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Research Article

Non-autoclaved Sterilization Procedures of Sugarcane Tissue *in vitro* Culture

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Abstract

Background and Objective: In traditional plant tissue culture, sterilization medium and laboratory tools were used the autoclave procedures but this way was time consuming if compared with NaOCl system. The objectives of this observation were to shorten the sterilization procedures and to study the effect of NaOCl on *in vitro* callus induction (CI) and plantlet regeneration (PR) of Sugarcane plants. **Materials and Methods:** The laboratory tools (LT), immature leaf segments of sugarcane as explant (EP) and Murashige and Skoog basal medium (BM) were sterilized by 5.25% active chlorine (NaOCl). The different concentrations of NaOCl treatments tested were ranging from 100-500 mL L^{-1} solute for LT and EP sterilization and 50-200 μL L^{-1} for BM sterilization. All treatments were arranged in completely randomized design (CRD) and the percentage of LT and BM contamination, CI and PR were the observed variables. The simple mean comparison analysis was applied and to judge the best treatment of BM and EP contamination, CI and PR. **Results:** The best concentration of active chlorine were 300 mL L^{-1} for LT and EP of contamination free and 100, 150 and 200 μL L^{-1} for BM contamination-free, sugarcane CI and PR. **Conclusion:** Conditional protocol for non-autoclaved *in vitro* media, explants and laboratory tools in life span of third and callus induction and plantlets regeneration of sugarcane had successfully been achieved under active chlorine sterilization.

Key words: Calli induction, laboratory tools, media contamination, plantlets regeneration, sugarcane

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

There was no report has successfully been published in sugarcane tissue *in vitro* culture without the use of the autoclave or using the NaOCl only during laboratory equipment and explants sterilization procedures, except in the sterilization procedure of media[1-3]. The traditional plant tissue culture practices become time-consuming due to the use of lengthy procedure and excessive additional time of autoclave to completely run treatment. This obstacle resulted in longer time to achieve the successful end product of some plant tissue culture activities like in producing new sources of material planting in some mass propagation of some crop plants. Consequently, this procedure became inefficient and an alternative technique is needed to replace that is more efficiently and effectively to solve the time-consuming problem in gaining good results for ranged plant tissue culture applications.

Recently, some researcher groups have reported the successful practices of plant tissue culture without the use of the autoclave in the medium sterilization on several crop plant species like *Musa paradisiaca* and *Ananas comosus* by Teixeira *et al.*[4] and Teixeira[5], *Eucalyptus pellita* by Teixeira *et al.*[6], Sugarcane (*Saccharum officinarum*) by Sawant and Tawar[1] and Tiwari *et al.*[2], *Eucalyptus benthamii* by Brondani *et al.*[7], *Phalaenopsis* by Thepsithar and Thongpukdee[8] and *Chrysanthemum* by Deein *et al.*[9,10]. All groups above were done by using NaOCl in media sterilization in several concentrations as treatments and an autoclave in sterilization medium as control treatment, except hot pepper (*Capsicum frutescens* L.) by Suaib *et al.*[11,12] without using autoclave at all. The use of NaOCl provided better result than the autoclave in the frequencies of contaminated media by bacterial and fungal-agents and other sources of *in vitro* medium contaminant agents.

Using bioside, such as sodium hypochlorite (NaOCl), in reducing or eradicating activities of micro-organisms as the contaminant agents of *in vitro* medium became elevated as reported by many groups of researchers[2,6,7,13,14]. The advantages of this bioside was its availability elsewhere as household disinfectant or as industrial bleach, less expensive, easily be obtained in general markets or household shops and less poisonous to people[12]. Also, some researchers have reported the positive effects of NaOCl in cells, roots, nodes, shoots and whole plantlets development during *in vitro* culture of some crop plant species like pineapple (*Ananas comosus* cv. Smooth Cayenne) by Teixeira *et al.*[4], *Anthurium* and *reanum* Lind. cv. Tropical Red by Peiris *et al.*[13] and two species of orchids (*Arundina bambusifolia* and *Epidendrium ibaguenses*) by Rodrigues *et al.*[14].

In some decades later, only limited researcher groups of *in vitro* plant tissue culture have reported the use of NaOCl as the only sterilization agent in implementing the pathogen free of explant material even though the effectiveness of this bioside has been reported in earlier years[15]. A significant, good, very good and satisfactory results of chemical agents in surface sterilization of some explants during *in vitro* culture applied recently were Hydrogen peroxide (H_2O_2), Silver nitrate ($AgNO_3$), Calcium hypochlorite (CaOCl), Sodium hypochlorite (NaOCl), Bromine water [$Br_2(H_2O)$] and Mercuric chloride ($HgCl_2$) covering the wide ranges of crops plant species[16].

From these positive effects of NaOCl in sterilization medium, laboratory tools, explants surface sterilization and the advantage responses in callus induction and plantlet regeneration of several crop plants, the application of active chlorine in whole *in vitro* procedures of plant tissue culture became another key factor of the successful implementation of modern plant tissue culture in the next future.

Sugarcane (*Saccharum* spp.) is an important industrial plant worldwide in general and in Indonesia it is specifically for household consumption such as sugar kitchen, pharmaceutical component, beverages and cosmetics industries. Comparing with two other crop plants explained in the next elaborations, various end products of sugarcane plant consumption in Indonesia has been rising from year to year so that the existence of government in managerial stock and distribution was always hands on. By this wide usefulness and importance of this crop plant, the intensive and extensive field plantations should be extended both in conventional and in non-conventional ways. The sugarcane development through non-conventional way by various techniques especially in tissue culture should be developed in modified traditional tissue culture.

From above objective explanations, the use of these plants was a main reason in choosing it as object in this observation. The development of this plant through non-conventional way by various techniques especially in tissue culture should be developed in a more advanced to get the efficient and effective procedure so as to get budget minimally and the simplest way due to the shortened procedures with the good result. The aims of this report were in composed a general protocol in sterilization medium, laboratory tools and explant sources of the sugarcane plant species for *in vitro* culture by using bioside (NaOCl) as an autoclave replacement in their sterilizations. The callus

induction and plantlet growth of sugarcane plant were also discussed in relation to its positive responses as nutritional component of *in vitro* culture medium.

MATERIALS AND METHODS

Location and plant material: The experiment was conducted in *in vitro* laboratory Faculty of Agriculture Halu Oleo University Kendari, Southeast Sulawesi, Indonesia from June to November, 2018. The sugarcane donor plant, PS-860 clone as explants sources in form of meristematic sheath leaf segments (MSS) was gathered from The Indonesian Sugar Experiment Station, Pasuruan Regency, East Java Province, Indonesia and was used in this observation. The MS (Murashige and Skoog) basal medium[17], fortified by table sugar, solidified with agar "walet" and some exogen plant growth regulators according to the treatments tested were used in this experiment.

Sterilization solution and MS basal medium preparations: Sterilization solution (SS) was prepared in mixing 100, 200, 300 and 500 mL of household bleach, HB (active chlorine, 5.25% NaOCl) in 900, 800, 700 and 500 mL of distilled water. This SS was used as explants and *in vitro* laboratory tools of sterilizations. Medium preparation was applied according to the general steps practiced elsewhere except in sterilization method i.e., the inclusion of 250-750 μL of HB in a litre basal medium. There were three treatments tested in the range of 250 μL between treatments, i.e., 250, 500 and 750 μL L^{-1}, respectively. The media pH was adjusted at 5.8 or corrected with 1.0 N NaOH or 1.0 N HCl. By using a hotplate with magnetic stirrer the medium was homogenized and boiled for 5 min, then poured into 150 mL jars each 20 mL and topped with transparent plastic and sealed by rubber ring.

Equipment and explants sterilization and household bleach concentrations tested: In the *in vitro* laboratory tools sterilization like jars, scalpel, scissors, erlenmeyer, etc., were surface sprayed or soaked in SS at 15 or 30 or 45% HB for 2-3 min. The MSS of sugarcane as explant was sterilized by SS. The sterilization procedures of the MSS was soaked it first in 100 mL erlenmeyer contained ethanol 70% for 60 sec, continued to ethanol normality with distilled water in 3 times each 5 min, then lastly soaked in 15, 30, 60% HB+2 drops of Tween 30 in non-sterilized aquadest for 15 min. The sterilized explants were put into another sterile petri dish for use in the next incubation step. All matters sterilization steps were conducted outside the room of laminar air flow cabinet condition.

Experimental design and statistical analysis: *In vitro* calli induction and plantlets initiation of MSS were incubated onto culture jars according to the different HB treatments tested. Four NaOCl treatments tested, one MSS each jar and 10 replications of each treatment, so that there were totally 40 segments of MSS culture jars as experimental units. Those treatments were arranged in completely randomized design (CRD) and the difference between treatments within each variable was judged according to the simple comparison mean by Fischer[18]. The observation variables were the percentages of contamination of laboratory tools, explants, media, calli induction and plantlets regeneration and development during 30 days of incubation. All data were analyzed through SAS statistical package for Windows[19] version 9.13.

RESULTS

Effects of NaOCl on *in vitro* laboratory equipments and explants contamination: The effects of different treatments tested of active chlorine (NaOCl) i.e., 100, 200, 300 and 500 mL of a litre of distilled water on laboratory tools contamination was expressed in Fig. 1, whereas explant contamination was expressed in Fig. 2. The results have shown that the best concentration of NaOCl on both laboratory equipments and explants free from pathogenic contamination was 300 mL L^{-1} of distilled water.

Effects of NaOCl on media contamination: Figure 3 has also shown the different percentages of media contamination of that from the fourth of HB concentration tested in media contamination were 25, 50, 100 and 150 μL of active chlorine in a litre of MS basal medium. The best concentration

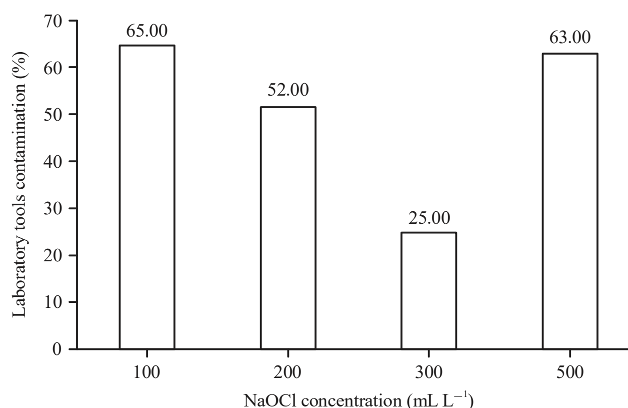

Fig. 1: Effects of different concentration of active chlorine (NaOCl) in distilled water on mean percentages of laboratory tools contamination

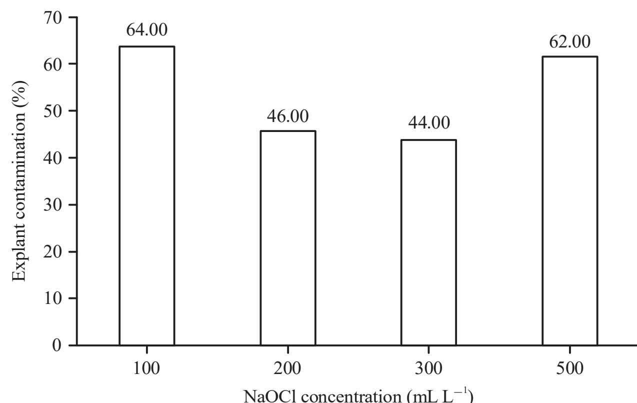

Fig. 2: Effects of different concentration of active chlorine (NaOCl) in distilled water on mean percentages of explants contamination

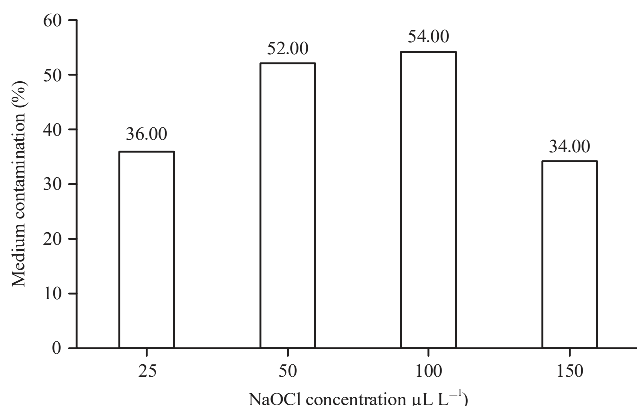

Fig. 3: Effects of different concentration of active chlorine (NaOCl) in MS basal medium on mean percentages of medium contamination

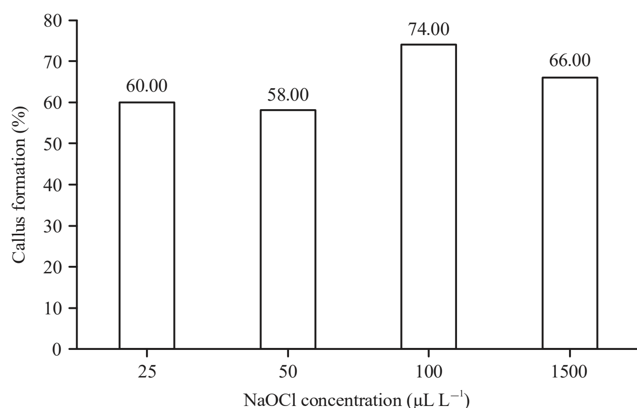

Fig. 4: Effects of different concentration of active chlorine (NaOCl) in MS basal medium on mean percentages of sugarcane callus derived explant formation

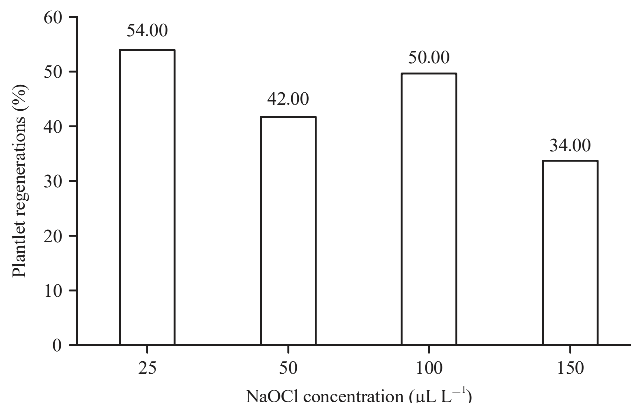

Fig. 5: Effect of different concentration of active chlorine (NaOCl) in MS basal medium on sugarcane plantlets regeneration

of HB on *in vitro* media free of contamination was 150 µL of active chlorine in a litre of MS basal medium, in which the mean contamination percentage of medium was 34.0%. The higher percentage of medium contamination was 54.0% of the 100 µL L^{-1} active chlorine in the MS basal medium observed.

Effects of NaOCl on plantlet derived callus and plantlet regeneration: Both callus induction and plantlet regeneration were observed in the same concentration of NaOCl in the media contamination, i.e., 25, 50, 100 and 150 µL of active chlorine in a litre of MS basal medium. Either in the callus induction or in the plantlets regeneration were responded in the different NaOCl concentrations. The higher percentage of callus induction have achieved in 100 µL of active chlorine in a litre of MS basal medium, i.e., 74.00% (Fig. 4), whereas the higher percentage of plantlets regeneration was indicated in 50 µL of active chlorine in a litre of MS basal medium, i.e., around 54.00% of callus transferred (Fig. 5).

DISCUSSION

As shown in Fig. 1 and 2, the best concentration of NaOCl in the solute on laboratory tools and explants lifespan was 300 mL L^{-1}. This concentration effectively prolonged the lifespan of *in vitro* laboratory equipment like erlenmeyers, jars, scalpels, scissors, magnetic stirrers, porceps and tweezers and *in vitro* MS basal medium as well. It means that the 300 mL L^{-1} of HB in the solute would be beneficial in preventing the activity of pathogenic micro-organisms as

contaminant sources. This finding have been reported the identical results to some several reports on various of tissue *in vitro* culture media of crop plant species of undergraduate researcher students[11,12], in which the use of active chlorine between the range from 250-500 mL L^{-1} solute (distilled water) was better in 300 mL L^{-1}. To summarize this, a single variable as an observed measure parameter, the lifespan of medium and of explants in free from contamination was achieved in 300 mL L^{-1} of solutes as the best concentration treatment of active chlorine in form of HB.

This data showed the inconsistency in the results of treatment ranges which no fixed patterns relating to the treatments elevation decreasing or increasing[12]. This observation showed that the highest and lowest concentration of active chlorine in the medium indicated the lowest percentages of contaminated media, whereas the medium which contained active chlorine in between the lower and the highest one showed the higher percentages of contaminated media. As indicated in Fig. 3, the lower percentages of contaminated medium was achieved in the higher concentration (150 μL L^{-1}) of HB tested in this observation.

Peiris *et al.*[13] have reported that the micropropagation of *Anthurium andreanum* Lind. cv. "Tropical Red" shoots were not fully preventing medium contamination in which 22-28% of medium used were contaminated. Compared to another lower concentration of NaOCl, Tiwari *et al.*[2] have reported that the use of 0.01-0.05% NaOCl in MS basal medium for sugarcane *in vitro* micro-propagation caused 75-46% medium were contaminated. Contrasted to the other higher concentration of NaOCl in the MS basal medium, Deein *et al.*[9,10] have indicated that the 6.0% of NaOCl was able to prevent medium contamination until 100% of the *in vitro Chrysantemum* explant incubation.

Similar findings have been reported in sugarcane micropropagation in which at the 14 days after *in vitro* incubation, the 0.01% NaOCl in MS medium were contaminated over 58% and the 0.2% of NaOCl was 56%, even though the 0.1% NaOCl was only 8% of cultures were contaminated[2]. The inconsistence frequencies of medium contamination in different concentration of NaOCl into MS basal medium i.e., the 5, 10, 15 and 20% resulting in 22, 27, 22 and 28% of culture medium were contaminated, respectively[13]. The departure responses of medium contamination between those reports above and in this report may be due to the difference of macro-climate conditions or the environmental behaviour between our laboratory and those laboratories explained particularly in kinds and amounts of pathogenic contamination agents[11,12]. These findings indicated the similar results that have been reported by Sawant and Meti[3] in which the use of NaOCl in freeing those contaminants on shoot multiplication and rooting media of the two varieties of sugarcane was achieved in better result. Adilakshmi *et al.*[20] also used NaOCl in sterilizing MS media to initiate both shoots and roots of sugarcane meristem tip culture which was better than the other sterilant agents like $HgCl_2$, H_2O_2 and $AgNO_3$.

Both calli induction and plantlets regeneration have different patterns in their responses on active chlorine in which the callus induction and plantlet regenerations showed the inconsistence patterns. The different concentrations of active chlorine in the MS basal medium, both callus induction and plantlets regeneration may be explained by the risk level of the material used in relations to the contamination opportunities. In the use of an explant derived from field growing of donor plant, the probability to have any pathogenic sources became increased, so that the active chlorine concentration should be contained in the highest in the SS. Meanwhile, the explants obtained from the callus derived explants which are healthy and free from pathogenic sources become the lowest concentration of active chlorine needed in the basal medium as a result of the callus clump clean and sterile from contaminant agents.

CONCLUSION

The 300 mL of NaOCl in a litre of distilled water successfully eradicated the pathogenic contaminant sources of *in vitro* laboratory tools and explants. The lower percentages of media contamination, the higher percentages of calli induction and plantlets regeneration were achieved in respective of 150, 100 and 50 μL of NaOCl in a litre of Murashige and Skoog basal medium.

SIGNIFICANCE STATEMENTS

This paper reported a study on *in vitro* tissue culture of Sugarcane (*Saccharum officinarum* L.) on non-autoclaved sterilization procedures of laboratory tools, media and explants. Results generated might help sugarcane propagator be applied in *in vitro* laboratories, media and explants without sterilization by using expensive and long procedural scheme and high consuming electricity of autoclave. The results presented give indication of the appropriate use of

active chlorine in sterilization *in vitro* laboratory tools, media and, explants and the normal callus induction and plantlets growth of sugarcane in using *in vitro* non-autoclaved laboratory tools, media and explants.

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Appendixes

General protocols proposed in non-autoclaved sterilization

Sterilizing solution preparation protocol

- Pouring 30 mL of household bleach (HB) contained 5.25% of active chlorine (NaOCl) in 100 mL vial glass
- Adding the distilled water until reaching 100 mL and softly shakes for 3 min
- Transfer this solution that so called "Sterilization Solution, (SS)" in to the another clean bottle with screw topping and attached the label with the name "SS 30% HB of 5.25% active chlorine (NaOCl)"

***In vitro* laboratory equipments sterilization protocol**

- Spraying those surface equipment (erlenmeyers, jars, scissors, scalpel, magnetic stirrer, tweezers, petri dish, etc. with SS
- Preparing 250 mL beaker glass with 200 mL SS
- Putting all equipments used in beaker glass during *in vitro* processes

Sugarcane leaf sheath segment sterilization protocol

- Preparing the sugarcane explant source with 30 cm of sheath leaf segment
- Washing with running tap water until clean of the sugarcane sheath leaf segment, praying the sheath leaf surface with 70% of analytical ethanol, then remove all non-meristematic part of sheath leaf segment until the meristematic part (±7-10 cm) was obtained
- Sterilizing the 120 mm Petri dish with SS by soaked it for 30 sec or surface sprayed it with SS
- Soaking the meristematic segment in the SS for 1 min or 60 sec
- Putting the sterile explant source in to the 12 cm sterile Petri dish
- The explant have to be sliced by sterile sharp scalpel blade in 2-3 mm thick
- Those slices are ready to incubate onto callus induction medium

***In vitro* medium sterilization protocol**

- Putting all elements medium as usual procedures elsewhere (macros, micros, irons, vitamins, plant growth regulators, sugar, organic substances, agar solidified and etc.) onto 1000 mL flask containing 600 mL sterile water
- Adding 100 µL of HB onto this flask, then pouring sterile water until reaching 1000 mL, justified medium pH at 5.8 by NaOH 0.1 N and HCl 0.1 N and putted the magnetic stirrer in it
- Putting this flask with medium onto hot plate and stirring and boiling for 5 min
- Pouring this medium onto each 100 mm Petri dish or each medium screw topping jars and putted them into the medium room for at least seven days before used

Sugarcane calli induction protocol

- Incubating a slice of sugarcane explant into a Petri dish or a jar containing callus induction medium and placed onto special box in the dark condition under standard culture room temperature for 7-10 days until callus have been appeared
- The dark condition of culture should be ended after 7-10 days and then continue lighting will go on until callus growth and development in the certain grade
- The callus is ready to go in the next step of regeneration procedure

Sugarcane plantlets regeneration protocol

- Splitting the 3-5 mm pooling callus with sterile sharped forceps and put onto sterile vertical jar containing regeneration plantlet medium with screw topping
- Initiating the plantlet in the culture room under light condition according to the standard culture application procedure elsewhere
- The plantlet growth and development will starting from callus embryogenic, embryo and plantlet phases which is takes place after 7 days of culture

Supplementary notes

- The A, B, C and D protocols should be done in outside of Laminar Air Flow Cabinet (LAFC) or onto preparation bench
- The E and F protocols must be done in the inside of non running LAFC without using the ethanol lamp or bunsen
- The sterile water used in this whole procedures was non-autoclaved one

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