transferred to cryotubes and stored at -18 °C, according to the OSSSU protocol (Orchid Seed Science and Sustainable Use Seed Bank (Seaton and Pritchard, 2008), at the *Universidade do Oeste Paulista* (*UNOESTE* – Presidente Prudente – SP).

Seed viability

Initial viability was assessed using the tetrazolium test, according to Hosomi et al. (2011). Portions of 5 mg seeds of each species were placed in microtubes at room temperature for 15 minutes, and then add 1.0 mL of 10% sucrose solution and kept for 24 hours at room temperature. After this period, the liquid was removed, and the seeds were rinsed twice with distilled water, followed by the addition of 1.0 mL of 1% tetrazolium salt (2,3,5 triphenyl tetrazolium chloride) solution and kept in a water bath at 40 °C for 24 hours. Finally, the solution was removed, and the seeds were rinsed with distilled water.

The seed samples were placed on glass slides to obtain the images. The seeds with red embryos were considered as viable, while those with white embryos were non-viable. Empty seeds without an embryo, were not considered.

Seed germination

Each species was divided into two subsamples for germination: one was pretreated for 24 hours in a 10% sucrose solution, and the other did not receive any treatment. Samples were placed in microtubes at room temperature before the pretreatment. All subsamples were disinfected with 3% sodium dichloroisocyanurate solution (DCCA), added with Tween 80 for 10 minutes in a laminar flow chamber, and shaken occasionally (Machado-Neto and Custódio, 2005). After this period, samples were rinsed twice with sterile water and distributed in Petri dishes containing nutrient medium.

The chosen media for germination were the half concentration MS media, with 1% agar and 2% sucrose, and the MM medium described by Malmgren plus MS vitamins, 1% agar and 1% sucrose. The pH for both media was 5.7 before autoclaving at 121 °C for 15 minutes and subsequently were distributed in 60 mm Petri dishes.

Pretreated and not-pretreated seeds were sowed in a laminar flow chamber according to Machado-Neto and Custódio (2005). Three Petri dishes were used per treatment, sealed with PVC film, and transferred to a growth room at 25 ± 3 °C with a 16 h light / 8 h dark. Germination was evaluated weekly in nine selected areas containing at least 30 seeds per area. Only seeds whose embryos were in stage 1 of germination, meaning swelled and rhizoid protrusion (Zettler and Hofer, 1998; Seaton and Ramsay, 2005; Pereira et al., 2011) were counted as germinated; the evaluation continued until germination stabilized. A camera attached to a stereoscope microscope obtained high-definition quality images for evaluation in Gnu Image Manipulation Program.

The germination speed index (GSI), described by Maguire (1962) and modified by Hosomi et al. (2012) for orchids, was also evaluated, as in the following formula:

$$GSI = G1 / N1 + G2 / N2 + \cdots + Gn / Nn$$

Table 1. *Catasetum* species, their place of origin and conservation status.

Catasetum species	Origin[1]	Conservation Status[1]
Ctsm.[2] *osculatum*	Pará, Rondônia, Mato Grosso do Sul, Mato Grosso	NE[3]
Ctsm. galeritum	Amazonas, Pará, Tocantins, Maranhão, Mato Grosso	NE
Ctsm. complanatum	Rondônia, Mato Grosso	NE

[1]Flora do Brasil 2020, Reflora Project (PETINI-BENELLI, 2020).

[2]Abbreviations of scientific names followed the rules of Royal Horticultural Society for orchid genera and hybrids (RHS, 2017).

[3]NE – Not evaluated

Where G is the number of germinated seeds per period, and N is the time in days after germination. Germination is evaluated weekly; therefore, the time intervals were 7, 14, 21, and so forth until germination stabilization.

Experimental design and statistical analysis

The work was carried out in triplicate in a completely randomized design with a triple factorial arrangement of species x preconditioning x culture medium (3 x 2 x 2).

Normality and homogeneity of the results were initially checked for the germination and GSI data so that it could be subjected to analysis of variance (ANOVA). Then the means were compared by the Tukey test at 5% probability. Statistical analysis was made using the software SISVAR 5.7 (Ferreira, 2011).

RESULTS AND DISCUSSION

Viability was assessed utilizing the tetrazolium test by the methodology proposed by Hosomi et al. (2011, 2012) (Figure 1). We could observe seeds with viable embryos stained in red (Figure 1, red arrows); non-viable embryos were not stained (white arrow, Figure 1) and empty testa (Figure 1, black arrows). The observed results shown that viability was 44.13% for *Ctsm. complanatum*, 21.13% for *Ctsm. galetirum* and 21.95% for *Ctsm. osculatum*. Although not considered for the viability test, we detected in the total amount of seeds 53.64% of empty testae for *Ctsm. osculatum*, 28.07% for *Ctsm. complanatum*, and 5.73% for *Ctsm. galeritum* (Table 2 and Figure 1).

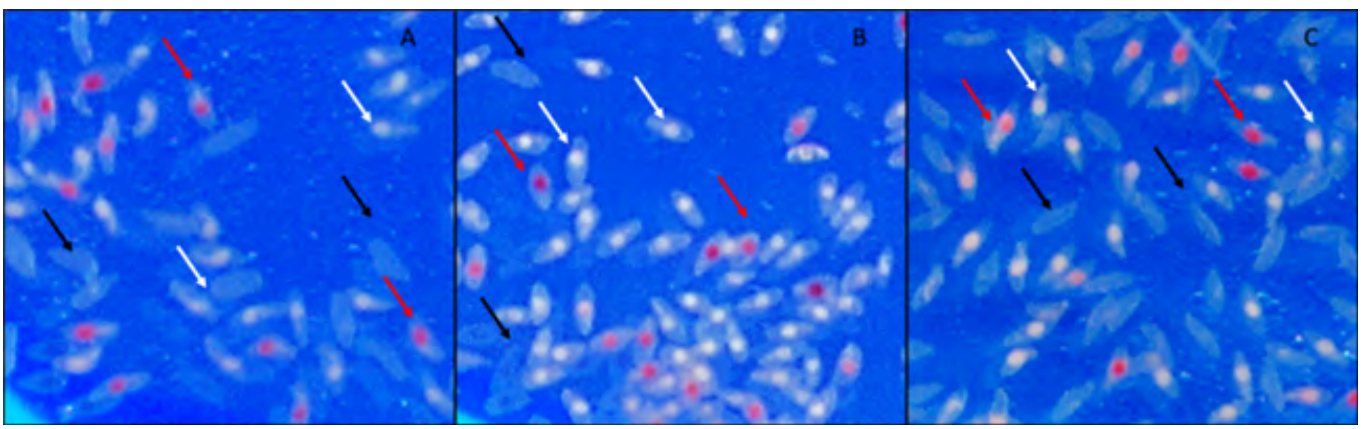

Figure 1. Stained seeds of *Catasetum complanatum* (A), *Catasetum galeritum* (B) and *Catasetum osculatum* (C), with the 2,3,5-triphenyl tetrazolium chloride (TZ). Viable seeds show embryo stained in red (red arrows), empty seed are indicated by black arrows and non-viable seed are indicated by white arrows.

Table 2. Tetrazolium test (TZ) results and empty testae percentages of *Catasetum* species. Values expressed as means and standard error.

Treatments	TZ	Empty testae* (%)
Ctsm. complanatum	44.13 ± 2.66	28.07 ± 2.24
Ctsm. galeritum	21.13 ± 1.56	5.73 ± 0.81
Ctsm. osculatum	21.95 ± 1.64	53.64 ± 11.87

*Empty testae percentages were based in the total count of seeds.

The analysis of the triple factorial of species, pretreatment, and culture medium showed no interaction between them (Table 3). Only the use of sucrose pretreatment significantly increased the germination rate (Table 3 and Figure 2). However, there was no difference in comparing the performance of the culture media MS and MM, as observed in not pre-treated seeds of *Cyrtopodium aliciae* (Oliveira et al., 2023).

The germination averages for all treatments can be seen in Table 4. The treatment without sucrose addition for *Ctsm. complanatum*, *Ctsm. galeritum* and *Ctsm. osculatum* germinated, 4.70%, 2.87% and 6.39%, respectively, while pretreatment with 10% sucrose solution, germination had higher means of 57.31% for *Ctsm. complanatum*, 25.80% for *Ctsm. galeritum* and 44.70% for *Ctsm. osculatum*.

Even though there is a difference between MS and MM media in not treated seeds (1.13% and 8.84%), the observed germination in pretreated seeds does not differ in both used media (Figure 3). The GSI supports the germination and media average, as the observed Speed Index from the pretreated seed is higher than those form the not pretreated seeds (Table 4).

Actual germination occurred from the second week of incubation, when seeds reach stage 1 of germination according to Pereira et al. (2011) and Zettler and Hofer (1998) in pretreated seeds of *Catasetum galeritum*, third week in *Ctsm*. *complanatum* and fourth week in *Ctsm*. *osculatum*. After the fourth week of cultivation, it was possible to observe germinated seeds in both pretreated and not pretreated seed of *Ctsm. complanatum*, fifth week of *Ctsm*. *galeritum* and seventh week of *Ctsm*. *osculatum*. Development was followed until protocorms reached stage 3 proposed by Zettler and Hofer (1998). These results imply that regardless of the species and the culture medium, the

Table 3. Germination average (%) of *Catasetum* in MS and MM media, with and without pretreatment and their interactions.

Species	Germination (%)
Catasetum complanatum	31.00 a
Catasetum galeritum	15.37 c
Catasetum osculatum	25.02 b
F	21.58*
Culture medium	
Murashige and Skoog	22.91 a
Malmgren	25.24 a
F	1.43 ns
Pretreatment	
With	42.25 a
Without	4.75 b
F	373.78*
F_{int} Species x Culture medium	0.48 ns
F_{int} Species x Pretreatment	24.50*
F_{int} Culture medium x Pretreatment	9.49*
F_{int} Species x Culture medium x Pretreatment	0.56ns
CV (%)	23.85

Means followed by equal letters do not differ by Tukey's test at 5% probability. *Significant at 1% probability by the F test. CV: coefficient of variation.

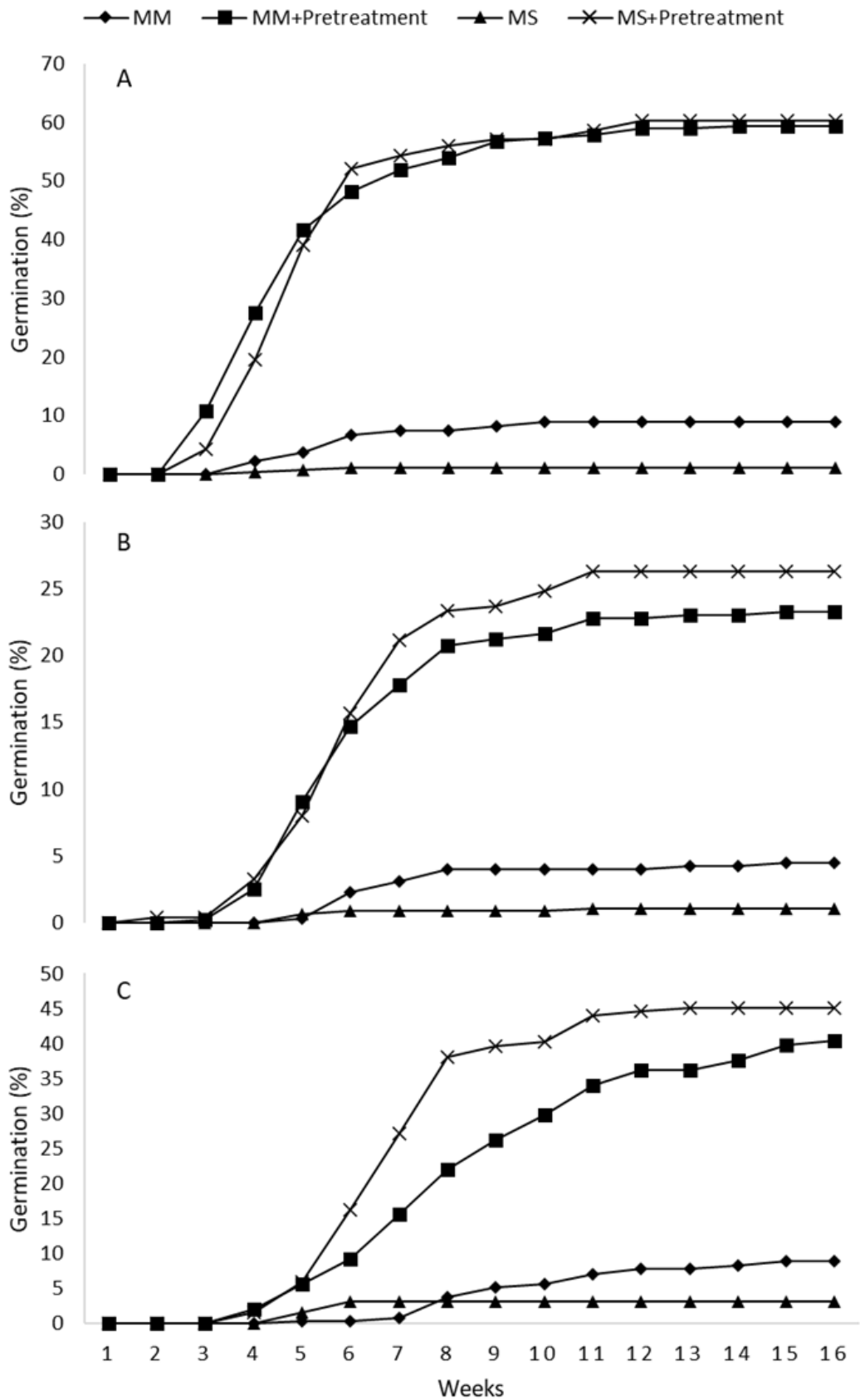

Figure 2. Germination percentage accumulated of *Catasetum complanatum* (A), *Catasetum galeritum* (B) and *Catasetum osculatum* (C) in MS and MM media, with and without pretreatment.

Table 4. Germination (G%) and germination speed index (GSI) of *Catasetum* species with and without pretreatment in MS and MM media. Values expressed as means and standard error.

Treatments	G%		GSI	
	Not Pre-treated	Pre-treated	Not Pre-treated	Pre-treated
Ctsm. complanatum	4.70 ± 1.64 aB	57.31 ± 3.39 aA	0.13 ± 0.05 aB	1.72 ± 0.10 aA
Ctsm. galeritum	2.87 ± 0.92 aB	25.80 ± 2.08 cA	0.06 ± 0.02 aB	0.62 ± 0.05 cA
Ctsm. osculatum	6.39 ± 1.78 aB	43.66 ± 3.16 bA	0.11 ± 0.03 aB	0.87 ± 0.07 bA
MS	1.13 ± 0.31 bB	44.70±2,52 aA	0.03±0.01 bB	1.13 ± 0.07 aA
MM	8.84 ± 1.02 aB	39.81±2.42 aA	0.19±0.02 aB	1.01 ± 0.09 aA

Means followed by the same letter, lowercase for column, uppercase for line, do not differ in Tukey's test at 5% probability.

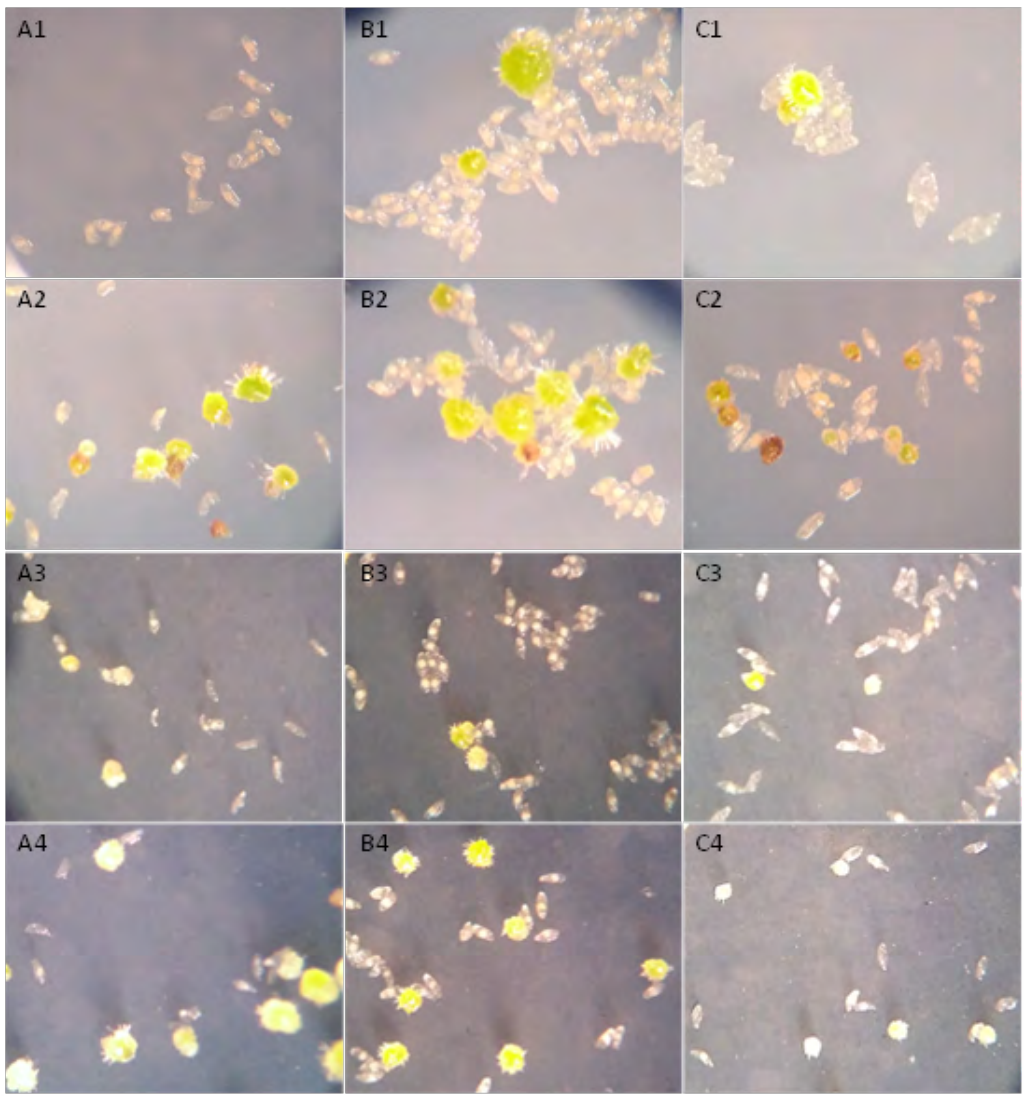

Figure 3. Germination images of 12 weeks old seeds of *Catasetum complanatum* (A), *Catasetum galeritum* (B) and *Catasetum osculatum* (C), in MS media without pretreatment (1), MS media with pretreatment (2), MM media without pretreatment (3) and MM media with pretreatment (4).

use of preconditioning with a 10% sucrose solution for 24 hours before sowing was of great value for the germination process (Figure 2). The GSI corroborates these results, exhibiting higher values in pretreated seeds. Deconninck and Gerakis (2021) presoaked *Anacamptis laxiflora* seeds with 10% sucrose solution, but for microorganism growth and increase sterilization susceptibility not for germination.

Comparing the pretreated seeds to not pretreated seeds, the observed increase in germination was 6.8 times for *Ctsm. osculatum* seeds, 9 times for *Ctsm. galeritum* up to 12.2 times for *Ctsm. complanatum*. This may be due to the osmoregulatory activity of the sucrose solution, reducing the osmotic potential and delaying the absorption of water by the seeds, avoiding damage to the embryo during hydration and activating the germination metabolism even before contact with the nutrient medium (Li et al., 2005; George et al., 2008; Hosomi et al., 2011).

According to Manning and Van-Staden (1987), a simple sugar exogenous source from the culture medium, would facilitate the energy cell machinery, supplying the enzyme system to activate the embryo germination. The pretreatment with sucrose 10% probably initiates the energy cascade, as imbibition occurs.

Usually, culture media with amino acids as a source of nitrogen are indicated for the germination of terrestrial orchids (Ponert et al., 2013; Figura et al., 2020) and some authors suggest that seeds and protocorms assimilate nitrogen more easily from the amino acids form than from nitric nitrogen which must be converted into amino acids before plant absorption (Malmgren, 1996; Majerowicz et al., 2000; Kauth et al., 2008; Gupta, 2016). Studies with *Ctsm. fimbriatum* demonstrated its preference for ammoniacal nitrogen form over nitric form or amino acids (Majerowicz et al., 2000). However, the results here are promising, with no difference in average germination rates between MM and MS medium. Comparing the usage of pretreatment, the observed germination comparison shows an increase in 4.5 times for MM and 39.5 times in MS medium, demonstrating that the use of sucrose 10% independent of the species was efficient.

Ferreira et al. (2018) proposed that the culture media are specific to the *Ctsm. macrocarpum* development stage. The ease of having seeds and plants available to evaluate the different asymbiotic conditions increase the capacity of reintroduced species in nature (Kitsaki et al., 2004).

The tetrazolium test presented inferior results than those observed in actual germination using pretreatment with 10% sucrose solution, which may indicate that despite being a good predictor of viability, the test does not substitute quantification for *in vitro* asymbiotic germination, or it might need adjustments to the *Catasetum* genus.

CONCLUSIONS

The pretreatment with 10% sucrose for 24 hours, regardless of the culture medium, significantly increases the germination potential of *Ctsm. complanatum*, *Ctsm. galeritum* and *Ctsm. osculatum* seeds.

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