Chemical sterilization in *in vitro* propagation of *Arundina bambusifolia* Lindl. and *Epidendrum ibaguense* Kunth

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ABSTRACT

There is a great demand for simpler and less costly laboratory techniques and for more accessible procedures for orchid breeders who do not have the necessary theoretical basis to use the traditional seed and clone production methods of orchids *in vitro*. The aim of this study was to assess the use of sodium hypochlorite (NaClO) as a decontaminant in the process of inoculating adult orchid explants of *Arundina bambusifolia* and *Epidendrum ibaguenses*. Solutions of NaClO (1.200, 2.400, 3.600, 4.800 and 6.000 mg L^{-1} - equivalent to 50, 100, 150, 200 and 250 mL L^{-1} of commercial bleach - CB) were sprayed on the explants (1.0 mL) and the culture medium (GB5), in the presence or absence of activated charcoal (2 g L^{-1}). The explants used were nodal segments of field-grown adult plants. The procedures for inoculating the explants were conducted outside the laminar flow chamber (LFC), except for the control treatment (autoclaved medium and explant inoculation inside the LFC). The best results for fresh weight yield, height and number of shoots were obtained using NaClO in solution at 1.200 mg L^{-1} (equivalent to 50 mL L^{-1} commercial bleach) with activated charcoal in the culture medium. Fresh weight figures were 1.10 g/jar for *Arundina bambusifolia* and 0.16 g/jar for *Epidendrum ibaguenses*. Spraying the NaClO solutions controls the contamination of the culture medium already inoculated with the explants. .

Key words: tissue culture, contamination, sodium hyplochlorite, orchid.

RESUMO

Esterilização química na propagação *in vitro* de A*rundina bambusifolia* Lindl. e *Epidendrum ibaguense* Kunth

Há uma grande demanda por técnicas de laboratório mais simples, e de menor custo, e por procedimentos mais acessíveis, por parte de orquidófilos que não têm o embasamento teórico necessário à utilização de métodos usuais de produção, seminífera e clonal, de orquídeas, *in vitro*. O objetivo deste trabalho foi avaliar a eficiência da aplicação de hipoclorito de sódio (NaClO), para a descontaminação, no processo de inoculação de explantes de orquídeas das espécies *Arundina bambusifolia* e *Epidendrum ibaguenses*. As soluções de NaClO (1.200; 2.400; 3.600; 4.800 e 6.000 mg L^{-1} - equivalentes a 50; 100; 150; 200 e 250 mL L^{-1} de água sanitária comercial - ASC) foram borrifadas (1,0 mL) sobre explantes e meio de cultura (GB5), na ausência ou na presença de carvão ativado (2 g L^{-1}). Os explantes utilizados foram segmentos nodais de plantas adultas, cultivadas a campo. Os procedimentos para inoculação dos explantes foram

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realizados fora da câmara de fluxo laminar (CFL), exceto no tratamento controle (meio autoclavado e inoculação dos explantes em condições de CFL). Máxima produção de matéria fresca, altura e número de brotações foi obtida com 1.200 mg L^{-1} de NaClO (equivalente a 50 mL L^{-1} de ASC), na presença de carvão ativado no meio de cultura, correspondente à matéria fresca de 1,10 e 0,16 g/frasco, para *Arundina bambusifolia* e *Epidendrum ibaguenses*, respectivamente. A pulverização de soluções de NaClO controla a contaminação de meio de cultura já inoculado com o explante.

Palavras-chave: cultura de tecido, contaminação, hipoclorito de sódio, orquídea.

INTRODUCTION

The production of orchid seedlings using tissue cultures has attracted increasing interest from orchid-growers for multiplying the best genotypes of the progenies of various crosses and, in particular, for increasing the availability of native plant species and varieties, some of which are under threat of extinction, making them more accessible at lower cost (Stacanto *et al*., 2001).

A number of studies have been conducted to find a way of making decontamination methods feasible for amateur growers and non-specialized people, some of them centered on assessing the need to use autoclaves and laminar flow chambers (LFC). Studies involving the use of sodium hypochlorite (NaClO) have clearly shown how it can be used as diluted solution, often promoting better growth of *in vitro* tissues, as verified in the germination of rice (Chun *et al*., 1997), orchid seeds (Alvarez-Pardo *et al*., 2006; Rodrigues *et al*., 2011), and the cloning of *Eucalyptus* (Teixeira *et al*., 2008).

Teixeira *et al*. (2006) used weak solutions of NaClO (1, 3, 5, 7 and 9 mg L^{-1}) added to culture media to control contamination in banana. In the autoclaved control, 25% of jars were contaminated, compared with only 10% using a medium containing 1 mg L^{-1} NaClO. At higher concentrations, there was no contamination and the tissues were unharmed. Sterilizing the culture medium using NaClO doubled the fresh weight yield (76 mg/jar) in comparison to the autoclaved medium (32 mg/jar), and results were similar regardless of the concentration of NaClO.

Sodium hypochlorite and calcium hypochlorite - $Ca(ClO)_2$ - are commonly used as sterilizing agents for explants grown *in vitro*. In experiments on apples, contamination control was improved using NaClO, but explant oxidation was higher by comparison with the use of $Ca(ClO)_2$ (Erig & Schuch., 2003).

Spraying seeds of *Bletilla striata*, *Cattleya loddigessi*, *Dendrobium kingianum*, *Habenaria radiata* and *Phalaenopsis* spp. with NaClO or H_2O_2 sterilizing solutions at different concentrations, Yanagawa *et al*. (1995) found 100% germination, even at high hypochlorite concentrations (5,000 mg L^{-1} active Cl), and seed mortality and medium contamination dropped to zero. They also obtained similar results in transplanted explants using the solutions in spray form, without impairment of seedling growth. The responses to H_2O_2 were similar when used at concentrations of 0.1 g L^{-1} (0.01%), both added to the culture medium and using spray application.

The use of activated charcoal in the culture medium, a form of carbon with a high surface area, is known to present elevated capacity to adsorb numerous toxic compounds during *in vitro* cultivation (Nunes *et al*., 2008), such as phenolic exudates and excess ethylene produced inside the culture jars (Ribeiro *et al*., 2000; Chagas *et al*., 2005; Thomas, 2008).

The aim of this study was to assess the response of inoculated explants in non-sterile culture media (no prior autoclaving) using solutions of NaClO at various concentrations, sprayed on the explants in the culture medium, in the absence or presence of activated charcoal.

MATERIALS AND METHODS

The experiments were conducted in the Plant Cell and Tissue Laboratory of the Departamento de Biologia Vegetal at Universidade Federal de Viçosa, in the state of Minas Gerais (MG), Brazil. The culture medium consisted of Gamborg 5 salts - GB5 (Gamborg *et al*., 1968), 20 g L^{-1} sucrose, 6 g L^{-1} agar and 100 mL L^{-1} of green coconut water, adjusting the pH to 5.5 in the control treatment, each culture jar contained 25 mL of the medium autoclaved at 121 ºC for 20 min.

All procedures of the sodium hypochlorite treatments were carried out outside the laminar flow chamber (LFC). Each treatment consisted of applying 1 mL of the respective sterilization solution in spray form over the solid (cooled) culture medium and explants. The control treatment consisted of using the conventional micropropagation method, conducting all procedures inside the LFC and not applying NaClO.

The experiment was arranged in a randomized block factorial design of (2 x 5) + 1: absence or presence of activated charcoal (2.0 g L^{-1}), five concentrations of sodium hypochlorite (1.200, 2.400, 3.600, 4.800 and 6.000

mg L^{-1}) in the form of commercial bleach (CB), at concentrations corresponding to 50, 100, 150, 200 and 250 mL L^{-1}, plus a control treatment with five replications.

The plant material consisted of *Arundina bambusifolia* nodal segments obtained from a home-grown clump in the municipality of Ouro Preto, Minas Gerais state, and nodal segments of aerial shoots of *Epidendrium ibaguenses*, grown in the ornamental plants section at Departamento de Fitotecnia, Universidade Federal de Viçosa. The plant material was washed with neutral detergent in running water, then surface sterilized in 70% alcohol for 1 min, and after discarding the alcohol, in commercial bleach (24 mL L^{-1}) for 15 min, and rinsed with autoclaved water.

The experimental unit was a 234 mL jar containing 25 mL of GB5 medium (Gamborg *et al*., 1968) inoculated with three nodal segments of *Arundina bambusifolia* Lindl. or *Epidendrum ibaguenses* Kunth. The culture jars were sealed with PVC-lined polypropylene plugs.

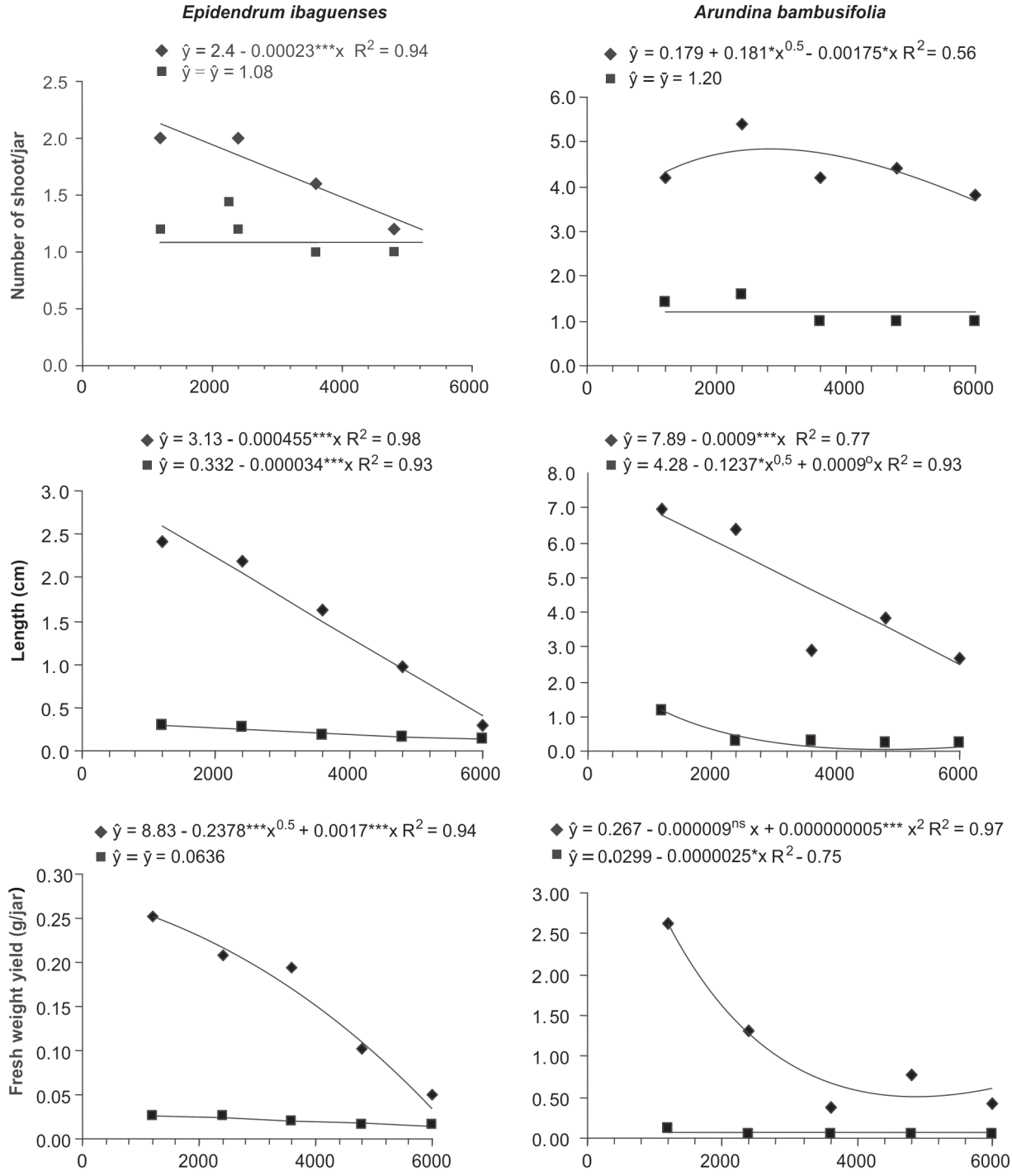

Figure 1. Responses of *Epidendrum ibaguenses* and *Arundina bambusifolia* to sprayed solutions of sodium hypochlorite, in the absence (■) or presence (◆) of activated charcoal.

The same procedures were used for the control treatment, except that after sterilization in alcohol, the explants were handled inside the LFC. For this trial, all jars containing medium were autoclaved at 121 ºC for 20 min. No chemical agents were added to sterilize the medium at this stage.

After treating with NaClO and inoculating the explants, the jars were kept in a culture room at 27 ± 2 ºC, with a 16/8 h (light/dark) photoperiod and irradiance of 48 μmol m^{-2} s^{-1}. The experiment was assessed in terms of explant oxidation, contamination by bacteria and/or fungi, and fresh weight yield, height and number of shoots.

The results were subjected to analysis of variance and the regression equations of the variables studied were adjusted according to the treatments (NaClO with or without activated charcoal), using the SAEG 9.0 software.

RESULTS AND DISCUSSION

The growth and development variables evaluated for *Arundina bambusifolia* and *Epidendrum ibaguenses* indicated that the best results were obtained in the presence of activated charcoal (AC). The results of number of side shoots, length and fresh weigh yield for *A. bambusifolia* and *E. ibaguenses*, are presented on Figure 1. Figure 2 shows the response of *Arundina bambusifolia* to sprayed solutions of sodium hypochlorite (NaClO) in the absence or presence of activated charcoal. These results are in agreement with those obtained by Kent *et al*. (2004), who reported the positive effects of adding 0.5 g L^{-1} AC on fresh weight yield and shoot length for nodal explants of *Anoectochilus formosamus*.

For *Catasetum fimbriatum*, the addition of 0.5 g L^{-1} of AC to the Knudson C medium led to an increase in the number of leaves per explant, in comparison with cultures containing no AC (Morales *et al*., 2006). In the presence of 0.2 g L^{-1} AC, green somatic embryos of *Rhynchostylis rubrum* exhibited strong development from embryogenic calluses in NDM and VW media; embryo development was 80% in the NDM medium and 19% in the VW medium (Te-chato *et al*., 2010).

Increasing the concentration of NaClO in the spray solution decreased the fresh weight yield, the number of shoots and shoot length for both species (Figure 1).

There was a decrease in medium contamination in jars treated with NaClO. However, in the control, we found an average of five bacterial colonies per jar for both species, as well as fungi, but only in the jars containing *A. bambusifolia*. Contamination was higher in the presence of AC, possibly due to adsorption of some chlorine, reducing the effect of the NaClO on microorganisms in the explant. In contrast, Ket *et al*. (2004) mentioned that the presence of AC in the culture medium reduced the effect of phenols released by *in vitro* cultured plant tissues, which in some cases can kill the explant in only a few hours.

Similar results were obtained for fungus contamination in the control (LFC) and in treatments with NaClO, whereas bacterial contamination was generalized for both species in the control (using the laminar flow chamber and without addition of NaClO to the jar), impairing tissue growth and development (Table 1).

Studies on NaClO have shown that the application of diluted solution often promotes superior growth of *in vitro* tissues of different plant species (Chun *et al*., 1997; Ervin & Wetzel, 2002; Teixeira *et al*., 2008). However, in our study, increased doses of NaClO had a harmful effect, most likely due to the decontaminant toxicity as the concentration

Table 1. Bacterial and fungal contamination in jars containing tissue of *Arundina bambusifolia* and *Epidendrum ibaguenses*, in response to doses of sodium hypochlorite (NaClO), in the absence or presence of activated charcoal in the culture medium. Numbers in parentheses refers to percentage of colonies per jar

NaClO	Activated charcoal	*Arundina bambusifolia*		*Epidendrum ibaguenses*	
		Fungal	Bacterial	Fungal	Bacterial
g L^{-1}	g L^{-1}	colonies/jars (%)			
0[(1)]	2[(1)]	1 (20)	5 (100)	0 (0)	5 (100)
1.200	0	1 (20)	0 (0)	0 (0)	1 (20)
2.400	0	0 (0)	0 (0)	0 (0)	1 (20)
3.600	0	1 (20)	0 (0)	0 (0)	1 (20)
4.800	0	0 (0)	1 (20)	0 (0)	1 (20)
6.000	0	0 (0)	0 (0)	0 (0)	0 (0)
1.200	2	3 (60)	3 (60)	2 (40)	1 (20)
2.400	2	0 (0)	3 (60)	0 (0)	1 (20)
3.600	2	0 (0)	1 (20)	0 (0)	0 (0)
4.800	2	0 (0)	1 (20)	0 (0)	1 (20)
6.000	2	0 (0)	1 (20)	0 (0)	0 (0)

[(1)] Control treatment.

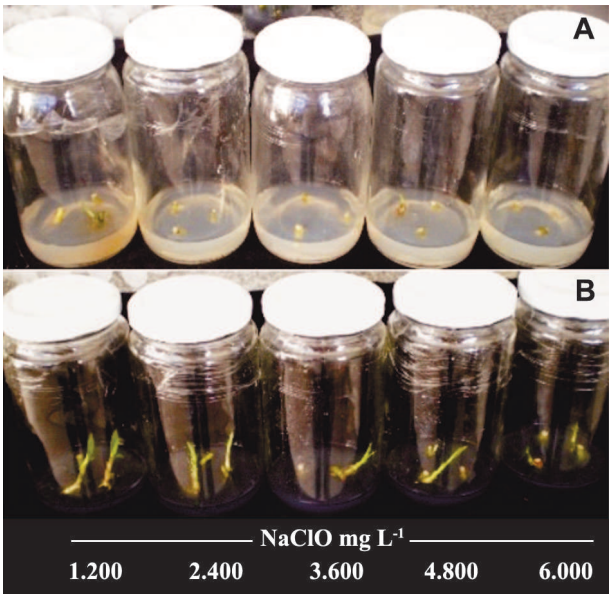

Figure 2. Response of *Arundina bambusifolia* to sprayed solutions of sodium hypochlorite (NaClO) in the absence (A) or presence (B) of activated charcoal, after 60 days in culture.

increased. On the other hand, the lowest NaClO concentration was effective in controlling microbial contamination and promoting explant growth (Figure 2), in contrast to the results obtained in the control with the experimental procedure conducted in the LFC and without NaClO spraying (Table 1).

CONCLUSIONS

Spraying NaClO solution over the culture medium after it has been inoculated with the explant (nodal segments) helps control contamination.

Higher concentrations of NaClO impair plant growth.

In terms of fresh weight yield, number of shoots and shoot length, the best results were obtained in the presence of activated charcoal. However, the charcoal treatment caused increase in bacterial and fungal contamination.

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