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Research Article
Isothiazolone Reduced Microbe Contamination of North Sumatra Local Garlic (*Allium sativum* L.) *in vitro*

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Abstract

Background and Objective: Sterilization of the explant is a very crucial stage in the tissue culture process because it determines the success to step into the process of induction of shoots or calluses. The purpose of this study was to get a sterile explant but does not suffer damage to plant cells or tissues due to sterile materials. **Materials and Methods:** The study used a Completely Randomized Design (CRD) factorial with 2 treatment factors. The 1st factor was the sterilization method with 3 methods consisting of P_1 (clorox 20% for 30 min then soaked in a solution of peracetic acid 0.5% for 5 min), P_2 (clorox 20% for 30 min then soaked in chloramphenicol 1000 ppm for 60 min) and P_3 (clorox 20% for 30 min then soaked in isothiazolone 1.5 mL L^{-1} for 60 min). The second was the addition of isothiazolone to the media, namely M_0 (medium MS without the addition of isothiazolone) and M_1 (medium MS+1.5 mL L^{-1} isothiazolone). The observed variables were the percentage of live explants and the level of explants contamination. **Results:** The results showed that the percentage of explant life observed reached 100% in all treatments, while the sterilization method had a significant effect on the level of sterilization, but the media type treatment gave an insignificant response and there was an interaction between the sterilization method treatment and the type of media. **Conclusion:** The best treatment to eliminate the contamination of local garlic was P_3M_0.

Key words: *In vitro*, local garlic, contamination, sterilization, isothiazolone

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Garlic (*Allium sativum* L.) is one of the herb plants which it usually applies for culinary and also in traditional medical practise[1]. Garlic is an essential commodity for the people of Indonesia. The dependence of Indonesian people on garlic, not balanced with sufficient production, has made Indonesia the largest garlic importer in the world.

Garlic is mainly propagated by vegetative methods and its improvement through breeding programs is limited due to difficulties in flower induction[2]. In Indonesia, farmers propagate garlic by vegetative division of the individual cloves of its bulbs from a previous crop as seeds in a small-scale production system. Almost all garlic bulbs that come from the field are contaminated with one or more pathogens, both on the surface and on the inside of the tissue.

The condition of plants contaminated with microbes is not easy to use in the activities of cultivation through plant propagation in tissue culture[3]. Therefore, sterilization of the explant is a very crucial stage in the process of garlic propagation and of all plants that come from the field in tissue culture, because the stage determines the success to step into the process of induction of shoots or callus. Standardization of explant sterilization protocols must continue to be developed to minimize explant mortality and to achieve a better survival rate of explants because plant tissues can be poisoned by disinfectants if the concentration, duration of exposure and the type of disinfectant are not suitable. Each tissue has a different sensitivity to different types of disinfectants[4]. Therefore, one of the most important aspects of successful micropropagation is the determination of an effective sterilization protocol, optimal types and proper concentrations.

Various methods had been used to eliminate bacterial and fungal contamination, including the application of biocide such as PPM™. Plant Preservative Mixture (PPM™) is a combination of 2 broad-spectrum industrial isothiazolone biocides, chloromethyl isothiazolone and methylisothiazolone[5], is a heat-stable preservative/biocide which can be used in the process of explant sterilization. Isothiazolone and its derivatives have been found as useful biological properties, such as antimicrobial, antibacterial, antifungal, antiviral, anticancer and anti-inflammatory activities[6]. It serves as an antibacterial and antimicrobial to effectively prevent or reduce microbial contamination in plant tissue culture. These compounds were described to be able to diffuse across the bacterial cell membrane and the cell wall of fungi[7].

Sterilization methods in tissue culture techniques vary greatly and depend on the type of plant, the explant used and the growing environment. The final result of the sterilization process is to obtain sterile explants that do not suffer from a plant cell or tissue damage due to sterile materials, so the research on the type, duration of soaking and concentration of disinfectants needs to be carried out because plants have specific natural microbes and also have distinctive tissue characteristics, which can only be eliminated by certain disinfectants.

By knowing the right protocol of explant sterilization, it will facilitate the process of seed propagation and development of local garlic plants in North Sumatra. This study aimed to find the right garlic sterilization materials and methods for *in vitro* plant propagation.

The optimization of techniques related to the explant sterilization procedure of Sumatra local garlic *in vitro* would enable further development of sufficient amounts of explants in a short time and as a consequence, the micropropagation of Sumatra local garlic should be optimized.

MATERIALS AND METHODS

Time and location: The research was conducted at The Tissue Culture Laboratory Agricultural Faculty, Universitas Sumatera Utara, Medan from May-July, 2021.

Experimental design: The study used a completely randomized design (RAL) factorial with 2 treatment factors. The 1st factor was the sterilization method with 3 methods consisting of P_1 (clorox 20% for 30 min then soaked in a solution of peracetic acid 0.05% for 5 min), P_2 (clorox 20% for 30 min then soaked in chloramphenicol 1000 ppm for 60 min) and P_3 (clorox 20% for 30 min then soaked in isothiazolone 1.5 mL L^{-1} for 60 min). The second was the addition of isothiazolone to the media namely, M_0 (medium MS without the addition of isothiazolone) and M_1 (medium MS+1.5 mL L^{-1} isothiazolone). Isothiazolone used in this research was PPM™ (Plant Preservative Mixture). The experiment was repeated 3 times and each replication consisted of 10 culture bottles.

Procedure: The garlic was peeled and the cloves were washed using detergent and rinsed with water, then soaked for an hour with Benlate fungicide solution with a concentration of 2 g L^{-1}, then rinsed with sterile distilled water 3 times then continued with sterilization in a laminar airflow cabinet. Thereafter, the cloves were soaked in 20% clorox solution for

15 min and rinsed with sterile distilled water 3 times then halved. The flesh cloves were removed and the shoots immersed in different disinfectant solutions according to the treatments, then cultured in M_0 and M_1 media. Parameters observed were the number of live explants, the number of dead explants, the number of contaminated cultures and the type of contamination, in addition to microscopic observations of the contaminated cultures, in the form of gram staining.

The sterile explants produced from treatment with the lowest contamination rate were transferred to media with various PGRs NAA and BAP to ensure the sterilization protocol truly resulted in free contaminant explant.

Statistical analysis: Parameters observed were the percentage of live explants and the level of contamination of the explants. The data obtained were analysed by ANOVA, followed by Duncan's Multiple Range Test (DMRT) at a 5% level of significance.

RESULTS

Percentage of surviving explants: Figure 1 showed there were no dead explants in all treatments. The survival rate reached 100% in all treatments and there was no bleaching or browning explant.

Contamination rate: Figure 2 displayed that there were different contamination rates in 1 and 2 weeks after culture. All treatments resulted from higher contamination rate in the 2nd week than in the 1st week after culture. The graphic illustrated that the sterilization methods showed different effectiveness. The highest percentage of contamination was in the P_1M_0 treatment and the lowest was in the P_3M_1 treatment. The immersion method with isothiazolone (P_3) had a lower level of contamination than peracetic acid and chloramphenicol. In this study, it can suppress contamination up to 19.31%.

According to the results of the ANOVA test, the sterilization method treatment had a significant effect on the level of sterilization, but the media type treatment gave an insignificant response between the treatment of M_0 (Medium MS without the addition of isothiazolone) and M_1 (media MS added by isothiazolone) and there was an interaction between the treatment of the sterilization method with the type of media. The combination of P_3M_0 was not significantly different from P_3M_1, indicating that there was no significant effect to add isothiazolone on the medium culture of local garlic *in vitro*.

Type of contamination: Based on the observations, this study found 2 types of fungi appeared in each treatment Fig. 3a-c and 4a-c, but the highest rate has resulted in chloramphenicol treatment. Figure 4 showed that the use of chloramphenicol was not effective in sterilizing garlic explants from contamination, especially bacteria. Fig. 3b displayed the microscopic appearance of the fungus with white mycelium, while Fig. 3c showed the microscopic observations of young spores suspected of being Fusarium. According to the result obtained, chloramphenicol was not effective to eliminate contamination in garlic explants, especially fungus.

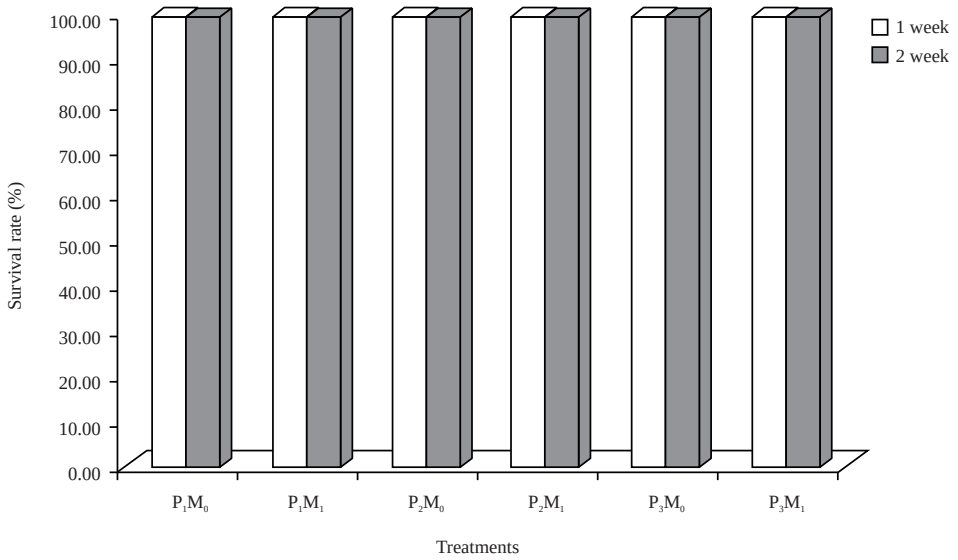

Fig. 1: Percentage of surviving explants

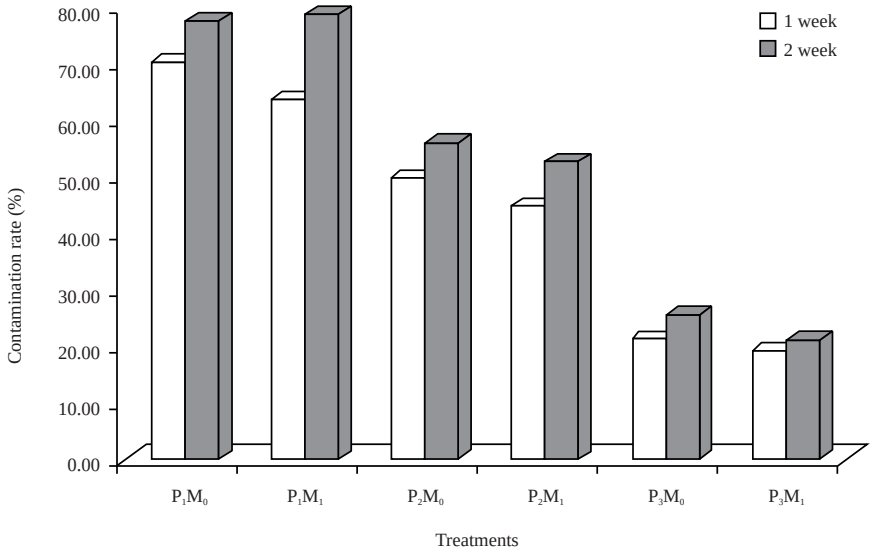

Fig. 2: Percentage contamination rate of garlic explant culture

Fig. 3(a-c): Garlic culture P_2M_0 and P_2M_1 treatments, macroscopic images of the fungus suspected of *Fusarium* and microscopic images of *Fusarium* spores (microconodium)
Bars: 1 cm

Figure 4a showed a culture contaminated by a fungus suspected of *Aspergillus* sp. It is characterized by a white macroscopic appearance with a blackish green in the centre, with round spores Fig. 3b and has insulated hyphae Fig. 3c. Figure 4d and e showed that the colour of bacterial colonies that contaminate the garlic culture was white. Microscopic observations showed that the contaminant was gram-negative bacteria. Most bacterial contamination was found in the peracetic acid (P_1) treatment and less in the chloramphenicol (P_2) and isothiazolone (P_3) treatments. The bacteria appeared on the 4th day after the culture. The lowest contamination was found in P_3M_0, indicating that isothiazolone is effective in controlling microbes, both fungi and bacteria, compared to the other 2 disinfectants.

Contamination on the second subculture: The result found that there was no contamination in a culture in which the explants derived from treatment P_3M_0, no presenting

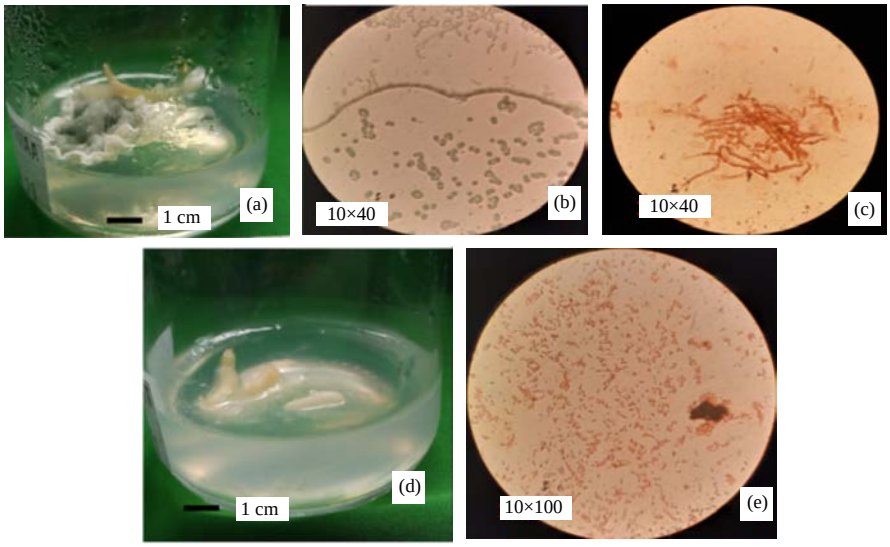

Fig. 4(a-e): Image of suspected *Aspergillus* spores, (a) Macroscopic image of *Aspergillus*, (b) Microscopic images of suspected *Aspergillus* spores, (c) Microscopic images of *Aspergillus* hyphae, (d) Macroscopic bacterial contaminated cultures and (e) Microscopic images of gram-negative bacteria
Bars: 1 cm

contamination fungus or bacteria. This indicates that there was no contamination inside the explants and the treatment was effective to control microbe's contamination in the establishment stage of garlic micropropagation.

DISCUSSION

Several factors affect the success of explants in culture establishment, among which the most important thing to note is the type and concentration of disinfectant that can be tolerated by plant tissues so as not to cause damage or death to the explants[8]. Surface sterilization is a sensitive matter that affects the success of plant regeneration in the tissue culture process, so it is necessary to do specific research on each type of plant by considering the sterilant material, concentration and duration of immersion that do not result in the death of plant cells but could eliminate contamination[9].

The results of the current study found that there was no contamination in the 1st week after culture however, the percentage of contamination in the 2nd Week After Culture (WAC) increased in all treatments. This indicated that the sterilant used mostly cleaned the contaminating agents on the surface of the explants. When the explants were cultured, the contaminating agents which were inside the explant tissues exposed to the outside, grew and developed. This result was supported by Putri *et al.*[10] which stated that most sterilant agents only eliminate the external microbe and the internal contaminant was hidden in plant tissue and would be active when the effect of disinfectant disappear. Lazo *et al.*[11] also found that establishment in grape showed sterilized grape explants appeared sterile up to the 3rd day after culture, but after 7 days all explants were contaminated. This is because the inside of the explant tissue contains microbes that are not damaged. After all, they are not exposed to sterile agents during the explant sterilization process. This could happen because the inside of the plant tissue contains microbes that do not die. After all, they do not come out during the explant sterilization process. Babaei *et al.*[12] said that internal microbes can grow and develop inside explant and destroyed plant tissue. In other studies, the contamination appeared after 8 week after culture because there was internal contamination which hided and active inside the explant during the culture periode[13].

Isothiazolone treatments resulted in the lowest explant contamination rate. However, the results showed that there was no significant difference in the level of contamination of explants cultured on media that was added with isothiazolone and those that were not given isothiazolone. These results indicate that isothiazolone is more effective in eliminating the microbes attached to the explants when the explants are immersed in isothiazolone solution before culture. Perhaps when the explants were immersed in a 1.5 ppm concentration of isothiazolone solution for 60 min, some of the isothiazolone seeps into the tissue and causes the microbes in the tissue to

die. However, when added to the media, the concentration of isothiazolone that can be absorbed into the explant tissue is limited so that only a few microbes in the tissue die.

The result showed that Isothiazolone was more effective than peracetic acid and chloramphenicol in eliminating garlic explant contamination. Isothiazolone with concentration 1.5 mL L^{-1} effectively controls the contaminating agent in garlic explants for it has a broad spectrum both against fungi and bacteria, making it more effective for successful sterilization. Research in *Clinacanthus nutans* sterilization reported that isothiazolone with a concentration of 1.25 mL L^{-1} was the optimum concentration that could increase the success of explant sterilization in plant cultures[14]. According to George and Tripepi[15] isothiazolone can controls microbes by sticking to microbial membranes and inhibiting enzyme reactions in the citric acid cycle which results in disruption of the electron transfer chain and inhibits the transport of monosaccharide's and amino acids from the medium to microbes, but does not affect metabolism and transport pathways in plant cells.

The study found that isothiazolone is more effective in controlling microbes, both fungi and bacteria, compared to the other 2 treatments. It indicated that isothiazolone is effective in controlling endophytic bacteria in garlic, which is in line with other research that the use of isothiazolone (PPM) can control endophytic bacteria in papaya explants[16].

The results showed that chloramphenicol was ineffective in sterilizing garlic explants from contamination, especially fungi (Fig. 4 and 5). This was because chloramphenicol is an antibiotic and generally antibiotics are specific. Chloramphenicol didn't control *Fusarium* and *Aspergillus*. *Fusarium* was reported as one of the fungi that attacks the bulbs of garlic. The fungus can survive on tubers and often appears as a post-harvest pathogen during tuber storage[17], although other research was reported as a broad-spectrum antibiotic but only specifically inhibits certain eukaryotes, namely the fungus *Magnaporthe oryzae*[18].

The current study also found that peracetic acid was not an effective sterilant to sterilize garlic. Every living organism has specific natural microbes that not all can be controlled by peracetic acid, even it is widely used as a disinfectant in the health sector. Qihua and Deng[19] mention that 0.1% peracetic acid is not toxic and can control microbes in the corneal tissue of the eye, however in this study, it was not effective in controlling contamination in garlic explants. Lazo *et al.*[11] also reported that peracetic acid is not effective in controlling contamination in the sterilization process of grape explants.

A suspected *Aspergillus* sp. a was found in this research. It is characterized by a white macroscopic appearance with a blackish green in the centre part with round spores (Fig. 4b) and has insulated hyphae (Fig. 4b). The other similar result of the research reported that *Aspergillus* sp. is common contamination found in abaca banana explants. The fungus has a green colony colour in the middle with insulated hyphae. It can stick to the explants or be in the air and grew in vessel[20].

Furthermore, the propagules produced by P_3M_0 treatment were sub-cultured to new media and as a result, all the derivatives 6 weeks after subculture were clean from the contamination. This indicated that the P_3M_0 treatment was successful in controlling the contamination of garlic, both external and internal microbes. It was the parameter of suitable protocol to gain sterile explant that could be used in further research.

CONCLUSION

The P_3M_0 treatment (20% Clorox immersion for 15 min and 1.5 mL L^{-1} isothiazolone immersions for 1 hr with media without isothiazolone) was an effective treatment to obtain sterile garlic explants.

SIGNIFICANT STATEMENT

This study demonstrated the effect of isothiazolone in removing fungal and bacterial contamination in local garlic explants. Isothiazolone which is used as a sterilizer with a concentration of 1.5 mL L^{-1} for 1 h of immersion, can eliminate external and internal microbial contamination of garlic explants *in vitro* about 78.8% and is not toxic to the operator and the environment. In addition, the propagules produced from these treatments were still sterile when transferred to other media for further tissue culture purposes. This study will help researchers and tissue culture workers to apply isothiazolone to garlic or other plants.

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