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Research Article

POTENTIAL OF AQUEOUS OZONE FOR THE *IN VITRO* STERILIZATION PROCEDURE OF *SOLANACEAE*

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ABSTRACT

Objective: This research aimed to verify the use of aqueous ozone as an alternative sterilizer for *in vitro* sterilization procedure.

Methods: Three species of *Solanaceae* were selected based on their trichomes. They are thorn apple (*Datura metel* L.), eggplant (*Solanum melongena*), and tomato (*Lycopersicon pimpinellifolium*). The treatment used four levels of concentrations; K0 (NaOCl – control), K1 (AO 0.1 ppm), and K2 (AO 0.5 ppm), K3 (1 ppm) and it employed three types of leaves, namely, D1 (thorn apple), D2 (eggplant), and D3 (tomato). The data were analyzed by analysis of variance univariate two factors and followed by Duncan's multiple comparisons test.

Results: This assay showed that the effect of ozone was almost immediately against microorganisms, and the optimum ozone concentration depends on the types of the leaf. Both levels of ozone concentration and types of leaves had a significantly different effect ($p<0.05$), and there is no interaction between aqueous ozone concentration and types of leaves ($p>0.05$). The level of sterility in K0 is 77, 78%, K1 is 22, 22%, K2 is 44, 44%, and K3 is 100%. Ozone 1 ppm had the highest result, but there is no viable explant because they got damage in their cells.

Conclusions: Aqueous ozone showed good potential to inhibit the growth of microorganisms for the *in vitro* sterilization procedure of *Solanaceae*.

Keywords: Aqueous ozone, Sterilization, Medicinal plant, *Solanaceae*, *In vitro*.

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INTRODUCTION

Plant tissue culture or also known as *in vitro* culture is an effort to multiply plants by isolating plant parts and grow them in aseptic conditions [1-3], then these parts can multiply and become self-propagating complete plants [4]. *In vitro* culture techniques have the advantages of producing larges of some plants in a relatively short time [5], can conserve a rare plant without disturbing the wild species in their natural habitat [6,7].

Contamination with microorganisms has considered being the single most important reason for losses during *in vitro* culture of plants [8]. Such microorganisms include viruses, fungi, bacteria, and yeast [9]. The presence of these microorganisms usually resulting in increased mortality and also invariable growth, necrosis, reduced proliferation, and reduced rooting. Efforts to maintain sterile conditions are necessary for a whole series of *in vitro* culture activities, so sterilization must be done to prevent and reduce contamination [10].

Sterilizer with a chemical solution such as calcium hypochlorite, sodium hypochlorite, and hydrogen peroxide is commonly carried out in the process of the *in vitro* culture sterilization [10-13]. During sterilization, the living material should not lose their biological activity and the only contaminant should be eliminated, therefore, explants are surface sterilized only by treatment with the disinfectant solution at suitable concentration and period [8,14].

Ozone is increasingly used as an alternative disinfectant to chlorine for water treatment because of its potent oxidative and antimicrobial properties and without disinfection by-product of trihalomethanes and haloacetic acids [15,16]. Vegetables and fruits washed with ozone water were proven to be more durable, do not change the color, taste, and nutritional content of fruits and vegetables [17,18]. The use of 1.5 ppm ozone has been proven to be effective in suppressing *Escherichia coli* bacteria up to 31.25% in postharvest handling of the spinach plants [19]. The mechanism of ozone in killing microbes is ozone will oxidize the cell wall, causing changes in the permeability of the cell, and then, lysis will occur in bacteria [16,18,20]. Based on the ability of ozone to kill bacteria is very good, so make it an appropriate tool for microbial decontamination [21,22]. Hence, this research objective is to verify the use of aqueous ozone as an alternative sterilizer for *in vitro* sterilization procedure. This is a novelty because the use of ozone as a sterilizer for *in vitro* procedure has never been done.

This research used leaves explant selected directly from field-grown medicinal plants of *Solanaceae*. The leaves of *Solanaceae* have typical trichomes in their anatomical structure [23,24]. There are so many types of trichomes on its surfaces so microbes are not easily sterilized because it is hidden in it and makes them being potential places for increasing microbes growth [19,25]. Several types of Solanaceae family used in this research were plants that have benefits for humans, such as medicines and food sources, namely, thorn apple (*Datura metel* L.), purple eggplant (*Solanum melongena*), and tomatoes (*Lycopersicon pimpinellifolium*).

METHODS

Plant material

The explants of the thorn apple plant leaves (*D. metel* L.), purple eggplant (*S. melongena*), and tomatoes (*L. pimpinellifolium*) were obtained from the City of Semarang, Central Java Province, Indonesia. Each leave explant was selected from the third leaf from the top of the plant using the cutter and put in a glass covered.

The making of culture media *in vitro*

A 300 mL of water was heated in a Bekker glass with a magnetic stirrer at 200°C, 300 rpm. Magnetic stirrer bar sprayed with 90% alcohol was put into it. After boiling, add 2215 g of Murashige and Skoog

Media (MS) into it. After the MS media dissolved, sucrose was added as much as 15 g. NAA and BAP each as much as 5 mL were added into the solution then add distilled water until the volume reaches 500 mL. The pH measurement carried out using a pH meter. If the pH is <6 add NaOH, but if more than 6 add HCl. Finally, add the Gelzan powder, and make sure it dissolved well. After that, the solution was poured out into the culture bottle much as 10 mL for each culture bottle, cover with aluminum foil, and reinforce with a sealer. The culture bottle was put into an autoclave, sterilize at 121°C for 20 min, and a pressure 1 of atm.

Equipment sterilization

All the tools used in this research washed with detergent, rinsed with running water, then dried, and then sprayed with 90% alcohol, then wrapped with paper. After that, it sterilized with an autoclave at a temperature of 121°C for 20 min, a pressure of 1 atm.

The making of ozone water (aqueous ozone)

Aqueous ozone made by the ozone generator with double dielectric barrier discharge plasma system. Pure oxygen used to produce an ozone-oxygen mixture. A magnetic stirrer used to make it easier. A 1200 ml aquadest ozonated for 80 min, 60 min, and 40 min using 300 ppm ozone to produced aqueous ozone 0.1 ppm, 0.5 ppm, and 1 ppm. Ozone concentration measured by Dissolved Ozone Meter Kit 0-5 ppm.

Explants surface sterilization

Treatment with sodium hypochlorite

The leaves washed with running water, then washed with detergent, and brushed slowly. Next, soaked with fungicide for 3 min and then rinsed with running water. The next step is the leaves soaked with sodium hypochlorite with a concentration of 5% for 5 min.

Treatment with ozone

A few strands of thorn apple, eggplant, and tomato leaves washed with running water, then washed with detergent and brushed slowly. Next, soaked with fungicide for 3 min and rinse with running water. After being rinse, each type of leaves grouped into three parts. The first part put into ozone water with a concentration of 0.1 ppm for 10 min, the second part is 0.5 ppm for 10 min, and the third part is 1 ppm for 10 min.

The planting of explants into culture media

Laminar air flow (LAF) sterilized first with 90% alcohol. Control leaves, as well as those that received sterilization treatment, put into the LAF along with the tools then irradiated with ultraviolet for 40 min. All pieces of equipment soaked in 90% alcohol and passed it into a fire before used to cut the leaves. The leaves cut into three parts. The part used as explants is the middle part which wounded on both sides of the leaves.

The leaf explants planted into culture bottles containing MS media and covered it again with an aluminum foil. Each culture bottle contains one explant. Each culture bottle labeled with a treatment description. Observations carried out every day for up to 2 weeks.

Statistical analysis

The data were analyzed with SPSS software using analysis of variance (ANOVA) univariate two factors and followed by Duncan's multiple comparisons test (DMRT).

RESULTS

The effect of leaf type on sterility

The effect of leaf types on the level of explants sterility is shown in Table 1.

The effect of ozone concentration on sterility

The effect of ozone concentration on the level of explant sterility is shown in Table 2

The results of the ANOVA test at a 95% confidence level indicate that there is no interaction between the leaf types with ozone concentration (p>0.05). This test also shows that the type of leaves has a significant effect on the level of sterility (p<0.05), as well as the ozone concentration, which shows a significant effect on the level of sterility (p<0.05). The results of the DMRT test show that the treatment K0, K1, K2, and K3 provide a real influence on the level of sterility.

Table 1: Statistical results of the effect of leaf type on sterility levels

Leaf type	Sterility level (%)
D1 (Thorn apple)	66.67[b]
D2 (Eggplant)	83.33[b]
D3 (Tomato)	33.33[a]

*Different letters indicate statistically significant differences (p<0.05)

Table 2: Statistical results of the effects of ozone concentration

Concentration level	Sterility level (%)
K0 (NaOCl)	77.78[b]
K1 (AO 0.1 ppm)	22.22[a]
K2 (AO 0.5 ppm)	44.44[a]
K3 (AO 1 ppm)	100[b]

*Different letters indicate statistically significant differences (p<0.05).
AO: Aqueous ozone

The data in Table 1 show that the leaves type as a single factor has a significant impact on the level of sterility, which is continue further test. It means the explants derived from eggplant leaves give the highest level of sterility when compared with thorn apple and tomato leaves. Nevertheless, eggplant leaves and thorn apple leaves have the same notification (b), it means that both the leaves of eggplant and thorn apple have the same impact on sterility. But, the tomato leaves have a different notification, which means that tomato leaves have a different impact on the level of sterility compared to eggplant leaves and thorn apple leaves.

Table 2 shows the influence of ozone concentration as a single factor is very significant, then continues with further tests. It explains that ozone at all concentrations can kill microbes directly. The use of sodium hypochlorite (control) as a sterilant also shows a good result in the level of sterility.

The effect of ozone concentration on this test is very significant (p<0.05). In general, the higher ozone concentration will make sterility level increase in all types of leaf, which also means that more microbes die.

DISCUSSION

The surface type of leaf is one of the factors that affect the level of explant sterility of *in vitro* culture [8]. The surface of eggplant leaf has typical trichomes, as shown in Fig. 1, which is *Stella* that allows the microbes or spores to hide there so that when it sterilized the microbes or spores may be not fully reached by sterilizer. The thorn apple itself has the type of trichomes headed unicell, as shown in Fig. 2. The results of the explant sterility of the leaf have the same value as the explant of eggplant leaf. On the other hand, tomato leaf shows different results. The level of sterility of the explant of tomatoes leaf is lowest when compared to eggplant or thorn apple leaf. Tomatoes leaf is very thin and fills with a trichomes shaped needle very tightly, as shown in Fig. 3. It allows the spores or microbial located hidden there, make it difficult cleaned by ozone. Based on these results, it can be concluded that the leaf of eggplant and thorn apple had better sterility levels than tomatoes.

This assay shows that the higher ozone concentration will make sterility levels higher also. It shows that the ability of ozone to kill microbes such as bacteria, fungi, or spores is great. According to Miller *et al.* [17], ozone can reduce the level of viability of the conidia of the fungus *Aspergillus flavus*, which could lead to contamination in Brazil nuts. Giordano *et al.*

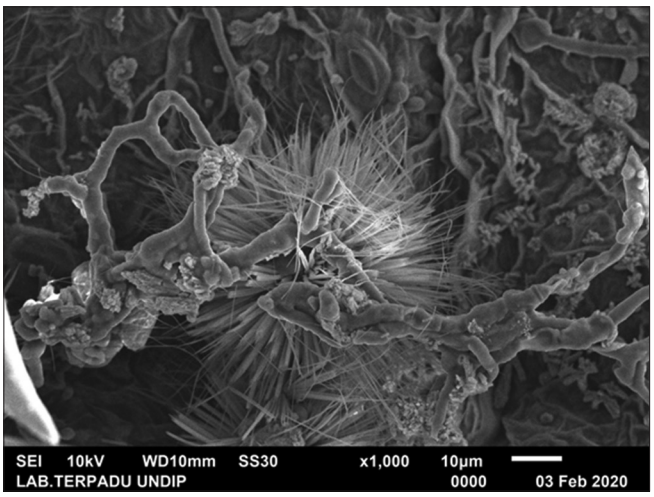

Fig. 1: Scanning electron micrograph of trichomes in eggplant leaf

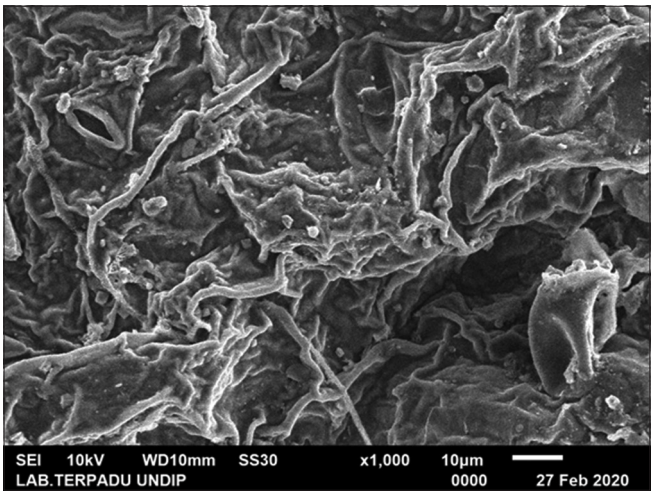

Fig. 2: Scanning electron micrograph of trichomes in thorn apple leaf

[26] proved that ozone with a concentration of 31 mg L-1 can inhibit the viability of fungi on the beans. Beber-Rodrigues *et al.* [27] found that ozone can reduce the fungus from 3.0×10^5 to 1.4×10^2 CFU/ml. The ability of ozone to inhibit microbial growth or even kill the microbes is due to the ability of ozone to damage the cytoplasmic membrane and increases the permeability of the membrane. Based on the research results from Zhang *et al.* [15] who obtained that almost all the cells of *Pseudomonas aeruginosa* given ozone, change shape, and occur expenses ion K^+, Mg^{2+}, and ATP indicates the damage to the cytoplasmic membrane.

K^+ channels are proteins transmembrane to mediate ion K^+ pass through the cell membrane on passive transport [28]. Treatment with ozone can lead to protein denaturation and a decrease in the enzyme activity of microbes [29]. The leakage of K^+ from microbes treat with ozone could be caused by denaturation of membrane proteins associated with K^+ channels, failed to hold the ion K^+, and increased the permeability of K^+ of the cell membrane.

The leakage Mg^{2+} could be indicated ozone can change the function of the membrane so that it can increase the permeability of the membrane to Mg^{2+}. According to Zhang *et al.* [15], leakage of K^+ and $Mg2^+$ proved ozone causes damage to membrane proteins in cells by degrading unsaturated fats in the sheath cells, hence the contents of the cells out.

The release of ATP from the cells due to the treatment of ozone also leads to cell death. In general, ATP used as an energy source by all organisms includes bacteria and fungi. If the cell membrane is broken and the ATP out of it, then microbes will lack the energy to survive. The fact ozone leads to leakage of ATP shows that the cytoplasmic membrane has changed its permeability characteristics [15].

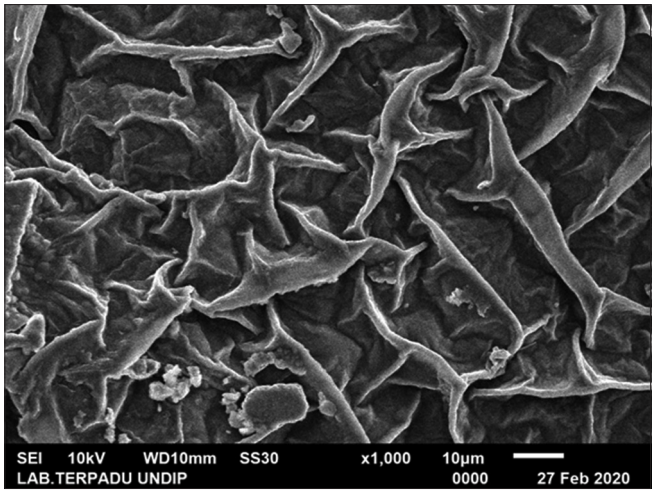

Fig. 3: Scanning electron micrograph of trichomes in tomato leaf

The leakage of ATP, K^+, and Mg^{2+} causes damage to intracellular components such as plasmid DNA, oxidation of proteins, and decrease the activity of the enzyme cytosolic. Agglutination of cytoplasm is an impact of the treatment of ozone. The death of these microbes can be caused by the increased permeability of the membrane and also by the agglutination of cytoplasm.

Based on the results of this research, ozone with a concentration of 1 ppm has very significant results, which is 100% sterile. Even it has a significant result, unfortunately, tissue necrosis and explant mortality were increased also.

CONCLUSIONS

Ozone is very potential as a sterilizer in the sterilization procedure of *in vitro* culture. However, it still needs to do more research to determine the ideal concentration of which can increase sterility and does not cause tissue damage on the explants.

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AUTHORS' CONTRIBUTIONS

Titis Yuliana designed and carried out the experiment, wrote the manuscript under supervised by Dr. Erma Prihastanti, M.Si and Dr. Yulita Nurchayati, S.Si, M.Si. All authors discussed the results and contributed to the final manuscript.

CONFLICTS OF INTEREST

There are no conflicts of interest in this research.

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REFERENCES

1. Dakah A, Zaid S, Suleiman M, Abbas S, Wink M. *In vitro* propagation of the medicinal plant *Ziziphora tenuior* L and evaluation of its antioxidant activity. Saudi J Biol Sci 2014;21:317-23.
2. Ikenganyia E, Anikwe M, Omeje E, Adinde J. Plant tissue culture regeneration and aseptic techniques. Asian J Biotechnol Bioresour Technol 2017;1:1-6.
3. Oseni M. A review on plant tissue culture, a technique for propagation and conservation of endangered plant species. Int J Curr Microbiol Appl Sci 2018;7:3778-86.
4. Suryanti E, Mellisa D. Perbanyakan tanaman tomat dengan menggunakan BAP dan NAA secara *in vitro*. J Bioterdidik Wahana Ekspresi Ilmiah 2017;5:1-7.
5. Kumar N, Reedy MP. *In vitro* plant propagation: A review. J For Sci 2011;27:61-72.
6. Sengar RS, Chaudhary R, Tyagi SK. Present status and scope floriculture developed through different biological tools. Res J Agric Sci 2010;1:306-14.
7. Vinoth A, Ravindhran R. *In vitro* propagation-a potential method for plant conservation. Int J Comput Algorithm 2013;2:268-72.
8. Singh V, Tyagi A, Chauhan PK, Kumari P, Kaushal S. Identification and prevention of bacterial contimination on explant used in plant tissue culture labs. Int J Pharm Pharm Sci 2011;3:160-3.
9. Omamor IB, Asemota AO, Eke CR, Eziashi EI. Fungal contaminants of the oil palm tissue culture in Nigerian institute for oil palm research (NIFOR). Afr J Agric Res 2007;2:534-7.
10. Goswami NK. *In vitro* sterilization protocol for micropropagation of *Musa amritsagar* banana. Indian J Appl Res 2013;3:51-4.
11. Winarto B, Da Silva JA. Sterilization procedure for *in vitro* culture of leather leaf fern. Int J Plant Dev Biol 2012;6:46-50.
12. Khatun M, Tanny T, Razzak A, Alam U, Amin YS. Standarization of *In vitro* sterilization procedures for micropropagation of ginger (*Zingiber officinale* Rosc.). Int J Appl Biol Pharm Technol 2016;7:131-7.
13. Bello O, Esan E, Obembe O. Establishing surface sterilization protocol for nodal culture of *Solanecio biafrae*. IOP Conf Ser Earth Environ Sci 2018;210:012007.
14. Firoz A, Udin E, Amin R, Razak A, Manik M, Khatun M. Studies on the effect of various sterilization procedure for *in vitro* seed germination and succesful micropropagation of *Cucumis sativus*. Int J Pure Appl Biosci 2016;4:75-81.
15. Zhang YQ, Wu Q, Zhang J, Yang X. Effects of ozone on membrane permeability and ultrastructure in *Pseudomonas aeruginosa*. J Appl Microbiol 2011;111:1006-15.
16. Rojas-Valencia MN. Research on ozone application as disinfectant and action mechanism on waste water microorganism. Sci Againts Microb Pathog 2011;1:263-71.
17. Miller F, Silva C, Brandão T. A review on ozone-based treatments for fruit and vegetables preservation. Food Eng Rev 2013;5:77-106.
18. Carletti L. Use of ozone in sanitation and storage of fresh fruits and vegetables. J Food Agric Environ 2013;11:585-9.
19. Shynkaryk M, Pyatkovskyy T, Yousef A, Sastry S. Gaseous ozone treatment of baby spinach within the existing production chain for inactivation of *Eishericia coli* O157:H7. J Food Eng 2016;191:10-8.
20. Fathima MK. Ozone Technology in Fruits and Vegetables. Kerala: Kerala Agriculture University Kelappaji College of Agriculture Engineering and Technology; 2013.
21. Matthes R, Bender C, Schlüter R, Koban I, Bussiahn R, Reuter S, *et al*. Antimicrobial efficacy of two surface barrier discharges with air plasma against in vitro biofilms. PLoS One 2013;8:70462.
22. Daeschlein G, von Woedtke T, Kindel E, Brandenburg R, Weltmann KD, Jünger M. Antibacterial activity of an atmospheric pressure plasma jet against relevant wound pathogens *in vitro* on a simulated wound environment. Plasma Proc Polymers 2010;7:224-30.
23. Jayanthi PD, Aurade RM, Kempraj V, Roy K, Shivashankara, KS Singh TH. Morphological diversity of trichomes and phytochemicals in wild and cultivated eggplant species. Indian J Hortic 2018;75:265-72.
24. Harisha C, Jani S. Pharmacognostical study on trichomes of *Solanaceae* and its significance. Univ J Pharm 2013;2:100-4.
25. Ali M, Boonerjee S, Islam MS, Mihir H, Imdadul SR. Endogenous bacterial contamination of plant tissue culture materials: Identification and control strategy. Plant Tissue Cult Biotechnol 2018;28:99-108.
26. Giordano B, Nones J, Scussel V. Susceptibility of the in-shell brazil nut mycoflora and aflatoxin contamination to ozone gas treatment during storage. J Agric Sci 2012;4:1-10.
27. Beber-Rodrigues M, Savi G, Scussel V. Ozone effect on fungi proliferation and genera susceptibility of treated stored dry paddy rice (*Oryza sativa* L.). J Food Saf 2014;35:59-65.
28. Hebert SC, Desir G, Giebisch G, Wang W. Molecular diversity and regulation of renal potassium channels. Physiol Rev 2005;85:319-71.
29. Komanapalli IR, Lau BH. Ozone-induced damage of *Escherichia coli* K-12. Appl Microbiol Biotechnol 1996;46:610-4.