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Chemical sterilization of culture medium: a low cost alternative to *in vitro* establishment of plants

Esterilização química do meio de cultura: uma alternativa de baixo custo para o estabelecimento *in vitro* de plantas

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Abstract

This study aimed at evaluating the *in vitro* establishment of nodal segments of *Eucalyptus benthamii* in a non-autoclaved culture medium supplemented with different concentrations of active chlorine (NaClO): P2 - 0.000%, P3 - 0.001%, P4 - 0.003%, P5 - 0.005%, P6 - 0.007% and P7 - 0.010%, v/v. The control consisted of an autoclaved culture medium without active chlorine (P1 - traditional preparation). The percentage of establishment, viable shoots, fungal and bacterial contamination, number of shoots and total shoot length were evaluated after 28 days. The *in vitro* establishment of nodal segments of *E. benthamii* was feasible in culture medium supplemented with active chlorine without autoclaving. Concentrations of 0.001% and 0.003% active chlorine produced results most similar to the traditional culture medium. We conclude that treatment with active chlorine is an effective method for tissue culture in *Eucalyptus*, and has considerable potential to decrease costs and energy expenditure in other plant-culture systems.

Keywords: active chlorine, micropropagation, contamination, aseptic cultivation, *Eucalyptus benthamii*.

Resumo

Objetivou-se avaliar o estabelecimento *in vitro* de segmentos nodais de *Eucalyptus benthamii* em meio de cultura suplementado com diferentes concentrações de cloro ativo (NaClO) visando eliminar a autoclavagem. Para tanto, utilizaram-se concentrações de cloro ativo suplementado ao meio de cultura não autoclavado (P2 - 0,000%, P3 - 0,001%, P4 - 0,003%, P5 - 0,005%, P6 - 0,007% e P7 - 0,010%, v/v). A testemunha constou de meio de cultura autoclavado sem a suplementação de cloro ativo (P1 - preparo tradicional). A porcentagem de estabelecimento, brotações viáveis, contaminação fúngica e bacteriana, número de brotos e comprimento total de brotos foram avaliados aos 28 dias. Como principais resultados, o estabelecimento *in vitro* de segmentos nodais de *E. benthamii* foi viável em meio de cultura suplementado com cloro ativo sem autoclavagem, sendo que as concentrações de 0,001% e 0,003% de cloro ativo foram as mais adequadas e assemelharam-se aos resultados do meio de cultura preparado tradicionalmente com autoclavagem, sendo uma importante prática para a redução de gastos energéticos na cultura de tecidos de plantas.

Palavras-chave: cloro ativo, micropropagação, contaminação, cultivo asséptico, *Eucalyptus benthamii*.

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INTRODUCTION

Biotechnology has driven numerous advances in commercial plant production laboratories. Among the greatest successes is the establishment of homogeneous clonal forests from elite trees that have been established in many different regions of the World. *Eucalyptus* is one of the most important genera that has benefited from these technologies, and commercial plantations are now vital for diverse industrial sectors such as energy, biomass, pulp and paper, among others. *Eucalyptus* is clonally propagated using techniques such as minicutting and microcutting of superior genotypes (XAVIER; COMÉRIO, 1996; WENDLING et al., 2010). These methods were developed to meet the demand for a sufficiently high quality and quantity of trees for the establishment of high value-added plantations, in which can provide the raw material for industrial applications.

Micropropagation is one of the most important techniques for commercial cloning of plants (DUTRA *et al.*, 2009). This method relies on *in vitro* culture of propagules (e.g., root, stem, leaf and buds) in a culture medium under appropriate environmental conditions for growth and development (GEORGE *et al.*, 2008; GREENWAY *et al.*, 2012). Among the main advantages of micropropagation is the rejuvenation of adult materials (BRONDANI *et al.*, 2012; JOSHI et al., 2003; NOURISSIER; MONTEUUIS, 2008), the production of plants with superior genotypes, phytopathological and nutritional control (DUTRA et al., 2009; GEORGE *et al.*, 2008; HARTMANN et al., 2011), and applications for molecular biology such as genetic transformation of plants (SILVA et al., 2011). More recently, there has been considerable interest in the production of micropropagated plantlets in clonal nurseries (in order to establish microstumps to replace ministumps) for planting in hydroponic systems (BRONDANI *et al.*, 2012) - this practice has been widely adopted by commercial forest companies.

Despite several clear advantages of micropropagation, some problems persist and do not satisfy the commercial interests, particularly the high costs of implementation and technical maintenance. In this context, one of the main problems is the very high energy expenditure and time taken for the preparation of material during the autoclaving phase of culture medium (MACEK et al., 1995). Autoclaving of culture medium could potentially be replaced by adding chemicals substances to the culture medium that eradicate pathogens such as fungi and bacteria (i.e., chemical sterilization of the culture medium). One of the most promising substances is sodium hypochlorite (NaClO), which is commonly used for asepsis of plant tissues (ALCÂNTARA *et al.*, 2011; BORGES *et al.*, 2012; BRONDANI et al., 2011; MALYSZ et al., 2011; NIEDZ; BAUSHER, 2002; TEIXEIRA et al., 2006) and has the added advantage of low toxicity when compared to other chemicals substances with similar effects, such as mercury ($HgCl_2$), fungicides and antibiotics (DUTRA et al., 2009; NIEDZ; BAUSHER, 2002; TIWARI *et al.*, 2012). Also, it is desirable to develop new alternatives of asepsis to replace chemicals products of impactful to environment.

To act as a viable alternative to autoclaving (or the use of more harmful chemicals), NaClO needs to be effective at small concentrations, eliminating (or at least reducing the activity) microorganisms while providing optimal conditions for the development of plant tissues. Another potential advantage of eliminating autoclaving of the culture medium, relates to the maintenance of the chemical properties of the culture medium (e.g., macro and micronutrients, plant growth regulators, carbohydrates, vitamins, amino acids and agar) which are disrupted during traditional culturing methods. Moreover, treatment with NaClO would avoid the formation of furfural - which is toxic to the tissues - from the degradation of carbohydrates at high temperatures and pressures.

Several studies have already reported the use of sodium hypochlorite added to the culture medium for *in vitro* culture of various plant species (CARDOSO; TEIXEIRA, 2012; SAWANT; TAWAR, 2011; TEIXEIRA et al., 2006; TIWARI et al., 2012), including *Eucalyptus* (RIBEIRO, 2006; TEIXEIRA et al., 2008). However, detailed information regarding the potential for NaClO interference during micropropagation is lacking. Thus, it is important to further evaluate this process to verify its efficacy during different micropropagation phases (i.e., establishment, multiplication, elongation and rooting). Moreover, the use of NaClO may have applications in bioreactor systems, where one of the main problems is the high rates of contamination by microorganisms during cultivation of plants (ETIENNE; BERTHOULY, 2002).

This study aims to assess the feasibility of re-

placing traditional autoclaving by the addition of active chlorine (NaClO) to non-autoclaved culture medium. Specifically, we evaluate the *in vitro* establishment of *Eucalyptus benthamii* nodal segments in different concentrations of active chlorine (NaClO) added to a standard culture medium.

MATERIAL AND METHODS

Source material and preparation of explants

Ministumps of *Eucalyptus benthamii* Maiden & Cambage clones (BP115 and BP137) propagated by minicutting technique and managed in semi-hydroponic system (BRONDANI et al., 2012) were sprayed with sulfur solution (2 g L^{-1}, Kumulus DF®, BASF) 7 days before the collection of shoots. Shoots were collected and immersed in a solution of ascorbic acid (10 g L^{-1}). After collection, the shoots were washed with deionized water to remove dust and other particles deposited on the surface of vegetative tissues, and the leaves were removed. Nodal segments from the middle of the shoot - containing a pair of axillary buds, with leaves removed, and 1.5 cm in size - were considered as explants.

Treatments and experimental design

Explants were immersed for 15 min in benomyl solution (0.5 mg L^{-1}, Benlate 500®, Dupont) supplemented with Tween 20 (0.05%, v/v). They were then washed with autoclaved deionized water, and immersed in hydro-alcoholic solution at 70% (water:alcohol, v/v) for 15 s and rinsed again with sterile deionized water. Explants were then immersed for 10 min in a sodium hypochlorite solution (1.5 % active chlorine v/v, NaClO) supplemented with Tween 20 (0.05 %, v/v). At the end of the aseptic treatment, the explants were rinsed three times with autoclaved deionized water and inoculated vertically in conical test tubes (2×10 cm) containing 10 mL of MS culture medium (MURASHIGE; SKOOG, 1962) without plant growth regulators. Different concentrations of active chlorine (NaClO) (P2 - 0.000%, P3 - 0.001%, P4 - 0.003%, P5 - 0.005%, P6 - 0.007% and P7 - 0.010%, v/v) were added to non-autoclaved culture medium. The control consisted of autoclaved culture medium without supplemental active chlorine (P1 - traditional preparation). The culture medium was boiled in order to homogenize the gelling component (agar) for distribution in tubes, regardless of the treatment. A completely randomized factorial design (2×7) was used, with the factors consisting of two clones and seven concentrations of sodium hypochlorite (14 treatments), with 20 replications. The experimental unit consisted of a test tube containing 10 mL of the culture medium and one explant.

Data collection

The percentage of establishment (EST), viable shoots (VS), fungal (FUN) and bacterial (BAC) contamination, number of shoots (NS) and total length of shoot (LS) were evaluated at 28 days. Nodal segments free of fungal contamination, bacterial or oxidation were considered established and viable shoots were characterized by normal growth from the axillary buds.

Preparation of culture medium and growth conditions

The culture medium was prepared with deionized water, and 7 g L^{-1} agar (Riolab®) and 30 g L^{-1} sucrose (Nuclear®) were added. The pH was adjusted to 5.8 with 0.1 N HCl and 0.1 N NaOH before adding the agar to the culture medium. The P1 treatment was autoclaved at 121 °C (≈1.0 kgf cm^{-2}) for 20 min. Explants were cultured in a growth room at 25±2 °C, with a photoperiod of 12 h and a light intensity of 40 µmol m^{-2} s^{-1}. The tubes used during the preparation of the culture medium were rinsed with a solution of 0.001% active chlorine, and then rinsed three times with sterile deionized water.

Statistical analysis

The data were analyzed by the Hartley ($P<0.05$) and Shapiro-Wilk ($P<0.05$) tests and transformed as necessary by Box-Cox test. An analysis of variance (ANOVA, $P<0.05$ and $P<0.01$) was subsequently performed. According to the results of the ANOVA, data from the qualitative factors were compared by the Duncan test ($P<0.05$).

RESULTS AND DISCUSSION

According to the statistical analysis, a significant interaction between the preparation of the culture medium and clone in relation at establishment of explants (EST), viable shoots (VS) and fungal contamination (FUN) was found. Bacterial contamination (BAC) only varied significantly in relation to the culture medium preparation.

As expected, both BP115 and BP137 clones showed the lowest percentage of establishment of explants (EST) in the non-autoclaved culture medium (P2), differing significantly from the other treatments (Table 1). The sodium hypochlorite supplemented in culture medium (i.e., P3 to P7) showed similar values of EST compared with the autoclaved culture medium (P1 - traditional preparation, Table 1). Generally the *in vitro* establishment of explants varies according to the genetic material (BORGES et al. 2012), culture conditions (GEORGE *et al.*, 2008; NIEDZ; BAUSHER, 2002), type of explant (DUTRA et al., 2009), asepsis and phytotoxicity (BRONDANI et al., 2011; CARDOSO; TEIXEIRA, 2012; MALYSZ et al., 2011; NIEDZ; BAUSHER, 2002), season (BORGES et al., 2012) and physiological aspects of the mother plant (BRONDANI et al., 2011; DUTRA et al., 2009). *Eucalyptus* explants were derived from a clonal mini-garden (i.e., protected, with nutritional and phytopathological control), where *in vitro* establishment is generally high (BRONDANI et al., 2011; NIEDZ; BAUSHER, 2002). Indeed, the results obtained in this study are similar to those reported in the literature (BORGES et al., 2012; BRONDANI et al., 2011, 2012; MALYSZ et al., 2011; TEIXEIRA et al., 2006). Most significantly, our data clearly demonstrate that adding NaClO to the culture medium results in adequate rates of *in vitro* establishment of nodal segments of *E. benthamii* - similar to that reported for Teixeira et al. (2006) for various plant species.

Despite the high percentage of established explants (Table 1), there was a significant loss of material when considering shoots emitted from the pre-existing axillary buds. The best values of VS were obtained in P1, P2, P3 and P4 treatments, whereas concentrations greater or equal to 0.005% active chlorine induced lower values of VS (0 to 17.3%); indicating a toxic effect. Besides being easy to acquire and cheap, sodium hypochlorite has germicidal action and is commonly used for disinfection in tissue culture (GEORGE et al., 2008; NIEDZ; BAUSHER, 2002). Ribeiro (2006) reported negative effects of NaClO on *in vitro* culture of *Eucalyptus pellita* in concentrations of 0.009%, while emphasizing the positive effects at lower concentrations (i.e., 0.005% and 0.007%). Thus, active chlorine added to a culture medium can both cause damage to tissues and be beneficial depending on the concentration, indicating the need to establish the optimal concentrations for each type of genetic material. For both clones, the percentage of VS for culture medium preparation P3 and P4 did not differ significantly from that of the traditional culture medium (P1), demonstrating the utility of using these treatments for *in vitro* establishment of nodal segments of *E. benthamii* (Table 1). Furthermore, oxidation in the basal region of explants was only observed at concentrations equal to or greater than 0.005% active chlorine.

Reduced concentrations of active chlorine resulted in higher fungal contamination (Table 1). Fungal contamination is responsible for the greatest loss of material during the *in vitro* establishment phase (DUTRA et al., 2009) with high losses reported for *Eucalyptus* species (BORGES et al., 2012; BRONDANI et al., 2011). Several aseptic treatments are used to reduce contamination by micro-organisms, and are commonly performed on the mother plant prior to *in vitro* introduction (HARTMANN et al., 2011). A rigorous aseptic treatment of the mini-stump of *E. benthamii* followed by introduction into a culture medium supplemented with low concentrations of active chlorine may

Table 1. Percentage of establishment (EST), viable shoots (VS) and fungus contamination (FUN) of nodal segments of *Eucalyptus benthamii* regarding preparation of the culture medium and clone, 28 days after inoculation

Tabela 1. Porcentagem de estabelecimento (EST), brotações viáveis (VS) e contaminação fúngica (FUN) de segmentos nodais de *Eucalyptus benthamii* em relação ao preparo de meio de cultura e clone, aos 28 dias após a inoculação

Preparation of culture medium	BP115 clone	BP137 clone	BP115 clone	BP137 clone	BP115 clone	BP137 clone
	EST (%)		VS (%)		FUN (%)	
P1 - MA	84.2±3.7 Aa	84.2±3.7 Aa	31.5±4.8 ABa	15.7±3.7 Aa	15.8±3.7 Aa	10.7±3.2 Ba
P2 - MNA	68.4±4.8 Ba	47.3±5.1 Ba	57.8±5.1 Aa	11.2±2.3 Ab	10.5±3.2 Ab	42.4±5.1 Aa
P3 - MNA + 0.001% CL	84.2±4.9 Aa	89.4±3.2 Aa	36.8±4.9 ABa	15.7±3.7 Aa	15.8±4.9 Aa	5.4±2.3 BCb
P4 - MNA + 0.003% CL	84.3±3.2 Aa	78.9±4.2 Aa	57.8±5.1 Aa	15.7±3.7 Ab	15.7±3.7 Aa	15.9±3.2 Ba
P5 - MNA + 0.005% CL	86.5±4.8 Ab	100.0±0.0 Aa	10.5±3.2 Ba	5.2±2.3 Ba	13.5±4.8 Aa	0.0±0.0 Cb
P6 - MNA + 0.007% CL	94.8±2.3 Aa	94.8±2.3 Aa	17.3±5.1 Ba	0.0±0.0 Bb	5.2±2.3 Ba	5.2±2.3 BCa
P7 - MNA + 0.010% CL	94.8±2.3 Aa	89.5±3.2 Aa	15.7±3.7 Ba	0.0±0.0 Bb	5.2±2.3 Ba	10.5±3.2 Ba

In columns, means followed by the same capital letter and in rows, means followed by the same lowercase letter not differ significantly by Duncan test ($P<0.05$). MA – autoclaved culture medium, MNA – non-autoclaved culture medium, CL – active chlorine. Values are means ± SD.

possibly provide better control of fungal contamination. Although P2 had some positive results for EST, VS and FUN, the loss of material was elevated, and the treatment alone was used for comparison and control, not being recommended for use.

Increasing of the concentration of active chlorine reduced the presence of bacteria (Table 2). The non-autoclaved culture medium without the addition of active chlorine (P2) was the most favorable for the development of bacterial cultures and differed significantly from all other preparations of culture medium. Treatments P3 and P4 gave similar values to the autoclaved culture medium (P1), indicating the viability of active chlorine supplementation for the control of bacterial contamination in nodal segments of *E. benthamii*, especially in concentrations between 0.001% and 0.003% (Table 2).

Table 2. Percentage of bacterial contamination (BAC) of nodal segments of *Eucalyptus benthamii* regarding preparation of the culture medium, 28 days after inoculation

Tabela 2. Porcentagem de contaminação bacteriana (BAC) de segmentos nodais de *Eucalyptus benthamii* em relação ao preparo do meio de cultura, aos 28 dias após a inoculação

Preparation of culture medium	BAC (%)
P1 - MA	2.6±1.2 B
P2 - MNA	15.7±6.9 A
P3 - MNA + 0.001% CL	2.6±1.2 B
P4 - MNA + 0.003% CL	2.6±1.2 B
P5 - MNA + 0.005% CL	0.0±0.0 B
P6 - MNA + 0.007% CL	0.0±0.0 B
P7 - MNA + 0.010% CL	0.0±0.0 B

In columns, means followed by the same capital letter not differ significantly by the Duncan test ($P<0.05$). MA – autoclaved culture medium; MNA – non-autoclaved culture medium; CL – active chlorine. Values are means ± SD.

In *in vitro* establishment of explants, bacterial manifestation typically occurs after the fungal one (GEORGE *et al.*, 2008; HARTMANN et al., 2011), but also results in a significant loss in productivity (DUTRA et al., 2009). The main cause of bacterial contamination is related to the presence of endophytic organisms (BORGES et al., 2012) which are exuded into the culture medium at the abscission region of the explant (GEORGE et al., 2008). In our study, the presence of active chlorine (from 0.001% to 0.003%) in the culture medium was effective in controlling bacterial manifestation. However, other studies should be conducted to evaluate the effectiveness of active chlorine supplementation during other phases such as the multiplication of buds and shoot elongation.

There was a significant interaction between the preparation of culture medium and clones for the number of induced shoots and the average total length of shoot per explant. Clone BP115 did not show significant differences in the number of shoots related to the preparation of the culture medium (Table 3). However, clone BP137 had the highest value of NS in P4, and lower values for P6 and P7, i.e., due to non-occurrence of shoot induction (Table 3). Generally, explants of *Eucalyptus* established *in vitro* conditions can be collected after 28 days (BRONDANI et al., 2012; DUTRA et al., 2009) and the number of shoots from axillary buds may vary from 1-3 shoots per nodal segment (BORGES et al., 2012; BRONDANI et al., 2011). Thus, regardless of genetic material, the explants of *Eucalyptus benthamii* that were established *in vitro* and which possessed viable shoots showed adequate values of NS compared to those reported in the literature for species of the same genus, except for clone BP137 in concentrations of 0.007% and 0.010% active chlorine (Table 3).

Total length of shoot (LS) was another characteristic that showed morphological variation (Table 3). The traditional culture medium (P1) provided the lowest value of LS for clone BP115 compared to other treatments, while the highest value was observed for the P3. Clone BP137 gave similar LS values for P1 and the active chlorine concentration of 0.005%. The highest values were recorded for P1, P2, P3 and P5, and the lowest values for P6 and P7 (explants with no viable shoots). Treatment P4 gave intermediate value of LS (Table 3).

Despite the toxic effects of active chlorine (i.e., 0.005%, 0.007% and 0.010%, Tables 1 and 3), when applied in lower concentrations (i.e., 0.001% and 0.003%) it can have a positive effect on LS of *E. benthamii* (Table 3). The highest values of LS (P3 and P4) may be related to the role of Cl as an essential trace nutrient (RAMAGE; WILLIAMS, 2002) and the possible influence in enhancing photosynthesis should not be discarded (TEIXEIRA et al., 2006). Ribeiro (2006) and Teixeira et al. (2008) also reported a greater number of shoots and greater shoot length for *Eucalyptus pellita* at concentrations of 0.005% and 0.007% of active chlorine supplemented in culture medium, demonstrating the positive influence of low concentrations of NaClO on morphological characteristics. Furthermore, biomass gain may be favored in non-autoclaved culture medium, considering that monosaccha-

Table 3. Number of induced shoots (NS) and total length of shoot (LS) of nodal segments of *Eucalyptus benthamii* according to preparation of the culture medium and clone, 28 days after inoculation
Tabela 3. Número de brotações induzidas (NS) e comprimento total das brotações (LS) de segmentos nodais de *Eucalyptus benthamii* em relação ao preparo de meio de cultura e clone, aos 28 dias após a inoculação

Preparation of culture medium	BP115 clone	BP137 clone	BP115 clone	BP137 clone
	------ NS (shoot explant^{-1}) ------		--------- LS (mm explant^{-1}) -------	
P1- MA	1.6±0.5 Aa	1.0±0.0 Bb	40.0±7.7 Cb	117.0±36.0 ABa
P2 - MNA	1.3±0.5 Aa	1.0±0.0 Ba	59.5±11.2 BCb	115.0±21.2 ABa
P3 - MNA + 0.001% CL	1.1±0.3 Aa	1.0±0.0 Ba	153.5±45.7 Aa	133.3±15.2 Ab
P4 - MNA + 0.003% CL	1.5±0.5 Ab	2.0±0.0 Aa	74.5±17.5 Ba	81.6±16.0 Ba
P5 - MNA + 0.005% CL	1.5±0.7 Aa	1.0±0.0 Ba	55.0±7.0 BCb	110.0±14.1 Aa
P6 - MNA + 0.007% CL	1.3±0.5 Aa	0.0±0.0 Cb	58.8±18.5 BCa	0.0±0.0 Cb
P7 - MNA + 0.010% CL	1.6±0.5 Aa	0.0±0.0 Cb	46.6±11.5 BCa	0.0±0.0 Cb

In columns, means followed by the same capital letter, and in rows means followed by the same lowercase letter not differ significantly by Duncan test ($P<0.05$). MA – autoclaved culture medium; MNA – non-autoclaved culture medium; CL – active chlorine. Values are means ± SD.

rides are thermally degraded during autoclaving at high pressures. This can negatively affect growth because the autoclaving not only results in the hydrolysis of sucrose to its monomers such as glucose and fructose (DRUART; DE WULF, 1993; WANN et al., 1997), but also results in the formation of other toxic products, including hydroxymethyl furfural, levulinic acid and hydroxyacetyl furan (WEATHERS et al., 2004). Non-autoclaved culture medium benefits from conservation of the chemical properties of the constituents (e.g., carbohydrates, amino acids, vitamins, plant growth regulators, and others), favoring culture growth in supra-optimal conditions (SAWANT; TAWAR, 2011; TEIXEIRA et al., 2006; TIWARI et al., 2012). Based on these observations, we found a similar effect with *Eucalyptus benthamii* where both clones (BP115 and BP137) cultured at concentrations of 0.001% active chlorine produced the highest value of LS (Table 3). This effect requires further investigation, particularly with respect to morphological and physiological aspects.

In general, concentrations of active chlorine between 0.001% and 0.003% favored the *in vitro* establishment of nodal segments of *E. benthamii*, and the responses were similar to those with the traditionally prepared culture medium (P1). *In vitro* establishment of nodal segments is thus possible without the need for autoclaving, through the use of reduced concentrations of sodium hypochlorite (NaClO). This avoids the high energy costs of autoclaving, and considerably reduces the time needed to prepare the culture medium. However, the impact of this procedure on other phases of micropropagation (e.g., bud multiplication, elongation and rooting of shoots) needs to be further investigated, in order to confirm the viability of sodium hypochlorite as an alternative to traditional cultivation. Alternative compounds should also be tested, taking into careful consideration the potentially toxic nature of many of these substances. Nevertheless, this study strongly indicates the potential for developing new, cost-effective methods in plant tissue culture, with important implications for the global *in vitro* cultivation.

CONCLUSION

The *in vitro* establishment of nodal segments of *E. benthamii* was possible in non-autoclaved culture medium supplemented with 0.001% and 0.003% of active chlorine.

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