Scientific Article

Easy and efficient chemical sterilization of the culture medium for in vitro growth of gerbera using chlorine dioxide (ClO_2) [1]

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ABSTRACT

Micropropagation techniques changed the production of clonal plantlets in the world. However, the high costs of micropropagated plantlets continue as the main constraint for the expansion of the technique. This paper aimed to test the use of the chemical sterilization of culture medium using chlorine dioxide (ClO_2) for *in vitro* cultivation of gerbera. There was used gerbera *in vitro* shoots in the stage of rooting for these experiments, using 0.0035%, 0.0070% and 0.0105% of chlorine dioxide in the culture medium. Also, peracetic acid was tested previously for sterilization, but resulted in microbial contamination. Chemical sterilization of the culture medium was successfully using ClO_2 at 0.0035% to 0.0105% (100% decontamination) at rooting and elongation stage of gerbera with production of plantlets with similar (number of leaves, total and root fresh weight) or higher quality (mainly aerial part) at rooting/elongation stage, compared with autoclaved culture medium. The increase of concentration of ClO_2 also resulted in increasing of height and fresh weight of aerial part of gerberas. The ClO_2 could replace the autoclaving with production of sterilized culture medium without phytotoxic problems to gerbera in vitro cultivation.

Keywords: *Gerbera jamesonii*, micropropagation, sterilization methods, cost reduction, chemical alterations, phytotoxicity.

RESUMO

Esterilização química do meio de cultura para o cultivo *in vitro* de gérbera utilizando dióxido de cloro (ClO_2)

As técnicas de micropropagação de plantas mudaram a produção de mudas clonais no mundo. Entretanto, os altos custos das mudas micropropagadas ainda continuam sendo o maior desafio para a expansão dessa tecnologia. O objetivo de realizar esse trabalho foi o de usar a esterilização química do meio de cultura utilizando o dióxido de cloro (ClO_2) para o cultivo *in vitro* de gerbera. Para isso foram utilizadas, nesse experimento, gérberas cultivadas *in vitro* no estágio de enraizamento, utilizando o dióxido de cloro nas concentrações de 0,0035%, 0,0070% and 0,0105% para a esterilização química do meio de cultura. Também, um produto a base de ácido peracético foi testado previamente, mas resultou em contaminação microbiana dos meios de cultura. A esterilização química do meio de cultura ocorreu de forma efetiva utilizando o ClO_2 entre 0,0035% e 0,0105% (100% descontaminação), com a obtenção de plantas de gérbera com qualidade similar (número de folhas, massa total e de raízes) ou superior (principalmente a parte aérea) na fase de enraizamento/alongamento, comparado aquelas obtidas em meio autoclavado. O aumento da concentração de ClO_2 resultou em incremento da altura e massa fresca da parte aérea de gérberas. O uso do ClO_2 pode substituir o método de autoclavagem, com a produção de meios de cultura livre de contaminantes e sem efeitos fitotóxicos as gérberas cultivadas in vitro.

Palavras-chave: *Gerbera jamesonii*, micropropagação, métodos de esterilização, redução de custos, alterações químicas, fitotoxicidade.

1. INTRODUCTION

Production of *in vitro* plantlets by micropropagation represents, actually, one of the most important applied technologies, which increase clonal propagation capacity and help breeders for accelerating conventional breeding programs or using biotechnological tools. However, the high costs of micropropagated plantlets resulted in limited application of this technology only for a species with high difficulties of propagation by other conventional methods and for species with important problems of systemic diseases (CARDOSO et al., 2018).

Gerbera (*Gerbera jamesonii*) is one of most important ornamental species for cut-flower production, in which conventional propagation is difficulty by the low rate of propagation and the increase of systemic diseases in plants obtained from these methods. In this way, the micropropagation is the only method that reliable the production of commercial clonal plantlets without phytosanitary problems (CARDOSO and TEIXEIRA DA SILVA, 2013).

The electrical energy is mainly used in plant tissue culture labs for control temperature and light in growth room, and for autoclaving the culture medium, which

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represents actually 20-25% of costs of micropropagated plantlets (CARDOSO and TEIXEIRA DA SILVA, 2013; CHEN, 2016). The culture medium is commonly autoclaved (120 °C and 1 kgf cm^{-2} for 20-30 min) for *in vitro* plant cultivation, but this technique delays the culture medium preparation and increases the cost of micropropagated plantlets (CARDOSO, 2009; TEIXEIRA et al., 2008). The autoclaving could also result in production of toxic compounds for plant cultivation, such as 5-(hydroxymethyl)-2-furaldehyde and phenolics (WANG and HSIAO, 1995).

The chemical sterilization of the culture medium is an alternative to autoclaving and represents the most easyand viable technique for microbial decontamination of the culture medium, besides autoclaving. However, the use of sterilizing chemicals could also result in no complete sterilization of culture medium and/or phytotoxicity for plantlet cultivation that reduces the efficiency of the propagation (TIWARI et al., 2012, VARGAS et al., 2016). Then, potential sterilizing chemicals needs be tested both for sterilization efficiency and to evaluate it phytotoxicity for plant cultivation, before the use in large-scale systems of micropropagation.

Chlorine based chemicals as ClO_2 (CARDOSO, 2009) and NaOCl (TEIXEIRA et al., 2006) was proved it efficiency for use on *in vitro* cultivation of plantlets. ClO_2 acts destroying cell membrane and oxidizing intracellular components of microorganisms (HUANG et al., 1997). Interestingly, autoclaving continues as the main sterilization method in tissue culture lab conditions because the long historical uses in plant tissue culture labs with excellent repeatability both for it successfully microorganism sterilization and low phytotoxicity.

Together, there are a high potential to test and uses of chemicals disposable in the market as disinfectant for chemical sterilization of culture medium using conventional micropropagation (agar-media), but also for bioreactor systems (liquid media with temporary-immersion systems), aiming the selection of a high efficient product for microbial sterilization and that not causes phytotoxicity effects on cultivated *in vitro* shoots and plantlets. The replacement of autoclaving by chemical sterilization could also result in cost reduction of micropropagated plantlets. The peracetic acid is a bleaching solution using commonly for fruit sanitation with similar effects of sodium hypochlorite (WALTER et al., 2009) and the dioxide chlorine have large applications as make water safe to drink, including plant cultivation without phytotoxicity reported problems (CARRILLO et al., 1996).

The aim of this paper was to test two types of chemicals, chlorine dioxide and peracetic acid, compared with autoclaving, and it efficiency in sterilization of semi-solid culture medium and for *in vitro* cultivation of gerbera in stage of rooting and elongation.

2. MATERIAL AND METHODS

Micropropagated shoots of gerbera cv. Basic, with rose inflorescences, previous cultured in multiplication culture medium, were used as explants for the experiment.

The culture medium of the actual experiment was used at rooting phase of gerbera micropropagation and consisted of Murashige and Skoog (1962) with the half of macronutrients (MS ½), 30 g L^{-1} of sucrose, 0.1 g L^{-1} of myo-inositol and 0.1 mg L^{-1} of indol-butyric acid (IBA).

The pH of the autoclaved (121 °C; 1 kgf cm^{-2}; 20 min) control culture medium was adjusted for 5.8 before the addition of 6 g L^{-1} agar, and after autoclaving the pH was reduced for 5.4. The pH of chemical sterilized culture media with ClO_2 were adjusted for 5.8 to 6.0 using chloridric acid (HCl) at 0.1 N (Table 1), depending on concentration and were realized after the addition of ClO_2 at different concentrations (0.0035%, 0.0070% and 0.0105%) (CARDOSO, 2009) mainly because addition of chemical (DioxiPlus®, Indaiatuba, Brazil) increase the pH value of the culture medium for 7.0 to 7.5 in these concentrations. At contrary, addition of the peracetic acid (Proxitane®, Cotia, Brazil) at 1.0, 2.0 and 3.0 ml L^{-1} reduces drastically the pH of the culture medium to 3.5 to 4.5 and the pH was adjusted for 5.8 using potassium hydroxide (KOH) at 0.5 N.

After adjusting the pH, electrical conductivity (EC) of each culture media was also measured before shoot inoculation (initial EC) (Table 1). The pH of the control was realized after culture medium autoclaving. The pH and the EC of the culture media were also measured after 28-d of gerbera *in vitro* cultivation (final pH and EC).

Around 10 repetitions (flasks) containing culture medium were maintained for 28-d in growth room at 25 °C without inoculation of gerbera, for previous evaluation of sterilization efficiency of peracetic acid, chlorine dioxide and autoclaved culture medium. In this essay, a control with none treatment of sterilization was used. This essay was evaluated by the contamination percentage of flasks. Also, bacterial or fungi contamination were quantified according each treatment.

The experiment with *in vitro* shoots of gerberas was realized only with 100% decontaminated treatments observed in the previous essay.

Individualized shoots with 2.6±0.2 cm in length and containing two leaves and none roots were used as explants. Each flask (repetition) with 200 ml capacity and 35 ml of the culture medium contains four individual shoots, and five flasks for each treatment were used. The experiments were realized in completely randomized design and were repeated twice.

The plantlet development was evaluated by the height of plantlets, number of leaves and roots and fresh weight (total, aerial part and roots) with 28-d after *in vitro* cultivation in 25±1 °C, 16-h photoperiod with white fluorescent lamps (30-35 µmol $m^{-2}s^{-1}$) (CARDOSO and TEIXEIRA DA SILVA, 2013).

The means were submitted to Analysis of Variance (ANOVA) followed by the mean comparison by Duncan's Test at 5% probability.

3. RESULTS AND DISCUSSION

The autoclaving treatment resulted in expected 100% sterilization, without common contamination caused by

fungi or bacteria microorganisms in culture medium. Also, the control without any treatment of sterilization showed 60% of contamination by microorganisms in the first 7-days of incubation after the culture medium preparation and at 28 days resulted in 100% of contaminated flasks with culture medium (Table 1).

Table 1. Contamination percentages of culture medium using autoclaving or chemical sterilization with different products for sterilization.

Treatments	Contamination percentage (days after sterilization)				Type of contamination	
	7	14	21	28	Fungi	Bacteria
Control	60	70	80	100	80	20
Autoclaved	0	0	0	0	0	0
ClO_2 [0.0035 %]	0	0	0	0	0	0
ClO_2 [0.007 %]	0	0	0	0	0	0
ClO_2 [0.0105%]	0	0	0	0	0	0
Pa [1 mL L^{-1}]	50	70	80	80	80	0
Pa [2 mL L^{-1}]	30	60	60	60	60	0
Pa [3 mL L^{-1}]	0	20	20	20	20	0

ClO_2 – liquid stabilized chlorine dioxide at 7% concentration (Dioxiplus®, Dioxide, Indaiatuba-SP, Brazil); Pa – Peracetic Acid (0.25%), peroxide hydrogen (5%) and acetic acid (4%) (Proxitane®, thech, São Paulo-SP, Brazil)

Either in control or with sterilization treatments that presented contamination, 80% to 100% of the total contaminated flasks were caused by fungi (Table 1), observed by the growth of different type of mycelia.

The chemical sterilization with peracetic acid added to the culture medium reduced but not complete eliminate the microorganism contamination, independently of the concentration used. The most effective concentration of peracetic acid was 3 ml L^{-1}, which reduced the total percentage of contaminated flasks from 100% (control) to 20% (Table 1). Higher concentrations could be resulted in 100% decontamination, but not were tested in this experiment, mainly due the excessive acidification of the culture medium.

As observed, the use of chemical sterilization with liquid stabilized chlorine dioxide (ClO_2) at 0.0035% to 0.0105% resulted in 100% of decontamination flasks, since after 28-days of incubation, the same time required for gerbera transplantation to new culture medium (Table 2). Although the mode of action of chlorine in microorganism is not complete elucidated, the ClO_2 acts destroying cell membrane and oxidizing intracellular components of microorganisms (HUANG et al. 1997). In *Anthurium andraeanum* there was possible to observe the complete decontamination of the culture medium after 90-d of *in vitro* cultivation of shoots, and the transplantation of these shoots to autoclaved rooting medium not resulted in any contamination (CARDOSO et al., 2009), showing the real effect of chlorine dioxide as sterilizing. Also, in the same work with *A. andraeanum*, the complete sterilization of the culture medium was not obtained using peracetic acid, with 100% of contaminated flasks after 21-d of *in vitro* cultivation of *Anthurium* (CARDOSO et al., 2009).

The results obtained in the first experiment, with different methods for sterilization, lead to conduct the experiment with *in vitro* development of gerbera only with different concentrations of chlorine dioxide compared to autoclaved culture medium.

In this second experiment, with chemical sterilization using different concentrations of chlorine dioxide, there was observed the total sterilization of culture medium, with any type of contamination or microorganism signals in the culture medium contained plantlets of gerbera. Also, there was not observed any signals of phytotoxicity caused in gerbera plantlets by the addition of chlorine dioxide to the culture medium, such as phenol production and liberation in the culture medium, yellowing of leaves, absence or necrosis of roots, or another signals that could be detected visually. The plantlets obtained with culture medium chemical sterilized have similar leaf morphology and green coloration intensity compared to obtained in conventional autoclaved culture medium (Figure 1).

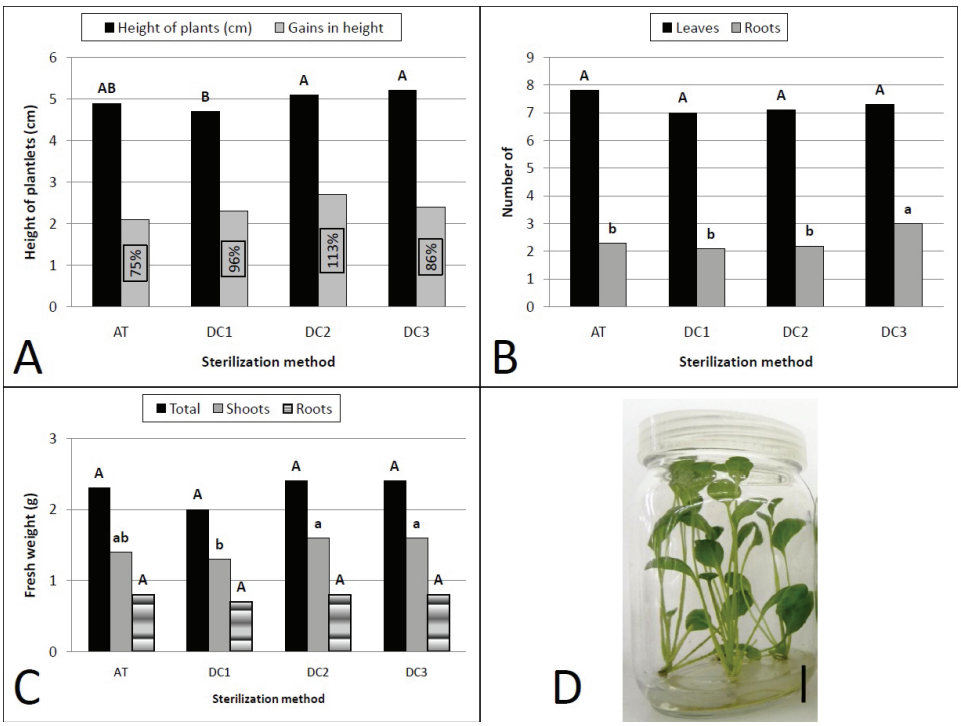

Figure 1. *In vitro* development of gerbera cv. Basic in autoclaved (AT) and chemical sterilized culture media with chlorine dioxide (ClO_2) at 0.0035 (DC1), 0.0070 (DC2) and 0.0105% (DC3): (A) Total Height (cm) and gains in height; (B) Number of leaves and roots; (C) Total, shoot and root fresh weight (g); (D) Morphological characteristics of plantlets of gerbera cv. Basic cultivated for 28-d in culture medium sterilized with 0.0070% of ClO_2 (chemical sterilized). Columns with the same letters for each evaluated characteristic not differs at 5% probability using Duncan's average test. Bar: 1.0 cm

Also, the shoots of gerbera presented good development using chemical sterilization, compared to autoclaved culture medium. The chemical sterilization of the culture medium resulted in similar height compared with autoclaved culture medium, with gains in height ranged from 86% and 113% after 28-d of *in vitro* cultivation (Figure 1). These gains represents the differences in height between the initial height of inoculated gerbera shoots and the height measured after 28-d of *in vitro* cultivation.

Also, the number of leaves and fresh weight of aerial part and roots were similar to observed with autoclaved culture medium (Figure 1). Only the number of roots presented significant difference with highest number of roots (3.0 plant^{-1}) at 0.0105% of chlorine dioxide, with 30% increasing, compared with autoclaved (2.3 plant^{-1}) culture medium (Figure 1).

The results obtained could be partially explained by the chemical characteristics of the culture medium that presented differences among the autoclaved and chlorine dioxide chemical sterilized. As example, the initial pH of the chemical sterilized culture medium maintained similar or few higher pH, compared to the first adjusted (near from 5.8), ranging from 5.85 (0.0035%) to 5.97 (0.0105%), while the pH of autoclaved culture medium reduced from 5.8 (before) to 5.4 (after the physical treatment) (Table 2).

Table 2. pH and electrical conductivity (EC) of the culture medium before and after 28-d of *in vitro* cultivation of gerbera .

Culture		Chlorine dioxide		
Medium	Autoclaved	0.0035%	0.0070%	0.0105%
	pH			
Initial pH	5.40	5.85	5.98	5.97
Final pH	4.04	3.89	3.90	3.87
Δ pH	-1.36	-1.96	-2.08	-2.10
	CE (dS m^{-1})			
Initial EC	2.40	2.30	2.20	2.40
Final EC	1.72	1.49	1.67	1.64
Δ CE	-0.68	-0.81	-0.53	-0.76

Interestingly, although the final pH (after 28-d of gerbera cultivation) was reduced for similar values among autoclaved or chemical sterilized treatments, ranging from 3.87 (0.0105% ClO_2) to 4.04 (autoclaved), a significative difference in ΔpH were observed among autoclaved (-1.36) and chemical sterilized culture medium (-1.96 to -2.1). At the same time, the ΔEC also ranged from -0.53 to -0.81 with chemical sterilization and -0.68 with autoclaved culture medium. The EC (electrical conductivity) of the MS culture medium with half concentration of macronutrients (MS ½) with sucrose at 3%, before the sterilization procedures, was 2.2-2.4 dS m^{-1} (Table 2).

Based on the observations of reduction of EC (direct correlated with nutrient consumption by the plantlets) to 1.49 (0.0035% ClO_2) and 1.72 (autoclaved), there was concluded that the most chemical limitation of the culture medium for *in vitro* gerbera plantlet development was the pH, because the stabilization of final pH for all treatments was around 3.9-4.0, independently of the reduction of EC value. Probably, this reduction of pH also affects the nutrient uptake or it availability to *in vitro* plantlets affecting the plantlets development, because the EC of 1.5-1.7 (observed at the end of *in vitro* gerbera cultivation) is considered a good reference for production of adult plants of gerbera under fertigation using semi-hydroponics system (LUDWIG et al., 2010). This observations lead to a conclusion of several lost of nutrients added to the culture medium that not participate of *in vitro* plantlet development processes due to pH reduction in culture medium.

Cardoso and Silva (2012) observed that chlorine dioxide at 0.0025% could be used for chemical sterilization of the culture medium in the *in vitro* cultivation of gerbera cv. AL101, and also observed similar development of shoots or plantlets when compared with autoclaved culture medium.

Labor and electrical energy together represents more than 80% of the total cost of each micropropagated plantlet in the lab

Table 3. Costs for preparation of culture medium based on labor and electrical energy costs.

Type of costs	Costs/unit (U$)	Perliter of culture media	
		Quantity	Costs (U$)
Autoclaved			
Labor	2.80 / hour	1/4 hour*	0.70
Electrical Energy	0.80 / hour	1/4 hour*	0.20
Total			0.90
Chlorine dioxide (ClO_2)			
Labor	2.80 / hour	1/8 hour	0.35
Tecsa-Clor (5%)	40.0 / liter	0.5 ml (0.0035%)	0.01
Total			0.36

*There was considered that the preparation of four liter of culture medium lead around 1-h of labor, using of 75-100 L autoclave capacity and potency of 4000 W.

The replacement of autoclaving by chemical sterilization reduced the use of electrical energy and the labor time for culture medium preparation from ¼ (autoclaving) to 1/8 hour. The use of chlorine dioxide also reduces the cost with sterilization, especially because the electrical energy for autoclaving have high cost compared with the chemical product used for sterilization (Table 3). The results obtained in actual experiment, based only in these two components showed that the use of chemical sterilization compared with autoclaving, represented a 3.5% to 5.0% of cost reduction in individual micropropagated plantlet of gerbera. Brondani et al. (2013) also agree that using of chemical sterilization could result in economy of electrical energy used for autoclaving and time required for culture medium preparation.

The chemical sterilization was used in the replacement of autoclaving using other products, as sodium hypochlorite (NaClO) in the *in vitro* cultivation of *Eucalyptus benthamii* (BRONDANI et al., 2013), gerbera cv. Essandre (PAIS et al., 2016), pineapple (TEIXEIRA et al., 2006), among others. Despite the use of other chemical products as hydrogen peroxide (VARGAS et al., 2017) and Plant Preservative Mixture®, the chlorine based chemical sterilization as sodium hypochlorite and chlorine dioxide was the most consistent for their low cost, high efficiency in sterilization, and low or no phytotoxycity to different *in vitro* plant cultivation species at adequate and very low concentrations (CARDOSO, 2009; CARDOSO and SILVA, 2013).

The most common concentrations of NaClO used were 0.003% of active chlorine, and the use of higher concentrations lead to phytotoxicity to the cultures, as observed in *Eucalyptus* in concentrations higher than 0.005% (BRONDANI et al., 2013) and in *Prunus mume* at 0.01% (VARGAS et al. 2016). These last authors also observed that the use of a culture medium chemical sterilized with chlorine dioxide and hydrogen peroxide showed higher percentage of survival (78,0-80,4%) and lower contamination rate (14,7-15,9%) of nodal segments of *Prunus mume*, compared with sodium hypochlorite sterilized (45% survival rate and 45,8% of contaminated explants). Cardoso and Silva (2012) also not observed phytotoxicity of chlorine dioxide when used for culture medium sterilization until 0.1% in different stages of

micropropagation, e.g., establishment, multiplication and rooting/elongation phases. Similarly, in this experiment the use of chlorine dioxide at 0.0035% to 0.0105% resulted in best *in vitro* plantlets of gerbera at rooting/elongation stage.

In micropropagation of sugarcane the concentration of 0.1% of NaClO resulted in best treatment for chemical sterilization, with good microbial decontamination (96%) and normally grown (TIWARI et al., 2012). However, the use of NaClO depends of the previous treatment of bottles and caps with sodium hypochlorite solution at 0.1% for 10 min (TEIXEIRA et al., 2008; TIWARI et al., 2012). Using ClO_2 there was reported no additional treatments for using chemical sterilization and the flasks used have had the same type of washing in all treatments, only with water and detergent, before sterilization procedures.

The chlorine dioxide also proved is efficient for *in vitro* growth of *Anthurium andraeanum* (CARDOSO, 2009). This author was observed that the use of chlorine dioxide resulted in 100% of sterilization of culture medium with no signals of phytotoxicity until the concentration of 0.010% of ClO_2, while the use of peracetic acid at the same concentration not sterilized the culture medium. The use of 0.05% of ClO_2 also resulted in increases in the leaf number and diameter (CARDOSO, 2009).

Chemical sterilization represents nowadays an interesting alternative for culture medium sterilization, but in most of micropropagation laboratories autoclaving is considered the most security way for culture medium sterilization, main because the long repeatability of results and that avoid any type of microorganism resistance. However, the use of chemical sterilization with chlorine products, as chlorine dioxide, also represents an important tool to be use in plant tissue culture labs for production of thousands of plantlets, and represents one way to reduction costs of micropropagated plantlets, one of the challenges of micropropagation techniques for this century (CARDOSO et al. 2018). Studies with chemical sterilization of also liquid culture media for temporary immersion bioreactor systems needs be realized to evaluate the use and efficiency of chlorine dioxide also for this system, actually used for large-scale micropropagation of plantlets by tissue culture.

4. CONCLUSIONS

The use of chlorine dioxide at 0.0035% to 0.0105% for chemical sterilization of the culture medium (100% decontamination) and to produces of *in vitro* plantlets is highly effective and replaced autoclaving methods conventionally used for gerbera micropropagation.

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AUTHORS CONTRIBUTIONS

J.C.C. 0000-0001-6578-1723: contributes with main idea, experimental design and writing and editing of the paper. A.C.P.I. 0000-0003-4116-5429: contributes with execution of the experiment, data collection and analysis of the experiments, and revision of the paper.

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