Contamination control conditions for the *in vitro* sowing of orchid seeds

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Abstract
Orchids are highly valued for their ornamental and economic importance, with increasing demand for their cultivation in horticulture and the floral industry. However, the in vitro sowing of orchid seeds presents considerable challenges, particularly regarding contamination control, which is critical for successful germination. This issue becomes even more pronounced in laboratories with limited infrastructure, where the absence of a laminar flow hood necessitates methodological adaptations. This study aimed to evaluate the effectiveness of various contamination control strategies by analyzing the impact of pH, culture medium composition, inoculation techniques, light regimes, and fungicide (Chlorothalonil) application on the contamination rate and germination potential of Cattleya perrinii and Cattleya bicolor seeds. Both species are endemic to Brazil and possess significant conservation and ornamental value. Three experimental conditions were tested: E1 (control), E2 (pH adjustment with a synthetic medium), and E3 (light regime alteration and fungicide application). The results indicated that E2, which involved pH optimization and the use of a synthetic medium, significantly reduced contamination, achieving a contamination index (CI) of 0%. In contrast, E3, which involved changes in light exposure and fungicide application, resulted in a CI ranging from 50% to 70%, with no significant reduction in contamination levels. These findings suggest that effective contamination control is achievable even in the absence of a laminar flow hood, particularly when pH and culture medium are appropriately adjusted. This highlights the feasibility of implementing the protocol in low-cost settings, such as small-scale laboratories or conservation initiatives. While pH and culture medium composition have proven effective without compromising seed germination, other factors, including light exposure and fungicide treatment, exhibited minimal or no influence on contamination control. Further methodological refinements are necessary, particularly regarding these variables' independent and combined effects, to enhance the reproducibility and consistency of the in vitro sowing process.
Keywords: *Cattleya perrinii*; *Cattleya bicolor*; fungicides; micropropagation; seed germination.

Condições de controle de contaminação para a semeadura in vitro de sementes de orquídeas

Resumo
As orquídeas são altamente valorizadas por sua importância ornamental e econômica, com crescente demanda por seu cultivo na horticultura e na indústria floral. No entanto, a semeadura in vitro de sementes de orquídeas apresenta desafios consideráveis, particularmente no que diz respeito ao controle da contaminação, que é crítico para o sucesso da germinação. Essa questão se torna ainda mais pronunciada em laboratórios com infraestrutura limitada, onde a ausência de capela de fluxo laminar exige adaptações metodológicas. Este estudo teve como objetivo avaliar a eficácia de diversas estratégias de controle de contaminação, analisando o impacto do pH, composição do meio de cultura, técnicas de inoculação, regimes de luz e aplicação de fungicida (clorotalonil) na taxa de contaminação e no potencial germinativo de sementes de Cattleya perrinii e Cattleya bicolor. Ambas

as espécies são endêmicas do Brasil e possuem significativo valor ornamental e de conservação. Três condições experimentais foram testadas: E1 (controle), E2 (ajuste de pH com meio sintético) e E3 (alteração do regime de luz e aplicação de fungicida). Os resultados indicaram que o E2, que envolveu a otimização do pH e o uso de um meio sintético, reduziu significativamente a contaminação, atingindo um índice de contaminação (IC) de 0%. Em contraste, o E3, que envolveu mudanças na exposição à luz e aplicação de fungicida, resultou em um IC variando de 50% a 70%, sem redução significativa nos níveis de contaminação. Esses achados sugerem que o controle eficaz da contaminação é alcançável mesmo na ausência de uma capela de fluxo laminar, particularmente quando o pH e o meio de cultura são adequadamente ajustados. Isso destaca a viabilidade da implementação do protocolo em ambientes de baixo custo, como laboratórios de pequena escala ou iniciativas de conservação. Embora o pH e a composição do meio de cultura tenham se mostrado eficazes sem comprometer a germinação das sementes, outros fatores, incluindo a exposição à luz e o tratamento com fungicida, apresentaram influência mínima ou nenhuma no controle da contaminação. Refinamentos metodológicos adicionais são necessários, particularmente em relação aos efeitos independentes e combinados dessas variáveis, para aumentar a reprodutibilidade e a consistência do processo de semeadura in vitro.
Palavras-chave: *Cattleya perrinii; Cattleya bicolor; fungicidas; germinação de sementes; micropropagação.*

1 Introduction

Orchids are widely recognized for their ornamental appeal and economic value, contributing to the significant expansion of their cultivation within the horticultural and floral industries (Hossain *et al*., 2013; Kindlmann; Kull; McCormick, 2023; Yuan *et al*., 2021). In addition to their aesthetic attributes, orchids are also valued for their medicinal and ecological significance (Hernández-Mejía; Rosa-Manzano; Delgado-Sánchez, 2024; Moreira *et al*., 2024; Singh; Duggal, 2009). However, these plants possess unique reproductive characteristics, most notably their extremely small seeds, which are incapable of autonomous germination and require an association with mycorrhizal fungi. Natural germination of orchid seeds is highly complex and characterized by low success rates in natural environments. Consequently, in vitro propagation has emerged as a vital strategy to overcome these reproductive limitations and facilitate seed germination and cultivation. By creating controlled environments, in vitro sowing allows the circumvention of natural barriers to seed development (Jolman *et al*., 2022; Knudson, 1922; Rasmussen *et al*., 2015). Nevertheless, microbial contamination remains a persistent challenge in in vitro protocols, significantly compromising germination success and seedling viability (Jolman *et al*., 2022; Leifert; Ritchie; Waites, 1991).

The *in vitro* sowing of orchid seeds is particularly susceptible to contamination, even under sterile conditions provided by laminar flow chambers. Contamination rates often exceed 30–50%, typically arising from airborne microorganisms or inadequate laboratory practices (Oliveira; Silveira; Silva, 2000; Setiaji *et al*., 2021; Wright; Alderson; Dullforce, 1986). These challenges underscore the need for improved protocols to reduce contamination, especially in contexts where laminar flow chambers are unavailable or impractical (Nims; Price, 2017). Moreover, micropropagation technology is generally more expensive than traditional methods of plant propagation (Abdalla *et al*., 2022). Although recent research has highlighted the effectiveness of laminar flow systems in controlling contamination, increased attention has been directed toward cost-effective alternatives that minimize reliance on expensive infrastructure (Dunleavy, 1986; Fallon; Mathews; Hinds, 2022).

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Although recent studies have highlighted the role of laminar flow systems as an option for effective contamination control, there is growing interest in cost-effective alternatives that lessen reliance on expensive infrastructure (Dunleavy, 1986; Fallon; Mathews; Hinds, 2022).

In response to these limitations, several methodological strategies have been tested to reduce contamination rates during in vitro cultivation. Parameters such as pH levels of the culture medium, medium type, inoculation technique, light exposure, and fungicide treatment have been explored for their potential to enhance contamination control (Abdalla *et al*., 2022; Tisserat, 1982). Among the various orchid genera, *Cattleya* species are recognised for their ecological and ornamental significance and exhibit high sensitivity to environmental and cultural conditions (Pant *et al*., 2020; Pinheiro *et al*., 2023). Some species thrive under more acidic conditions, which promotes growth and minimises contamination (Nadal *et al*., 2023). The use of synthetic culture media and dry inoculation methods has also been linked to improved germination rates and reduced contamination risks (Stancato; Faria, 1996). Nevertheless, the impact of other factors, including light exposure and fungicide application, remains uncertain, with some studies indicating only minimal effectiveness in curbing microbial growth (Jolman *et al*., 2022; Laezza; Salbitani; Carfagna, 2022).

Given the ongoing challenges in controlling contamination, this research is vital for improving in vitro propagation techniques that do not depend on laminar flow chambers. By exploring specific methodological refinements, this study aims to increase the efficacy and reliability of orchid propagation in more accessible conditions. The results may provide more practical and economically viable protocols for conservation efforts and large-scale orchid production, where minimising the reliance on specialized equipment is essential (Zaynagabdinov *et al*., 2020). Furthermore, procedural optimization will encourage the wider adoption of sustainable propagation methods among conservationists, researchers, and commercial growers alike (Vilcherrez-Atoche; Iiyama; Cardoso, 2022).

The present study aims to evaluate the influence of selected variables on contamination control during the in vitro sowing of *Cattleya perrinii* Lindl. and *Cattleya bicolor* Lindl. seeds. Specifically, the study examines the effectiveness of a slightly alkaline pH, synthetic culture media, and dry inoculation methods in reducing microbial contamination. The limited efficacy of light regimes and fungicide treatments may be linked to their selective action: while some fungal genera preferentially develop under constant light, others thrive in darkness. This combination of conditions may restrict the growth of a broader range of fungal species (Giladi; Altman; Goren, 1979). Furthermore, the study assesses whether light conditions and fungicide treatments meaningfully contribute to contamination control or seed germination. The evaluation focuses on the contamination index and germination rate. This investigation seeks to establish more robust and efficient in vitro protocols suitable for non-laminar flow environments, with potential applications in both conservation and commercial orchid propagation.

From this point onward, the rest of this paper is structured into three main sections: Section 2 details the materials and methods, including seed origin, cultivation conditions, experimental design, and contamination control strategies. Section 3 presents the results and discussion, focusing on the effectiveness of the tested variables in reducing contamination. Finally, Section 4 outlines the study's conclusions, emphasizing its contributions and potential applications in orchid propagation systems.

2 Material and methods

This study was conducted at the Multidisciplinary Laboratory of Dietary Techniques and the Microbiology and Food Hygiene Laboratory of the Centro Universitário de Barra Mansa, as well as at the Plant Clinic Laboratory of the Agronomy Institute at the Universidade Federal Rural do Rio de Janeiro. Two mature seed capsules, one from *Cattleya perrinii* and one from *Cattleya bicolor* (Figure 1), aged between 9 and 12 months, were used for the experiments. Three independent experiments, designated as E1, E2, and E3, were carried out. The methodological variations employed in each experiment are summarized in Table 1.

Figure 1 – Photographs of the orchid species used in this study. (a) *Cattleya perrinii* and (b) *Cattleya bicolor*.

(a) (b)

Source: Image a by Reginaldo Baião (source: https://www.orchidspecies.com/); image b by Luiz Menini Neto (source: Flora e Funga do Brasil)

Table 1 – Variables tested across the experiments

Experiment	Seeds	Culture medium	pH	Light	Fungicide	Seeds inoculation	Bunsen burner	Sterilization capsule	Number of samples	Amostral groups
E1	–	MS	5.8	Artificial light	Chlorothalonil	–	–	–	40	4
E2	*C. perrinii*	Organic	5.7	Natural light	–	Aqueous solution	Sterile field	5’	50	1
E3	*C. bicolor*	MS	5.8	Artificial light	Chlorothalonil	Dry inoculation	Sterile field	10’	40	4

Source: research data

Experiment E1 served as the control and was performed using the culture medium without seeds, allowing for comparison with the variables tested in experiments E2 and E3. The specific characteristics of the sample groups across all experiments are detailed in Table 2. In each experiment, glass bottles manufactured by Samavidros®, equipped with "Bio Sama" specialized lids, were used. These containers were sterilized at 120 °C for 20 minutes, both before and after the addition of the culture medium.

Table 2 – Sample group diversity in the experimental design

Experiments	Sample groups	Number of samples	Number of total samples
E1 / E3	With fungicide + constant light	10	40
E1 / E3	With fungicide + no constant light	10	
E1 / E3	Without fungicide + constant light	10	
E1 / E3	Without fungicide + no constant light	10	
E2	Single group	50	50

Source: research data

The culture medium was prepared using 3 liters of autoclaved distilled water (ADE), 1.5 g of NPK 12-12-36 (Kristalon), 2.25 g of NPK 20-20-20 (Plantprod), 3 g of NPK 15-15-30 (Plantprod), 15 g of agar, and 4.5 g of activated carbon powder. Ammonia (25%) and phosphoric acid (10% v/v) were used for pH adjustment. For the alternative organic culture medium, 150 g of banana, 150 g of potato, and 30 g of sugar were added.

In experiment E1, the culture medium was evaluated without seed inoculation. For experiments E1 and E3, the pH was adjusted to 5.8. A total of 40 vials were used across these experiments, utilizing MS medium (Murashige; Skoog, 1962). While the original preparation method was conducted in an aluminum pan, as proposed by Komorizono (Kikuchi; Bevilacqua, 2005), it was modified in this study to use glass flasks. The total volume of culture medium was reduced to 1 liter, maintaining the original component proportions, and subsequently divided into two equal portions of 500 ml. One portion included a fungicide; the other did not, resulting in 20 vials per treatment condition. Each vial contained 25 mL of culture medium, with 0.2 g of Bravonil Utrex® (Chlorothalonil) added to the fungicide-treated group, according to Colombo *et al.* (2004).

The vials were acclimated under two different lighting conditions, resulting in four groups (10 samples per group) based on the presence or absence of fungicide and constant light exposure (as shown in Table 2).

In experiment E2, a single group of 50 samples was prepared using the organic culture medium, with a final pH of 5.2. Seeds from mature capsules of *Cattleya perrinii* were disinfected using a 30% sodium hypochlorite (NaClO) solution. After opening the capsule, the seeds were extracted, rinsed in distilled water containing two drops of neutral detergent for 5 minutes, and then suspended in 0.5 mL of distilled water. A sterile syringe was used to inoculate 0.1 mL of the seed solution into each vial.

Experiment E3 utilized seeds from *Cattleya bicolor*. The disinfection procedure was the same as in E2, except the rinsing time was increased to 10 minutes (George; Ravishankar, 1997). For inoculation, a platinum inoculating loop (dry sowing) replaced the syringe and was sterilized with 70% ethanol and flame before and after each procedure. Before seed manipulation in both E2 and E3, the laboratory environment was sterilized using ultraviolet (UV) light exposure for one hour. Sowing was performed over a surface approximately 0.20 cm in diameter between two Bunsen burner flames. Glass flasks containing the culture media and seed solutions were maintained in this sterile

environment until sealing. All vials were labeled with the species name, date, and treatment group designation.

Regarding lighting conditions, groups assigned to constant light exposure remained under continuous illumination (24 hours/day) using Grow Lux daylight lamps. Groups without constant light were kept in a translucent environment covered with aluminum foil. Across all experiments, contamination was monitored for 15 days, followed by germination observation in the non-contaminated samples. Germination potential was evaluated as the percentage of germinated samples among the sterile vials.

The Contamination Index (CI) was calculated for each experiment (E1, E2, and E3) as the proportion of contaminated samples relative to the total number of replicates (%). The results of each experiment were analyzed independently and comparatively using the Chi-square test at a 0.05 significance level (95% confidence). Statistical analyses were performed using R software, version 4.4, while the Bray-Curtis correlation index was calculated using Fitopac. The Bray-Curtis index is widely employed in biotechnology studies to quantify the similarity between samples based on species composition and abundance. In the context of the present study, it was instrumental in assessing the similarity of contamination levels and germination patterns across different experimental conditions. The Bray-Curtis index ranges from 0 (complete similarity) to 1 (complete dissimilarity), providing a robust measure for comparing community structures. Its application in this study enabled a nuanced understanding of the influence of different treatments on microbial contamination and seed germination outcomes (Bray; Curtis, 1957).

Contaminated samples from experiment E3 were analyzed at the Plant Clinic Laboratory of the Department of Phytotechnics, Universidade Federal Rural do Rio de Janeiro (UFRRJ). Fungal identification was conducted through direct morphological observation under an optical microscope and isolation on Potato Dextrose Agar. This culture medium is commonly used to isolate fungi and yeasts, with supplements or acidification to inhibit bacterial growth. The morphological criteria used to characterize the fungal genera included colony diameter, texture, surface and reverse coloration, zoning, and fungal sporulation (Kheyrodin; Rajabi; Kianian, 2018).

3 Results and discussions

In the first experiment (E1), the aseptic conditions of the culture medium, procedures, and environment were assessed. After seven days of observation, no contamination was detected in any sample group. Observations were extended to 15 days, during which no fungal or bacterial colonies appeared, resulting in a contamination index (CI) of 0% (Table 3). These results indicate that the aseptic techniques applied were effective in preventing microbial contamination.

Table 3 –Contamination Index (CI) for E1, E2, and E3

Experiment	Sample group	Number of samples	Contaminated samples	Uncontaminated samples	CI (%)
E1	With fungicide/Constant light	10	0	10	0
	With fungicide/ Without constant light	10	0	10	0
	Without fungicide/Constant light	10	0	10	0
	Without fungicide/Without constant light	10	0	10	0
E2	Single group	50	50	0	100

E3	With fungicide/Constant light	10	5	5	50
	With fungicide/ Without constant light	10	6	4	60
	Without fungicide/Constant light	10	7	3	70
	Without fungicide/Without constant light	10	6	4	60

Source: research data

In experiment E2, signs of contamination were observed in several flasks seven days after inoculation. To confirm the extent of contamination, the observation period was extended by an additional eight days. By the end of 15 days, all 50 flasks exhibited contamination, rendering seed germination unfeasible and resulting in a CI of 100% (Table 3). These findings underscore the critical importance of rigorous sterilization protocols during seed sowing procedures and demonstrate the need to adjust pH levels to restrict the development of microorganisms (Montarroyos *et al*., 2007).

Similarly, in experiment E3, contamination was noted by the seventh day post-inoculation. CI values ranged from 50% (with fungicide/constant light) to 70% (without fungicide/constant light) by the 15th day (Table 3). Despite the application of fungicide and the use of continuous light exposure, fungal colonies developed, corroborating the findings of Brown *et al*. (1982). Therefore, at the concentration used, chlorothalonil was not effective in inhibiting fungal development. Moreover, other environmental factors may influence interactions with the environment (Lemes *et al*., 2020).

A diversity of fungal genera was identified (Table 4), suggesting the presence of multiple contamination sources. The variation in contamination rates indicates that, although fungicide application and constant light exposure may have contributed to reducing contamination, they were insufficient to fully eliminate microbial presence in the culture environment.

Table 4 – Fungal genera identified in contaminated samples from E3

	Preliminary diagnosis of group in optical microscopy			
Contaminated samples	***With fungicide/ Constant light***	***With fungicide/ Without constant light***	***Without fungicide/ Constant light***	***Without fungicide/ Without constant light***
1	*Cladosporium sp*	*Cladosporium sp*	*Cladosporium sp*	*Cladosporium sp and Rizoctonia sp*
2	*Cladosporium sp*	*Cladosporium sp*	*Cladosporium sp and Rizoctonia sp*	*Cladosporium sp*
3	*Cladosporium sp*	*Cladosporium sp*	*Cladosporium sp and Rizoctonia sp*	*Cladosporium sp*
4	*Cladosporium sp*	*Cladosporium sp*	*Cladosporium sp and Rizoctonia sp*	*Cladosporium sp*
5	*Cladosporium sp*	*Cladosporium sp*	*Cladosporium sp and Rizoctonia sp*	*Cladosporium sp*
6	–	*Cladosporium sp*	*Cladosporium sp and*	*Cladosporium sp and*

			Rizoctonia sp	*Rizoctonia sp*
7	–	–	*Cladosporium sp and Rizoctonia sp*	–

Diagnosis of group by isolation in P.D.A.

Contaminated samples	***With fungicide/ Constant light***	***With fungicide/ Without constant light***	***Without fungicide/ Constant light***	***Without fungicide/ Without constant light***
1	*Cladosporium sp and Aspergilus sp*	*Cladosporium sp and Rizoctonia sp*	*Cladosporium sp*	*Cladosporium sp and Rizoctonia sp*
2	*Cladosporium sp*	*Cladosporium sp*	*Cladosporium sp and Rizoctonia sp*	*Cladosporium sp and Penicillium sp*
3	*Cladosporium sp*	*Cladosporium sp*	*Cladosporium sp and Rizoctonia sp*	*Cladosporium sp, Penicillium sp and Aspergilus sp*
4	*Cladosporium sp*	*Cladosporium sp*	*Cladosporium sp and Rizoctonia sp*	*Cladosporium sp and Penicillium sp*
5	*Cladosporium sp*	*Cladosporium sp*	*Cladosporium sp and Rizoctonia sp*	*Cladosporium sp and Aspergillus sp*
6	–	*Cladosporium sp*	*Cladosporium sp and Rizoctonia sp*	*Cladosporium sp and Rizoctonia sp*
7	–	–	*Cladosporium sp and Rizoctonia sp*	–

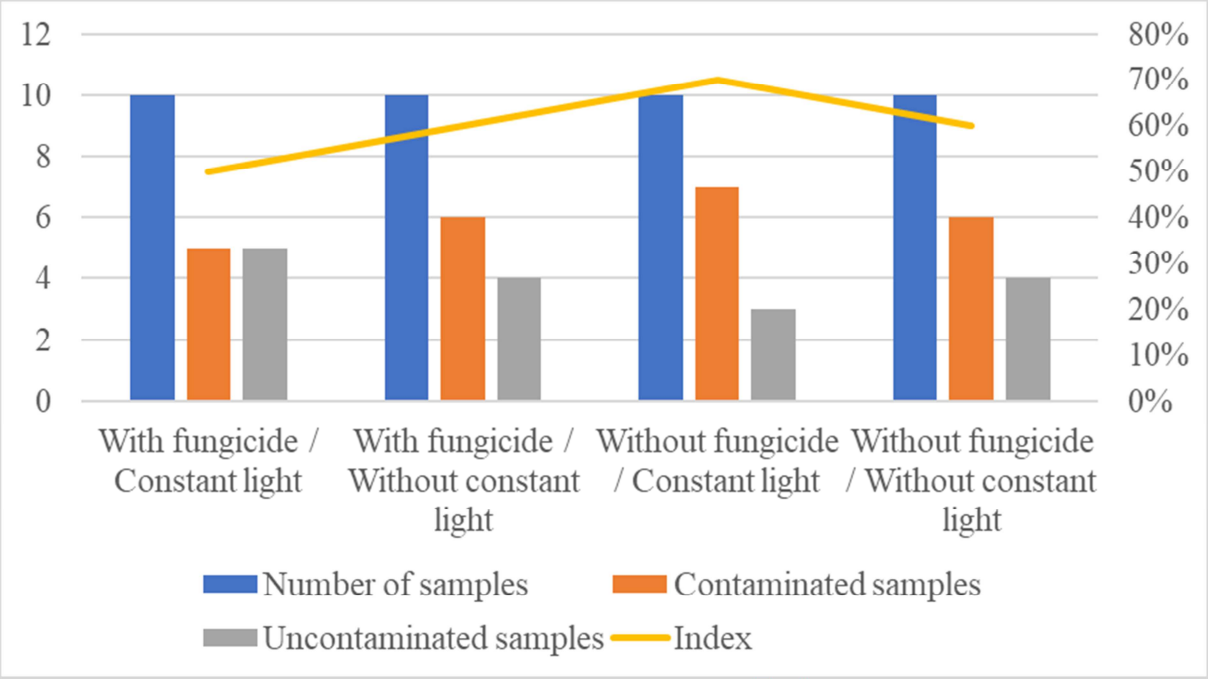
Source: research data

These results align with the findings of Oliveira *et al.* (2011), who reported a reduced occurrence of *Cladosporium* sp. and *Botrytis* sp. after 90 days of seed storage; however, no variation in seed germination was observed. Furthermore, the absence of light inhibited the mycelial growth of *Fusarium solani* across most culture media, while continuous light promoted fungal proliferation. Silva and Teixeira (2012) also demonstrated that fungal species associated with native seeds exhibit diverse physiological responses.

The observed contaminations were exclusively fungal, with a predominance of the genera *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp., and *Rizoctonia* sp. These genera are commonly associated with native forest seeds from the Atlantic Forest biome, where they are considered potentially pathogenic for several species. Additionally, the presence of phytopathogenic microorganisms in in vitro culture media may negatively affect seed germination potential (Parveen; Wani; Bhat, 2019).

The Chi-square test indicated no statistically significant differences among the contamination indices (CIs) of the various treatment groups ($p = 0.05$), suggesting that none of the four combinations significantly reduced contamination (Figure 2). The Student's t-test corroborated this finding, confirming that neither the isolated nor combined effects of fungicide application and light exposure significantly decreased the contamination rate. These results contribute to the optimization of protocols aimed at in vitro propagation without the use of a flow chamber, enabling large-scale production of native or endangered species (Lemes *et al*., 2020).

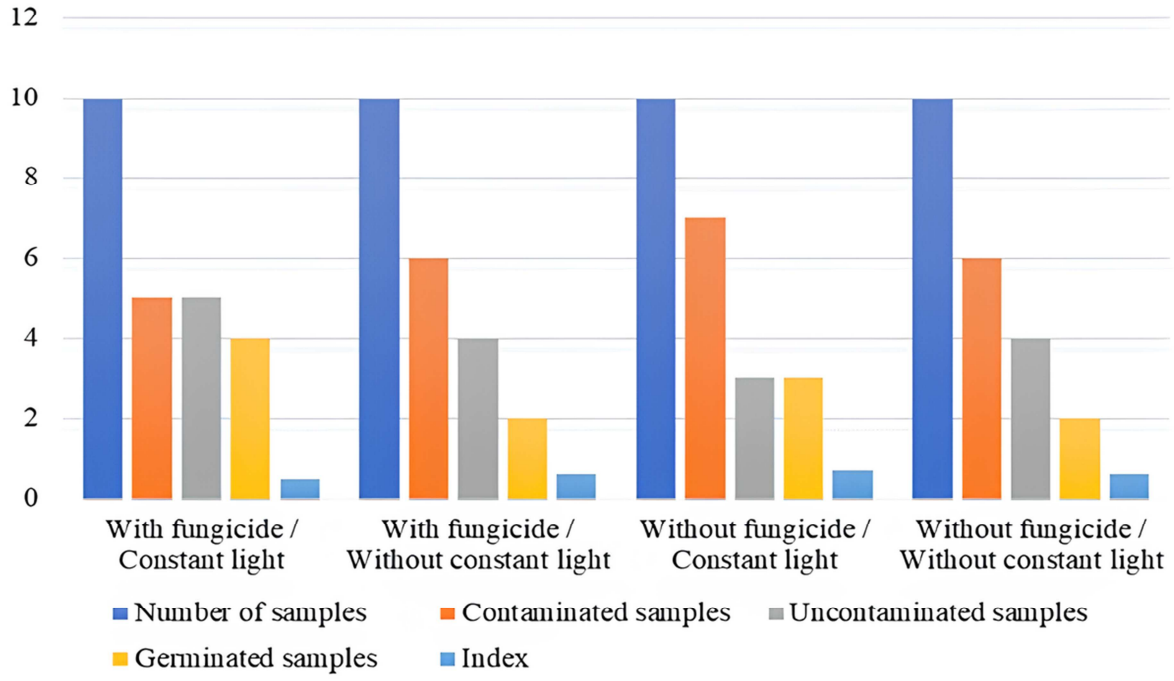
Figure 2 – Sample groups in E3 with analysis of culture medium variables



Source: research data

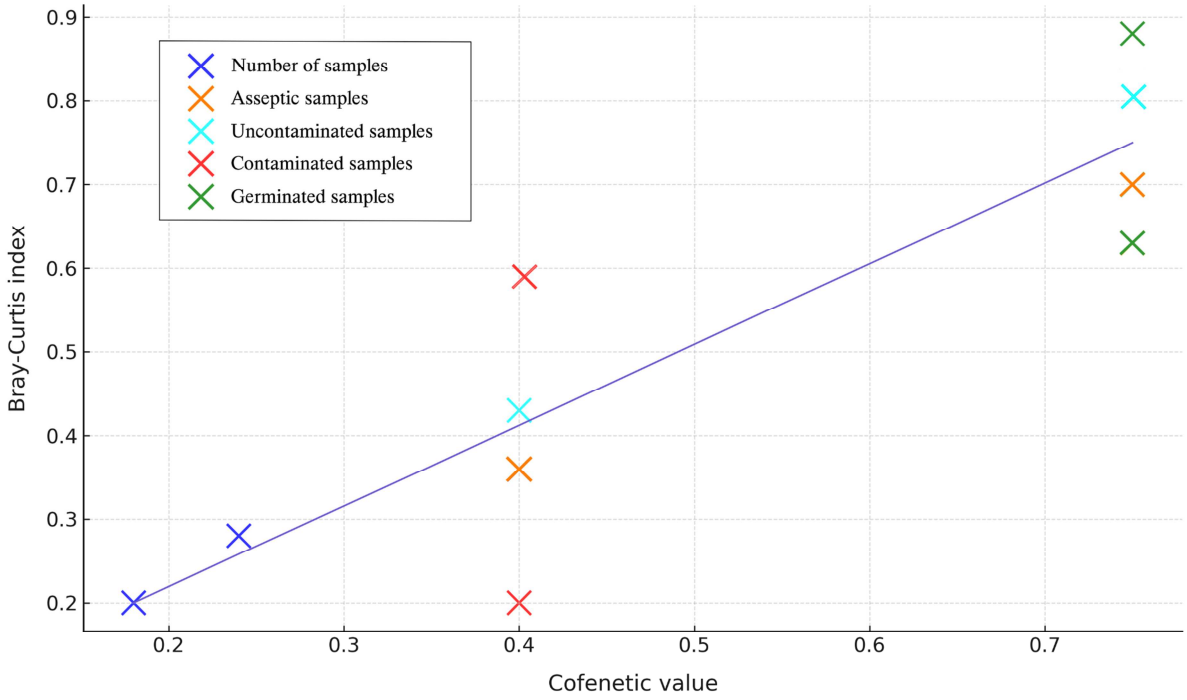
Seed germination was observed 30 days after sowing in all uncontaminated samples (Figure 3), underscoring the effectiveness of aseptic conditions in promoting germination in controlled environments. Moreover, Figure 4 presents the relationship between E3 sample groups and germination potential, yielding a cophenetic correlation coefficient of 0.91. This high correlation supports the Chi-square findings and indicates that contamination did not impair the inherent germination capacity among the sample groups.

Figure 3 – Germinative potential of E3 sample groups



Source: research data

Figure 4 – Cophenetic correlation between the E3 sample groups and the germination potential observed in each group



Source: research data

Adjustments to the culture medium, such as pH modulation, not only facilitate seedling development but also help suppress fungal and bacterial proliferation. Montarroyos *et al*. (2007)

reported that *Mycosphaerella musicola* exhibited optimal radial growth within a pH range of 4.9-5.7, while minimal mycelial growth was observed under constant light conditions. Similarly, Caovila; Gianini; Pedroso-De-Moraes (2016) found that a pH of 5.8 enhanced root development in *Oncidium flexuosum* seedlings. These findings are consistent with the outcomes observed in the E3 experiment, where the combination of constant light and fungicide application appeared to reduce contamination and favor germination.

A comparative analysis between E1 and E3 indicates that factors such as culture medium composition, flask sterilization methods, specific light conditions, and fungicide use significantly influenced the results seen in E3. This suggests that the contamination detected in E2 may not have been solely due to insufficient aseptic procedures. According to Tisserat (1982), the absence of light is crucial for the development of underground organs. In this context, activated charcoal was incorporated to inhibit light penetration and reduce oxidation caused by phenolic compounds released by the tissue, thereby encouraging root formation.

However, light conditions can significantly influence plant physiology, affecting both those cultivated under high light intensity, whose development relies on reserve compounds stored in the pseudobulbs, and those grown under low light, which depend on both these reserves and active photosynthetic capacity. Therefore, the photoperiod should be adjusted according to the specific requirements of each species (Stancato; Mazzafera; Buckeridge, 2002).

The procedural adjustments between E2 and E3, particularly the extension of disinfectant exposure from 5 to 10 minutes, likely contributed to the decrease in the Contamination Index (CI) observed in E3. A Chi-square test indicated a statistically significant difference in CI between E3 and E2 ($p < 0.05$), suggesting that the adjustments improved the effectiveness of aseptic techniques.

Campos (2002) recommends that seed or capsule disinfection be performed using distilled water with bleach in ratios ranging from 300 mL to 700 mL, followed by treatment with 70% aqueous alcohol. The importance of adequate lighting for plant development, such as Grow Lux lamps, is also emphasized. Couto *et al* (2004) reported minimal bacterial contamination in mahogany seeds treated with 2.5% sodium hypochlorite for 10 to 30 minutes. Similarly, Suzuki *et al*. (2009) recorded only 6.6% contamination in *Cattleya tenebrosa* protocorms through specific culture medium modifications.

Although none of the individual variables evaluated yielded a statistically significant reduction in contamination, the combination of fungicide and continuous light resulted in the lowest CI observed in this study, a reduction of 10% compared to the groups without fungicide. Future research should investigate whether this reduction is primarily attributable to the fungicide, the lighting condition, or their interaction.

Leifert, Morris, and Waites (1994) reported contamination rates as high as 35% in European laboratories, while Oliveira, Silveira, and Silva (2000) observed rates exceeding 30% in Brazil, primarily due to fungal and bacterial agents. Contamination in tissue cultures may also originate from microorganisms present in the air or inadequate laboratory procedures (Debergh; Zimmerman, 1991). Effective fungicide application, including considerations regarding concentration, method, and timing, is essential for contamination control (Brown *et al*., 1982). Colombo *et al*. (2004) demonstrated that the fungicide chlorothalonil, at concentrations of 0.1–0.2 g/L, decreased the diversity of contaminants in *Cattleya loddigesii* cultures, a factor that may have contributed to the lower contaminant diversity recorded in E3 (see Table 4).

Oda *et al*. (2003) reported no phytotoxic effects in Gomesa varicosa cultures treated with chlorothalonil or sodium hypochlorite. While fungicides can be valuable for mitigating contamination during the germination of native seeds, further investigations into their potential phytotoxicity are warranted (Su; Chen; Hsieh, 2018). Dimmock and Gooding (2002) also reported favorable outcomes from the use of fungicides and bactericides in both in vitro and in vivo plant propagation protocols.

Nonetheless, Giladi, Altman, and Goren (1979) emphasized that fungal diversity present in native species is often associated with the fungal load in the surrounding environment, particularly in the soil. Influenced by pH variation, genera such as *Fusarium*, *Aspergillus* sp., and *Cladosporium* sp.

were among the most frequently identified contaminants under different light, temperature, and habitat conditions (Laezza; Salbitani; Carfagna, 2022).

Agar remains one of the most effective gelling agents for in vitro culture, providing optimal conditions for seedling development (Stancato; Faria, 1996; Waes, 1987). Activated charcoal also enhances growth promotion and root induction (George; Ravishankar, 1997). Nutritional requirements vary depending on the genotype, and media formulations can be optimized by including plant growth regulators, organic additives, and mineral nutrients (Leifert; Morris; Waites, 1994). Rasmussen *et al.* (2015) emphasized that orchid germination depends on factors that generally influence plant success, such as biotic and nutritional factors, with nitrogen and sucrose being essential for supporting in vitro culture growth and secondary metabolite production, along with abiotic factors such as light and shading. Nitrogen sources like urea (45% N), ammonium sulfate (21% N), and Chilean saltpeter (16% N) are critical for various cellular functions in culture media (Stancato; Faria, 1996).

In summary, this study underscores the importance of carefully optimizing culture medium composition, pH levels, light conditions, and fungicide use in order to minimize contamination and enhance the germination process in *Cattleya* species. The findings reinforce the need for a multifactorial approach in developing effective aseptic culture protocols.

4 Conclusions

The findings of this study demonstrate that in vitro orchid seed germination can be successfully conducted without using a laminar flow chamber. Adjustments to specific parameters, including a slightly alkaline pH, using a synthetic culture medium, and implementing the dry inoculation technique, were associated with reduced contamination rates, indicating the potential viability of this approach (E2). Conversely, incorporating fungicides and alterations to light regimes (E3) did not significantly affect contamination levels.

These outcomes underscore the need for additional research to elucidate the individual effects of each variable and their possible interactions under different conditions and across various orchid species. Moreover, the degree to which seed asepsis alone contributes to contamination control remains unclear and warrants further investigation. A more refined understanding could improve protocol effectiveness and enhance the reproducibility of in vitro germination procedures under non-laminar conditions. Future studies may also evaluate the long-term effects of these optimized conditions on seedling viability and development, offering valuable insights for practical applications in orchid propagation and conservation.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

SCHELB, I. G.: study conception and design; data analysis and/or interpretation; final critical and intellectual review of the manuscript. **ARAÚJO, J. S. P.:** contamination analysis and fungal identification. **ZUÑE, F.:** final critical and intellectual review of the manuscript. **LOPES, R. C.:** data analysis and/or interpretation. All authors contributed to the writing, discussion, review, and approval of the final version of the manuscript.

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