

Seed disinfection methods for *in vitro* cultivation of epiphyte orchids from Southern Brazil¹

Veronica M Alvarez-Pardo⁵; Alfredo Gui Ferreira^{2,3}; Valter F Nunes⁴

²Department of Botany, UFRGS, Av. Bento Gonçalves 9500, Campus do Vale, prédio 43423, 91501-970 Porto Alegre-RS, Brasil; E-mail: ferreira@unb.br (corresponding author); ³CNPq scholarship; ⁴Technician from the Department of Horticulture, UFRGS; ⁵CAPES scholarship

ABSTRACT

Disinfection of orchid seeds is necessary when the germination is performed *in vitro*. In such case, cultures are supplemented by a source of sugar and salts in order to obtain energy and other requirements. However, the presence of sugar and salts easily allows the development of unwanted microorganisms. To avoid such phenomenon, an efficient procedure must be done as a pretreatment of the seeds with liquid or gaseous substances. In this study with several Brazilian orchids, the seeds were disinfected with sodium hypochlorite solutions containing 0.4% or 0.8% active chlorine for five minutes and sodium hypochlorite or formaldehyde fumes up to two hours, this procedure being efficient for such purpose and making it possible to be used routinely.

Keywords: orchids, *in vitro* culture, germination, sodium hypochlorite, formaldehyde.

RESUMO

Métodos de desinfestação de sementes para o cultivo *in vitro* de orquídeas epífitas do Sul do Brasil

A desinfestação de sementes de orquídeas é necessária quando a germinação é efetuada *in vitro*. Neste caso, as culturas são suplementadas por uma fonte de açúcar e de sais, para prover energia e outras necessidades. A presença de açúcares e sais facilmente permite o desenvolvimento de microorganismos indesejáveis. Para evitar estes microorganismos, um procedimento eficiente deve ser usado como pré-tratamento das sementes com substâncias líquidas ou gasosas. Neste ensaio com várias espécies de orquídeas brasileiras, as sementes foram desinfestadas com solução de hipoclorito de sódio 0,4% ou 0,8% de cloro nascente por cinco minutos ou, por vapores de formol ou hipoclorito de sódio por até duas horas, sendo eficientes estes procedimentos para desinfestação e possível seu uso rotineiramente.

Palavras-chave: orquídeas, cultivo *in vitro*, germinação, hipoclorito de sódio, formaldeído.

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Epiphyte orchids have been multiplied routinely from seeds, using a non-symbiotic culture as a propagation method (Knudson, 1922). The inoculation *in vitro* is the main technique for micro-propagation without symbiotic mycorrhizae association. In order to properly develop this technique, sugar and salts are required in a sterile culture medium, where seeds are free from microorganisms.

Orchid embryos can be cultivated *in vitro*, using immature seeds (Kerbaux & Handro, 1981). In such case, the seeds are taken from the closed capsule to prevent contamination when no other inhibition of germination is found (Van der Kinderen, 1987; Rasmussen *et al.*, 1990). This procedure is not suitable for a germplasm bank, because seeds must reach maturity and have low water

content to be stored (Pritchard & Seaton, 1993). For germination, the disinfection is mandatory before inoculation.

The disinfectant liquid should remove or kill all microorganisms from the seeds' surface without damaging the embryos. For the purpose of disinfecting orchid seeds, calcium hypochlorite is the most used one (Wilson, 1915; Thompson, 1980; Arditti *et al.*, 1982), besides hydrogen peroxide (Snow, 1985) and sodium hypochlorite (Pierik, 1990). Calcium hypochlorite needs to be filtered before utilization, and cannot be stored over 12 hours (Arditti *et al.*, 1982). Sodium hypochlorite also needs to be fresh, since both are alkaline and lose chloride, the active oxidizing ion, which captures oxygen, killing the aerobic microorganisms and fungi spores, which in turn, are mainly

responsible for most of the contamination. These chemicals can improve somehow the germination in a few species by light scarification of the seeds (Haas-von Schmude *et al.*, 1986; Van Waes & Debergh, 1986).

Sodium and calcium hypochlorite are prescribed to be used at different concentrations and times of exposure to disinfest seeds (Singh, 1988; Butcher & Marlow, 1989; Seaton & Hailes, 1989; Oddie *et al.*, 1994; Chu & Mudge, 1994). In spite of it, some contamination may appear due to small cracks on the surface of seeds that may keep a few spores. Applying sodium hypochlorite fumes for 5 to 30 minutes can solve this problem, allowing a complete disinfection (Arditti & Ernst, 1992).

In the attempt to attain an efficient method for several Brazilian epiphyte

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Table 1. Initial viability and orchid seeds germination percentage, disinfested with two sodium hypochlorite concentrations with 0.4% or 0.8% of active chlorine for 5; 15; 30 and 60 minutes periods. Porto Alegre, UFRGS, 2004.

Species	Viability (%)	Seed germination (%)							
		0.4% NaCl				0.8% NaCl			
		Time in minutes*							
		05	15	30	60	05	15	30	60
<i>Bifrenaria inodora</i>	84± 2	79a	47b	24c	03d	77a	52b	0c	0c
<i>Brassavola tuberculata</i>	97± 2	98a	97a	90a	06d	91a	34b	30c	02
<i>Cattleya bicolor</i>	99± 1	97a	89b	58c	10d	97a	63c	0e	0e
<i>C. intermedia</i>	99± 1	100a	97a	95a	03c	99a	56b	0c	0c
<i>C. intermedia pallida</i>	99± 1	100a	99a	95a	06d	100a	27b	11c	0e
<i>Encyclia pygmaea</i>	99± 1	88a	60b	0c	0c	90a	53b	0c	0c
<i>Epidendrum fulgens</i>	86± 3	74a	53b	04c	0d	52b	02c	0d	0d
<i>Laelia purpurata</i>	96± 2	94a	77b	34d	0e	88b	71c	05e	0e
<i>Miltonia regnellii</i>	88± 2	81a	76b	22d	0e	75b	38c	01e	0e
<i>Oncidium pumilum</i>	98± 3	98a	69b	02c	0c	75b	0c	0c	0c
<i>Pleurothallis glumacea</i>	74± 2	60a	26b	11d	0f	18c	04e	0f	0f

*Different letters indicate statistical differences among treatments. Tukey test, $p < 0.05$.

orchid seeds inoculation, four procedures were devised: two with liquid (0.4% and 0.8% of active chlorine), and two with gaseous substances (5.5% sodium hypochlorite and 99% formaldehyde fumes).

MATERIAL AND METHODS

Bifrenaria inodora Lindl., *Brassavola tuberculata* Hook., *Cattleya bicolor* Lindl., *C. intermedia* R.Grah., *C. intermedia* var. *pallida* Lindl., *Encyclia pygmaea* (Hook.) Dressler, *Epidendrum fulgens* A.Brongn., *Laelia purpurata* Lindl. & Paxt, *Miltonia regnellii* Reichb., *Oncidium pumilum* Lindl., *Pleurothallis glumacea* Lindl. were collected from native stands in the Northern and Eastern regions of the Rio Grande do Sul State, Brazil, in 1999 and 2000. Ripe capsules were dehydrated (Seaton & Prichard, 1989) and stored at $5^{\circ}\text{C} \pm 1^{\circ}\text{C}$, in the laboratory of the Federal University of Rio Grande do Sul. The disinfestation was carried out in two ways, by a moisten bath and gaseous exposition.

Moisten bath: Twenty milligrams of seeds from each of the eleven species were packed in 10 centimeters of plastic net (60 μm mesh) closed by plastic clips. On a laminar flowhood using a beaker, the material was dipped in 15 ml of Sodium hypochlorite 0.4% or 0.8% of active chlorine for 5; 15; 30 and 60

minutes. The vials were sealed to avoid the loss of chlorine. After that, the samples were washed three times with distilled water and placed on a paper filter to dry off the excess water. The seed viability was evaluated by germination on Knudson C (Knudson, 1946) plus micronutrients (Arditi *et al.*, 1982) and 0.05 mg/L of thiamin and nicotinic acid, 20 g/L of sucrose and 6 g/L of agar. The pH was adjusted to 5.7 with NaOH (Milanese, 1997). Ten milliliters of medium were autoclaved for 20 minutes at 120°C in glass tubes. The tubes were tilted; thus, when the medium cooled down a larger surface was formed, where the seeds were sown. The tubes were covered with plastic film and placed at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in a growth room lit for 16 hours ($30 \mu\text{mol}^{-1} \text{m}^{-2}$). The swollen seeds with green embryos were observed through a stereomicroscope after 30 days of incubation and were considered as germinated (Hailes & Seaton, 1989). A randomized design was used and the data for analysis were transformed in $\arcsin(x + 10)^{1/2}$ before submitted to ANOVA, and followed by Tukey test ($p < 0.05$).

Gaseous disinfestation: Twenty milligrams of *Cattleya intermedia* seeds were placed on a small piece (2x2cm) of aluminum foil on a nine-centimeter diameter Petri dish. Two milliliters of sodium hypochlorite solution (5.5% of

active chlorine) in a small vial or one tablet of formaldehyde (Rioquímica-Brasil) were utilized as disinfestant. The Petri dishes were sealed with parafilm and the disinfestant was applied ranging from 30 minutes to six hours in each of them every half hour. The seed viability was evaluated as done in the first assay, by germination in Knudson C or by tetrazolium test. For these tests, the seeds were water embedded for 24 hours and then placed in 1% tetrazolium solution (2,3,5-triphenyl tetrazolium chloride) for 24 hours at 30°C (Singh, 1981). The seeds were counted through a stereomicroscope, the red ones being viable. Randomized design was used, and the coefficient of correlation was calculated from the data, then the regression curve with best adjustment was determined.

RESULTS AND DISCUSSION

The eleven species examined showed over 75% of initial viability (Table 1). The disinfestation, with increased concentration of sodium hypochlorite solution, decreased the germination, and is related to the exposure time (Table 1). The species *B. tuberculata*, *C. intermedia* and *C. intermedia pallida* were the most tolerant to the effects of the disinfestant. This may be related to the size of the seeds, the structure and ornamentation of the seed's wall (Milanese, 1997). Overall, the most effective treatment was the five-minute with 0.4% sodium hypochlorite solution (Figure 1), which reached an average of 87% germination. At this concentration and time, the treatment was effective to avoid contamination in most of the cases. The use of a small package made disinfestation more efficient, therefore for larger quantities of seeds several packages should be used.

Several protocols for orchid seed disinfestation (Thompson, 1980; Arditi *et al.*, 1982; Snow, 1987; Pierik, 1990) were carried out using a sodium hypochlorite solution, which is simpler and less expensive (Butcher & Marlow, 1989; Seaton & Hailes, 1989; Chu & Mudge, 1994; Vujanovic *et al.*, 2000). Fresh solution and good reliable

chemicals are required, followed by several rinsing with sterile water. These procedures are time consuming, mainly when a large quantity of seeds should be processed.

The utilization of gaseous disinfection devised in this paper showed good results. The disinfection period was not as restricted as that of sodium hypochlorite, and besides less manipulation was required. Thirty minutes to four hours can be used without the loss of viability (Figure. 2A). The tetrazolium test used as a fast viability test could show a decrease of viability after 3 hours in the presence of formaldehyde fumes, probably by acidification of seed surface (Figure 2B). The use of buffered tetrazolium solution should overcome this trouble. After one hour of treatment, no further contamination was observed. This method is recommended because of its efficacy and ease. Formaldehyde fumes can be applied also when a vial with several explants shows a localized contamination. For rare orchid materials, the recovery of *in vitro* contaminated explants or plantlets can be very suitable.

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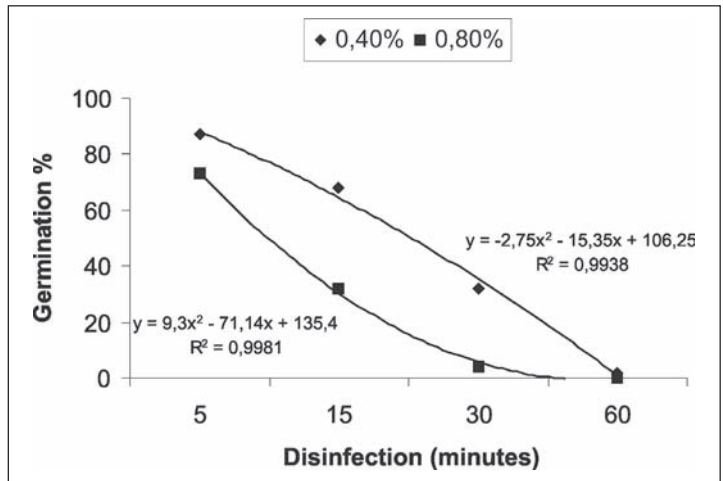


Figure 1. Germination percentage of eleven orchid species compared through polynomial regression. Disinfection with solution in two concentrations of sodium hypochlorite with 0.4% and 0.8% of active chloride for 5; 15; 30 and 60 minutes periods. Porto Alegre, UFRGS, 2004.

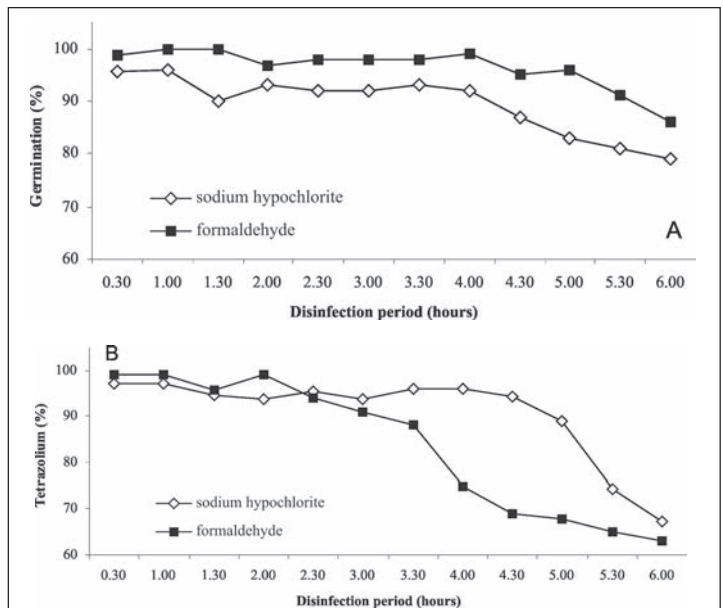


Figure 2. Germination curves (3A) and viability curves according to the tetrazolium test (3B) of *Cattleya intermedia* (Orchidaceae) seeds after several periods of disinfection with formaldehyde (99%) and sodium hypochlorite (5.5%) fumes. Porto Alegre, UFRGS, 2004.

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