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ORIGINAL ARTICLE – Conservation of Nature

Initial *in vitro* Establishment of the Native Cerrado Orchid *Miltonia Flavescens*

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Abstract

The situation of native orchids from natural environments becomes more vulnerable each day, especially those not protected by conservation units. Thus, studies on the propagation of species, especially on *in vitro* germination and initial establishment, are necessary for maintaining species in their habitat and reintroduction in restoration areas. This study verifies the influence of the nutritional composition of the culture media on *in vitro* germination and initial establishment of *Miltonia flavescens*. Seeds from natural pollination were sown in four different culture media and put in a growing room under controlled photoperiods and temperature. The germination was evaluated 30 days after sowing, and the initial growth of the seedlings at 120 and 180 days after sowing. The results indicated the Murashige and Skoog medium, complete and with half of the salt concentrations, promoted a higher germination percentage and were more effective in the initial development of *M. flavescens* seedlings.

Keywords: Orchidaceae, Cerrado, *in vitro* culture.

1. INTRODUCTION AND OBJECTIVES

The Orchidaceae family is widely distributed in Brazil with 2,475 species distributed among 220 genera, with confirmed occurrences in 27 states in the North, Northeast, Midwest, Southeast and South regions (JBRJ, [2018?]). Several species of this botanical family have medicinal and pharmacological properties and have being used for centuries in the traditional Chinese medicine (Lam et al., 2015; Silva et al., 2015; Tsering et al., 2017). Moreover, epiphytic species of this family can act as bioindicators of the successional stage of the forest, considering that communities in secondary phases have less epiphytic diversity than those in dynamic equilibrium (Ramalho & Pimenta, 2010); thus, they can be used in studies of ecological processes and reintroduced in environments under restoration.

Forming germplasm banks with genetic variability maintenance can contribute to restore natural populations (Gale et al., 2018). Thus, *in vitro* germination of native species of the Orchidaceae family is an important tool for producing plants to be used in reintroduction programs of species in natural areas (Gale et al., 2018; Schneiders et al., 2012).

Based on studies related to the *in vitro* establishment of orchids for both commercialization and conservation, *in vitro* sowing and cultivation techniques have been used for the propagation of several species. The Knudson C (KC) medium formulated by Knudson in 1946, VW, established by Vacin and Went in 1949, and the MS culture medium developed by Murashige and Skoog in 1962 are some of the most used for *in vitro* cultivation of orchids (Silva et al., 2015; Suzuki et al., 2010; Vudala & Ribas, 2017). The culture medium formulation is essential for the seed, since it provides the necessary constituents (minerals, vitamins, growth regulators, among others) for their development, and it can be composed of different combinations according to the requirements of each species (Silva et al., 2017).

Recent studies have shown that the *Miltonia flavescens* Lindl. species produces bioactive compounds with pharmacological properties, including antifungal and anticancer properties (Porte et al., 2014). This is an epiphytic species, native to

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Atlantic Forest, Caatinga and Cerrado environments, common in the Southeastern and Southern states of Brazil, and also in Pernambuco, Bahia and Mato Grosso do Sul, widely distributed and well represented in protected areas, which makes it a species whose situation in nature could be considered less worrying. However, this species is subject to great pressure due to deforestation, being on the red list of the National Center for Plant Conservation (*Centro Nacional de Conservação da Flora*), in addition to the Appendix II of the *Convention on International Trade in Endangered Species of Wild Fauna and Flora* (CITES) (JBRJ, [2018?]; Muller et al., 2007). Due to this fact, the National Center for Plant Conservation recommends larger population and exploration studies, which, in addition to *in vitro* propagation and development, could lead the species to a new evaluation in the future.

Thus, the objective of this study was to verify the influence of the nutritional composition of the culture media on the *in vitro* germination and initial establishment of the seedlings of the native *M. flavescens* species.

2. MATERIALS AND METHODS

Mature fruits of *Miltonia flavescens* Lindl. from natural pollination were used as the study material (Figure 1), from 12-year-old plants set in trees located in the Ornamental Plants Cultivation Area of the Universidade Federal da Grande Dourados – Faculty of Agrarian Sciences (UFGD/FCA).

The fruits were detached from the matrices with a pruning shear and taken to the FCA *in vitro* cultivation laboratory, where they were disinfested with 70% ethanol solution. Then, four fruits of different plants were opened with a scalpel and the seeds were removed, homogenized and conditioned in a desiccator with silica gel (25 ± 2 °C, 75% RH) for 14 days. The seeds were packed into aluminum foil and stored for up to 15 months under refrigeration (5 ± 2 °C) in opaque polypropylene vials with a silica gel cap.

A tetrazolium test was performed after this period following the methodology proposed by Soares et al. (2014), in which three portions of seeds of 5 mg each were placed in test tubes and each received 3 mL of aqueous triphenyl-tetrazolium chloride solution (0.5%). The seed suspensions were conditioned away from the light at room temperature (25 ± 2 °C). After 24 hours, 7 mL of distilled water were added and shaken into the tetrazolium suspensions, and 1 mL was pipetted into a Peters counting chamber to identify and estimate viable seeds using a stereoscopic binocular microscope with transmitted and reflected illumination. Three readings were performed out for each sample, and the average between them was then calculated. *In vitro* sowing was then carried out after confirming the viability of seeds.

Next, 10 mg of seeds were immersed in sterile distilled water for 15 minutes under aseptic conditions, and the water was separated from the seeds using a filter paper of 7.5 µm total porosity. The seeds were then disinfested for 15 minutes in 45 mL of 0.8% sodium hypochlorite solution and rinsed three times with sterile distilled water (50 mL per wash). After discarding the rinsing water, the seeds received 80 mL of sterile distilled water and this seed suspension was used for *in vitro* sowing.

The studied culture media were Murashige and Skoog (MS), MS medium with half of its components (MS½), Knudson C (KC) and Vacin and Went (VW). All media were solidified with 7 gL^{-1} of bacteriological agar (Himedia®, India) supplemented with 30 gL^{-1} sucrose. The pH was measured and adjusted to 5.8 using KOH (0.1M) before autoclaving. Transparent polypropylene containers (height = 5 cm, mouth diameter = 5 cm) with a capacity of 50 mL with a screw cap containing 20 mL of culture medium (according to the treatment) were used as culture vials, which were autoclaved at 120 °C and 1atm pressure for 20 minutes.

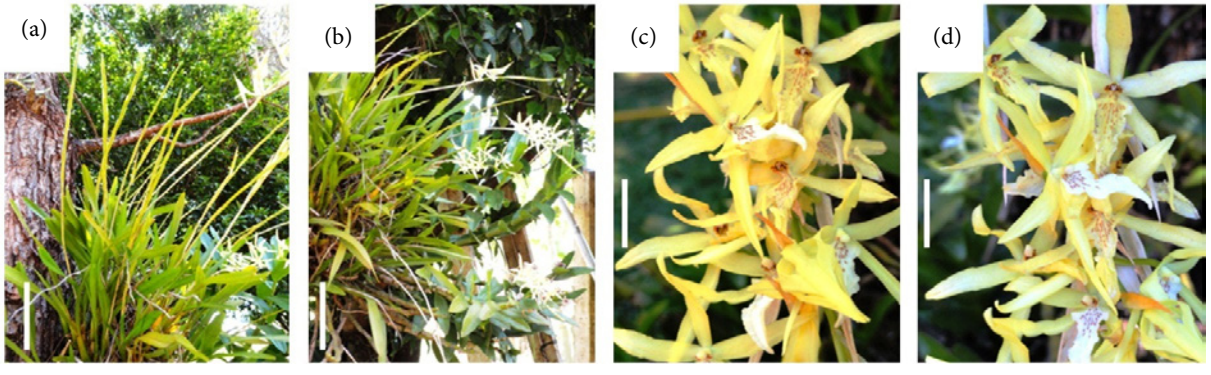

Figure 1. *Miltonia flavescens* Lindl. (a) and (b): Morphological aspect of the plant; (c) and (d): Morphological aspect of the flower. Scale bar: (a) and (b): 10 cm; (c) and (d): 1 cm. Photo: Camila S. R. Lemes.

After cooling and solidification of the medium in an aseptic environment, the seeds were sown using an automatic pipette using 1,000 µL of the seed suspension per bottle. The flasks were then closed and the cultures were stored in a growing room at 25 ± 2 °C with a photoperiod of 16 hours and light intensity of 20.0 µmol m^{-2} s^{-1} Photosynthetic Photon Flux Density (PPFD) from two fluorescent white lamps of 20 W each, approximately 30 cm distant from the culture flasks.

For the seed germination study, three flasks of each culture medium were opened 30 days after sowing, and 3 mL of sterile water were added. Then, the plant materials were transferred to Petri dishes, and this process was repeated until total removal of the material from the culture flask. Next, the number of chlorophyll embryos (CE) and number of non-germinated seeds (NGS) (Figure 2) were counted using a binocular stereoscopic microscope with transmitted and reflected illumination, with the germination percentage (G%) determined based on the equation: (CE / NGS + CE) × 100.

The evaluations for the initial growth of protocorms and seedlings occurred at 120 and 180 days of cultivation, according to the methodology described by Suzuki et al. (2009), considering the following morphological classes (Figure 3): Stage 1 = chlorophyll swollen protocorm; Stage 2 = seedling with formation of the first leaf; Stage 3 = seedling with two leaves; Stage 4 = seedling with leaves and one or more roots. The percentage of protocorms and dead seedlings was also determined. Three vials of each studied nutrient medium were used for each growing time.

The experimental design was completely randomized, and the treatments were arranged into subdivided plots, with the four culture media being allocated in the plots and the two cultivation periods in the subplots, with three replications of each culture vial. The results from discrete variables were transformed into $\sqrt{x + 1}$ and then submitted to analysis of variance and the means were compared using the Tukey's test (α 5%) with the Sisvar program (*Programa de Análises Estatísticas* v. 5.3., Universidade Federal de Lavras, MG).

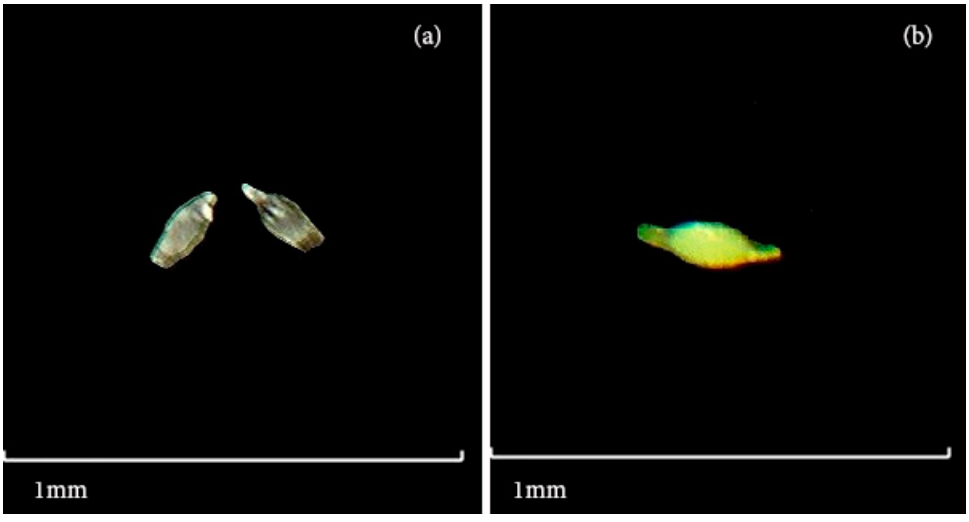

Figure 2. Morphological aspect of *Miltonia flavescens* Lindl seeds. (a): seed soaked in distilled water for 15 minutes, and (b): chlorophyll seed 120 hours after *in vitro* sowing.

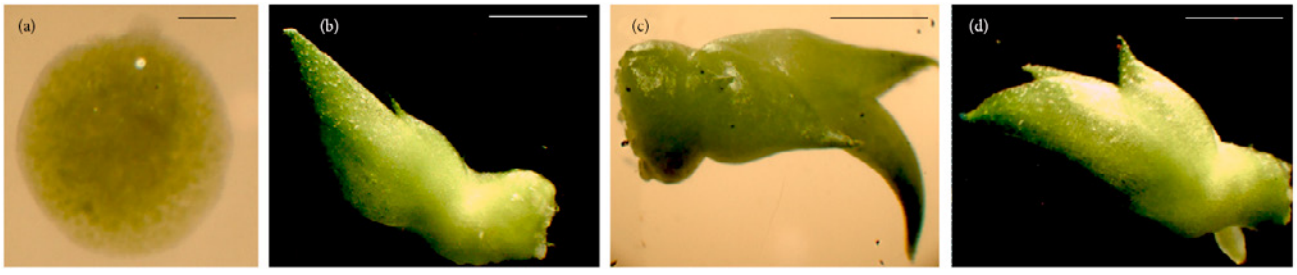

Figure 3. General morphology of the development stages of protocorms until the formation of *Miltonia flavescens* Lindl. Plants. (a) Stage 1: chlorophyll swollen protocorm; (b) Stage 2: seedling with formation of the first leaf; (c) Stage 3: seedling with two leaves; (d) Stage 4: seedling with leaves and one or more roots. Scale bars: (a): 100 µm; (b), (c), (d): 1 mm.
Photo: Camila S. R. Lemes.

3. RESULTS AND DISCUSSION

The different culture media had an effect ($p < 0.01$) on the *in vitro* germination of *M. flavescens* at 30 days after sowing. Seed germination was visually observed approximately 20 days after sowing, when the plant structures were chlorophyllated (Figure 3). The highest germination percentages (G%) were 74.2% for the MS medium, 70.7% for MS½, followed by VW (59.7%) and KC (59.4%), where the first two statistically differed ($p < 0.05$) from the last two.

Analyzing KC and MS culture media in the germination of *Gongora quinquenervis* Ruiz & Pavón, Martini et al. (2001) found that the embryos grown in the MS medium germinated 20 days after sowing, whereas the total of the embryos grown in the KC medium necrosed. Lo et al. (2004) also observed that the MS and MS½ media were the most appropriate for the germination of *Dendrobium tosaense*, while K and VW media were less effective. However, other authors concluded that the MS medium was not the most appropriate for seed germination depending on the orchid species, as in the case of Freitas et al. (2014), who found that the Knudson C culture medium was better than the MS medium for germination and *in vitro* development of *Cattleya intermedia*; and Suzuki et al. (2010) while studying the *Cattleya bicolor* species, who reported that the ideal culture medium for the *in vitro* germination of the seeds and for the initial seedling development was the VW medium.

The most suitable medium for a particular species is directly related to the nutrients supplied to the plants and their influence on germination. According to Stewart (1989), orchid species can be divided into two large groups according to their basic nutritional needs. One of the groups is composed of species that germinate in culture media with lower nutrient concentration, mainly nitrogen and potassium, such as KC and VW, while the other group is composed of orchid species that germinate best in media with higher concentrations of these nutrients, such as MS. The results presented here suggest *M. flavescens* belongs to the second group, requiring culture media with a broader and greater supply of nutrients to initiate seed germination.

In addition to the nutritional composition of the medium, the ammonium:nitrate ratio ($NH4^+$:NO_3-) should also be considered. Gamborg (1970) suggests that the germination of most orchids is more favored in media with higher content of ammonium compared to nitrate. The results presented by *M. flavescens* contradicts this idea, since MS and MS½ were the media with the lowest ammonium:nitrate ratios (Table 1).

The analysis of these results allows to infer that studies on the germination of the species are important, not only to help to understand the species, but also its interaction with the medium in which they will be cultivated, since the literature shows there is no behavior pattern that can be generalized. Such knowledge allows for elaborating *in vitro* multiplication protocols that guarantee their large-scale production, in addition to the reproduction of endangered species, aiming at the repopulation of their habitats or even their commercialization (Cardoso, 2014).

A combined effect of time factors and culture media ($p < 0.05$) was observed on initial seedling growth at 120 and 180 days after sowing. According to Suzuki et al. (2009), the developmental stage 3, when the seedling presented two leaves, was observed at 120 days (Figure 3). At this stage, K, MS½ and VW media stood out for having the highest percentage of seedlings (83%, 81% and 73%, respectively), statistically exceeding the value observed for the MS medium (44%) (Figure 4).

Table 1. Nutrient composition of the culture media used for the asymbiotic seed germination and initial growth of *Miltonia flavescens* Lindl.

Nutrients	Knudson C (KC)	Murashige and Skoog (MS)	Murashige and Skoog (MS½)	Vacin and Went (VW)
	mM	mM	mM	mM
Ammonia (NH_4^+)	3.79	20.62	10.31	3.79
Nitrate (NO_3^-)	4.24	39.43	19.72	5.20
Phosphate (PO_4^{---})	1.84	1.25	0.625	2.48
Potassium (K)	1.84	20.06	10.03	7.04
Sulfate (SO_4^{--})	4.84	1.50	0.75	4.80
Calcium (Ca^{++})	4.24	3.01	1.505	0.65
Magnesium (Mg^{++})	1.02	1.50	0.75	1.02
Chlorine (Cl^-)	–	6.03	3.015	–
Sucrose	87.72	87.72	87.72	87.72
Total nitrogen	8.03	60.05	30.025	8.99
NH_4^+:NO_3^-	0.89	0.52	0.52	0.73

At 120 days, in relation to the other developmental stages, MS medium presented 32% of protocorms (stage 1) and 24% of seedlings at stage 2. Regarding the seeds grown in the MS½ medium, 13% were protocorms (stage 1) and 6% of seedlings were at stage 2. In the KC medium, 10% were in stage 1 and 7% of seedlings were at stage 2. The VW medium had 14% at stage 1 and 13% of seedlings at stage 2 (Figure 4).

Seedlings at stage 4 (Figure 3) were observed in the cultures at 180 days after sowing, where the MS medium had the highest percentage of seedlings with roots (19%) and was statistically similar to the percentage found for the MS½ medium (14%). The other culture media achieved a percentage of seedlings with roots below and equal to 2.5% in the VW medium, and 1% in the KC medium, which in turn had the highest percentage of seedlings at stage 3 (90%) (Figure 4).

The composition of the culture medium is one of the factors influencing *in vitro* development. Among its components, the inorganic nitrogen forms act in a remarkable way on the growth and development of tissue cultures (Nadarajan et al., 2011). It is known that nitrate provides higher growth rates in many species; however, some do not develop well in the presence of this chemical compound. The ammonium:nitrate ratio ($NH4^+$:NO_3^-) was reported by Gamborg (1970) as one of the factors that contribute to the *in vitro* germination of orchids. In our study, it was also observed that it may influence the initial establishment of *M. flavescens*.

K and VW media have a total N content ranging from 8.0 to 9.0 mM, while the MS and MS½ media respectively contain 60.0 and 30.0 mM (Table 1). It is worth noting that seedling development with roots at 180 days after sowing was even observed in the media where the total N content was lower (Figure 4).

In relation to root formation and emission, the MS and MS½ media provided good root system development of *M. flavescens* (Figura 4). The nitrate concentration in relation to the ammonium is higher in these media, which in turn benefited the rooting. The harmful effect of ammonium on root formation was also reported by Tavares et al. (2012) in *Phalaenopsis amabilis* plants cultivated for one year in different nitrate and ammonium concentrations.

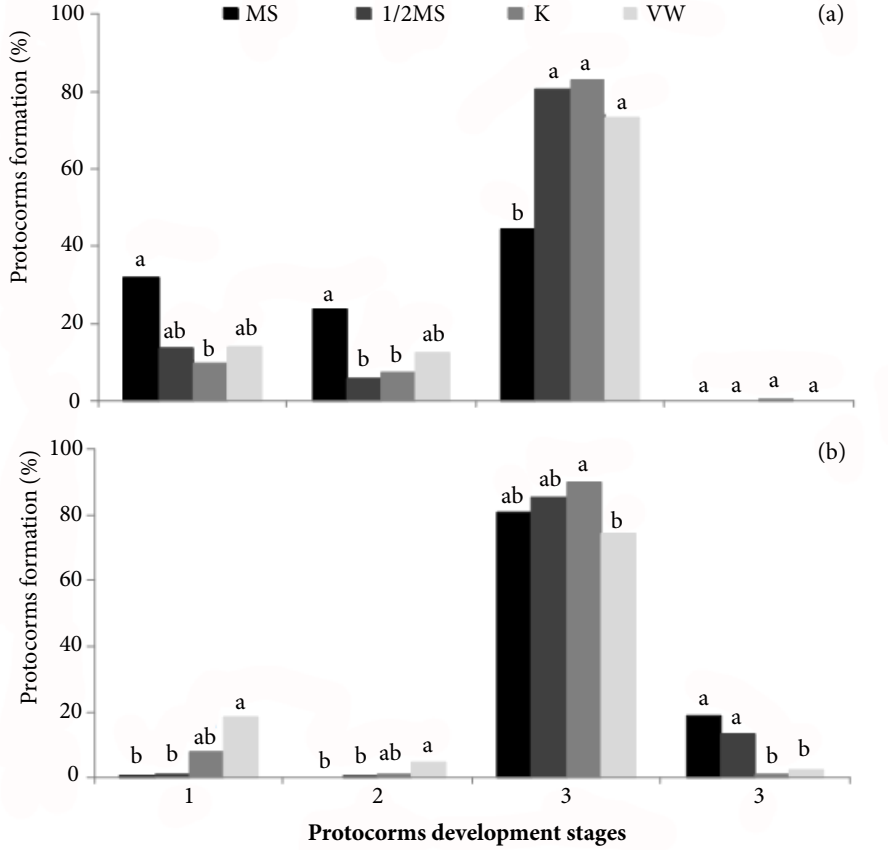

Figure 4. Initial development of protocorms of Miltonia flavescens Lindl. in different culture media. (a) 120 days after sowing *in vitro* and (b) 180 days after sowing *in vitro*. Stage 1: chlorophyll swollen protocorm; stage 2: seedling with formation of the first leaf; stage 3: seedling with two leaves; stage 4: seedling with leaves and one or more roots.
MS: Murashige and Skoog; KC: Knudson C; VW: Vacin and Went.

The benefits of the MS and MS½ formulation were also observed by Lo et al. (2004), who found that the maximum number of seedlings developed from seeds collected from *Dendrobium tosaense* capsules at 12 weeks of age was obtained with cultivation in MS½ medium at 112 days after sowing, in addition to the greater number of chlorophyllated protocorms observed in MS and MS½ media. On the other hand, the KC and VW media did not even allow for protocorm formation. These results are in contrast to those observed by Suzuki et al. (2010) in Cattleya bicolor plants, which had the highest percentage of individuals with roots when grown for 180 days in VW medium, followed by KC medium, and MS medium. Suzuki et al. (2009) did not find the formation of plants with roots for *Hadrolaelia tenebrosa* in the MS medium at 180 days after *in vitro* seeding.

These results contribute to optimize protocols that aim at the *in vitro* propagation of this species, enabling its reintroduction and its commercialization, and generating the expectation that the predatory collections will be minimized, thus preserving the natural populations of this botanical family.

4. CONCLUSIONS

The results observed in this study indicate that the Murashige and Skoog culture medium, either complete (MS) or with half of the salt concentration (MS½), provided the highest germination percentage and were also the most effective in the initial establishment of M. flavescens.

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