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Sodium hypochlorite sterilization of culture medium in micropropagation of *Gerbera hybrida* cv. Essandre

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Micropropagation requires controlling contamination that might compromise the success of the process. Thermal sterilization is traditionally used; however, costs deriving from equipment acquisition and maintenance render this technique costly. With the purpose of finding an alternative to thermal sterilization, this research aimed at assessing the efficiency and ideal concentration of sodium hypochlorite for sterilization of culture media and glassware used during rooting of micropropagated *Gerbera hybrida* cv. Essandre. Two experiments were carried out. In the first one, treatments consisted of control I (no sterilization), control II (thermal sterilization), and total active chlorine concentrations of 0.0005, 0.001, 0.002 and 0.003%. In the second experiment, based on the results observed in the first experiment, treatments consisted of control I (thermal sterilization) and II (chemical sterilization), and total active chlorine concentrations of 0.002, 0.0025 and 0.003%. Plant behavior was assessed based on the length of aerial part and roots, number of roots, and dry biomass of plants. Results showed that the addition of an active chlorine concentration of 0.003% to culture media provided total control of contaminants, and there were no significant differences regarding the variables analyzed between plants obtained with thermal sterilization and with sodium hypochlorite sterilization. Thus, chemical sterilization can be used as a replacement for thermal sterilization of nutrition media for rooting of gerbera *in vitro*.

Key words: Sodium hypochlorite (NaOCl), tissue culture, contamination, chemical sterilization, autoclaving.

INTRODUCTION

In the commercial production of gerberas, micropropagation enables the large-scale production of plants in a limited space, as well as the obtaining of disease- and pest-free uniform plants (Bhargava et al., 2013). This technique also ensures precision in production schedules and product quality, regarding plant homogeneity and vigor. Nevertheless, the occurrence of contamination in an *in vitro* culture is frequent, and might result in high damage. The method traditionally used in contamination control consists of autoclave-sterilization of

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culture media and glassware. Nonetheless, it is costly due to equipment (autoclave) acquisition and too high electric power consumption, which might lead to nutrient decomposition in culture media (Ribeiro, 2006; Weber et al., 2015).

Due to the advantages of micropropagation and to the need for reducing the costs involved, some alternatives to thermal sterilization have been examined. Latimer and Matsen (1977) suggested microwave sterilization, although they observed this method was inefficient in the sterilization of liquids due to medium overflow with elevation of temperature (Tisserat et al., 1992; Teixeira et al., 2005b). Another alternative is the sterilization of culture media by filtering, but it has proven to be inadequate when used for large volumes because it increases consumables and labor costs, and is time-consuming (Tisserat et al., 1992).

Chemical sterilization with sodium hypochlorite (NaOCl), proposed initially by Teixeira et al. (2005a, b), provided satisfactory results for pineapple (*Ananas comosus* L. cv Smooth Cayenne) (Texeira et al., 2006), sequoia (*Sequoia sempervirens* L.) (Ribeiro et al., 2011), and eucalyptus (*Eucalyptus pellita* L. and *Eucalyptus benthamii* Maiden et Cambage) (Teixeira et al., 2008; Brondani et al., 2013). Recently another chemical compound, chlorine dioxide (ClO_2), a stabilized gas, used by Cardoso (2009) in the sterilization of culture media for anthurium (*Anthurium andraeanum* Lind.) and gerbera, eliminated contamination without causing phytotoxicity to plants (Cardoso, 2009; Cardoso and Silva, 2012).

Among all chemical products tested, sodium hypochlorite stands out because it is of low cost and easy to acquire. Thus, this research aimed to determine the ideal NaOCl concentration and its effectiveness in culture media sterilization when growing *in vitro Gerbera hybrida* cv. Essandre.

MATERIALS AND METHODS

The influence of active chlorine in the sterilization of culture media and glassware in *G. hybrida* cv. Essandre cultivated *in vitro* was assessed during the rooting phase. Sodium hypochlorite (NaOCl) was used as a source of active chlorine, available in Qboa®, a marketed household bleach with 2% total active chlorine in its composition.

Two experiments were carried out with the purpose of assessing the most efficient total active chlorine concentration to obtain culture media sterilization. In the first one, treatments consisted of control I (no sterilization), control II (thermal sterilization), and total active chlorine concentrations of 0.0005, 0.001, 0.002 and 0.003% (m/v). In the second experiment, which was conducted based on the results observed in the first experiment, treatments consisted of control I (thermal sterilization) and II (chemical sterilization), and total active chlorine concentrations of 0.002, 0.0025, and 0.003%. The preparation of culture media and sterilization of glassware and water in NaOCl sterilized treatments followed the protocol developed by Teixeira et al. (2006), with addition of 0.003% active chlorine to the medium. In control II, autoclaving was used for a period of 40 min to sterilize glassware and water and 20 min to sterilize the culture medium at a temperature of approximately 121ºC at 1 kgf/cm^2. In control I (sterilization), the glassware was washed with detergent and rinsed with distilled water. The water used in the preparation of the culture medium was distilled and deionized, and no active chlorine was added to the culture medium.

The culture medium consisted of inorganic salts MS (Murashige and Skoog, 1962), White's vitamins (White, 1943), 100 mg L^{-1} i-inositol, 30 g L^{-1} sucrose, solidified with 10 g L^{-1} of agar. When autoclave was used to sterilize, the media used had pH 5.7 ± 1, and in NaOCl sterilized treatments pH was 6.0 ± 1.

After preparation, the nutrient medium was distributed in 20 ml aliquots per culture flask (250 ml), which were then left open inside the laminar flow cabinet for 10 min for chlorine to volatize. Explants were inoculated in the flasks that did not show visible microorganism growth after 48 h. Three-leaf gerbera shoots deriving from stock culture, in the stage of multiplication, were placed in the culture flasks and left in the growth room for 30 days at a temperature of 26 $\pm$ 1°C, with photoperiod of 16 h and irradiance of 19 mol.m^{-2}.s^{-1}.

Plant behavior was assessed based on length of aerial parts and roots, number of roots, and plant dry biomass. The flasks with visible growth of microorganisms were considered only as presence of contamination.

Experiments were conducted in a completely randomized design, with six treatments in experiment I and five in experiment II, both with five replications and three experimental plots; each plot was represented by one flask with one explant. Data were submitted to a variance analysis and means were compared using Tukey's test with 5% of significance with the help of the SAS program for Windows, version 9.2, 2002-2008.

RESULTS AND DISCUSSION

Contamination rate decreased as active chlorine concentration increased and the 0.003% concentration provided total sterilization of the culture medium (Table 1). In the treatment with no sterilization, there was 100% of contamination after 24 h, thus preventing the inoculation of explants.

Regarding mean length of aerial part, mean root length, mean number of roots, mean number of leaves, and plant dry biomass, there was a significant difference only in plant dry biomass, which was higher in the treatment containing 0.0005% of total active chlorine. However, this concentration did not totally prevent contaminants. In the other NaOCl concentrations, plant behavior was similar to the autoclaved control.

The contamination data in Table 1 showed that the ideal concentration for the total sterilization of the culture medium would be between 0.002 and 0.003% of active chlorine in the medium, and should be no less than 0.002%, as this concentration resulted in 13.3% of loss from contamination. Hence, experiment II (Table 2) was conducted with the purpose of determining a more precise sterilizing concentration, using shorter intervals than the ones used in experiment I.

In the second experiment data (Table 2), it was observed that all chlorine concentrations achieved efficient sterilization, but the 0.002% concentration showed 13.3% of losses in the first experiment. Therefore, contaminant control was obtained starting at 0.0025% of active chlorine in the culture medium.

Table 1. Contamination (number), mean length of aerial part (MLAP), mean root length (MRL), mean number of roots (MNR), mean number of leaves (MNL), and plant dry mass (PDM) in different active chlorine concentrations added to culture medium in experiment I for *Gerbera hybrida* cv. Essandre.

Active chlorine concentration (m/v)	Contamination		Variables				
	Flasks before inoculation	Flasks after inoculation	MLAP (cm)	MRL (cm)	MNR	MNL	PDM (g)
No sterilization	15	0	-	-	-	-	-
Autoclaved	0	1	4.5[a]	2.1[a]	1.2[a]	6.46[a]	0.027[b]
0.0005%	5	2	5.27[a]	2.18[a]	2[a]	8.53[a]	0.044[a]
0.001%	5	-	4.9[a]	2.34[a]	2[a]	7.47[a]	0.024[b]
0.002%	2	-	4.53[a]	2.67[a]	2.26[a]	8.2[a]	0.025[b]
0.003%	-	-	4.99[a]	2.18[a]	1.8[a]	5.67[a]	0.028[b]
CV (%)			24.3	15.3	46.2	31.0	35.3

Mean values followed by the same letter in the column do not differ statistically in Tukey's test with 5% significance.

Table 2. Contamination (number), mean length of aerial part (MLAP), mean root length (MRL), mean number of roots (MNR), mean number of leaves (MNL), and plant dry mass (PDM) in different active chlorine concentrations added to culture medium in experiment II for *G. hybrida* cv. Essandre.

Active chlorine concentration (m/v)	Contamination		Variables				
	Flasks before inoculation	Flasks after inoculation	MLAP (cm)	MRL (cm)	MNR	MNL	PDM (g)
No sterilization	15	0	-	-	-	-	-
Autoclaved	0	1	3.63[a]	3.45[a]	1.47[a]	6.27[a]	0.025[a]
0.002%	0	0	3.23[a]	2.86[a]	1.73[a]	6.33[a]	0.026[a]
0.0025%	0	0	3.82[a]	3.18[a]	1.8[a]	5.93[a]	0.027[a]
0.003%	0	0	3.28[a]	2.84[a]	1.33[a]	6.07[a]	0.022[a]
CV (%)			6.7	18.7	30.4	15.7	28.8

Mean values followed by the same letter in the column do not differ statistically in Tukey's test with 5% significance.

Regarding the variables analyzed, there were no significant differences between the results obtained with thermal sterilization and the three active chlorine concentrations of chemical sterilization.

However, since the 0.003% concentration provided to plants the same development in relation to autoclaved treatment in three experiment replications, this concentration can be recommended for gerbera micropropagation. The concentration 0.003% also was effective in sterilizing culture media for sequoia propagation (Ribeiro et al., 2011). However, Teixeira et al. (2006) working with *in vitro* pineapple tree cultures (*Ananas comosus* cv Smooth cayenne), obtained successful sterilization with a lower active chlorine concentration (0.0003%) with no damage to the plant's development; on the contrary, it promoted an increase in biomass and in the number of shoots. On the other hand, when sterilizing media to obtain Brazilian ginseng (*Pfaffia glomerata*) calluses, Ribeiro et al. (2009) obtained satisfactory results with 0.05% NaOCl concentration and did not observe any changes in callus biomass.

Differences observed among several authors regarding the ideal sterilizing concentration of sodium hypochlorite occurred according to laboratory asepsis conditions and the plant species used. This becomes evident in studies in which two species of the genus *Eucalyptus* were reported; whereas 0.005% concentration was effective in sterilizing *E. pellita* (Teixeira et al., 2008); it caused the number of shoots to decrease in *E. benthamii* (Brodani et al., 2013).

The effects of NaOCl sterilization are related to chlorine ions, which trigger oxidative reactions responsible for enzymatic inactivation and lipid and fatty acid degradation; hence, its biocide properties (Saran et al., 1998; Estrela et al., 2002; Emmanuel et al., 2004).

Regarding plant behavior in chemical sterilization, Cardoso and Silva (2012) observed a higher vigor in gerbera sprouts when the medium was sterilized with chlorine dioxide (ClO_2). In sodium hypochlorite (NaOCl) sterilization, the same behavior was observed in rooted plants either in hypochlorite- or autoclave-sterilized media.

The present results corroborate data observed by other authors, who verified that chemically-sterilized tissue

culture proved to be effective in the micropropagation of different species (Brondani et al., 2013; Weber et al., 2015). Weber et al. (2015) stated that laboratories in Kenya, Africa, routinely use this technique in potato micropropagation, which proves that there is a practical and promising use for chemical sterilization.

Conclusions

1. Chemical sterilization can replace thermally-sterilized nutrition media in rooting of gerbera *in vitro*.
2. Addition of total active chlorine concentration of 0.003% to the nutrition medium completely eliminates contaminations and allows for the satisfactory development of rooting of gerberas *in vitro* without causing phytotoxicity.
3. Gerberas grown *in vitro* in chemically sterilized nutrition media using 0.003% of total active chlorine resulted in plants with mean length of aerial parts, mean root length, mean number of roots, mean number of leaves, and plant dry biomass similar to plants grown in autoclave-sterilized media.

Conflict of interests

The authors have not declared any conflict of interests.

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