JSMARTech
Journal of Smart Bioprospecting and Technology
April 2022, VOL 03 NO 01
p-ISSN: 2686-0805 **e-ISSN**: 2714-7894
DOI:https://doi.org/10.21776/ub.jsmartech.2022.003.01.16

Surface Sterilization of Mango's Mistletoe Leaves (*Dendrophtoe pentandra* (L.) Miq.) for Endophytic Fungi Isolation

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Submission: 20 March 2022; **Revised**: 20 April 2022; **Accepted**: 30 April 2022

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ABSTRACT. *Mango mistletoe (Dendrophthoe pentandra L. Miq) is known as a parasitic plant that has been scientifically proven to have the potential as an antihypertensive adjuvant herbal. The content of secondary metabolites in plants is produced by plants andby microorganisms that grow in plant tissues. One of them is endophytic fungi which can induce the host to produce secondary metabolites. The ability of endophytic fungi to synthesize secondary metabolites is an opportunity for the large-scale production of phytopharmaca raw materials in a short time without causing ecological damage. Before isolating endophytic fungi from the plant tissue, one needs to research surface sterilization to remove various kinds of microorganisms that are not desired. This study aims to determine the best method's surface sterilization of the mango's mistletoe leaves (Dendrophthoe pentandra (L.) Miq) to isolate endophytic fungi. This study used a two-factor completely randomized design. The concentration of Sodium Hypochlorite (NaOCl) was 5% and 10% as the first factor, and the immersion time of 1, 2, 3, 4, and 5 minutes was the second factor. There is a total of 10 treatments. In each treatment, there were three replications and one control. Observational data were analyzed descriptively. The results showed that application of NaOCl 10% for 3 minutes is the best and recommended method's surface sterilization of the mango's mistletoe leaves because the percentage of live explants is 100%, the percentage of contamination is 0%, and the percentage of explants with endophytic fungi is 100%.*

Keywords: *Dendrophthoe pentandra; endophytic fungi; Sodium Hypochlorite; surface sterilization*

INTRODUCTION

The problem of disease development that is increasing rapidly every time, resistance to disease or pathogens to drugs, and also negative side effects due to the use of synthetic chemical-based drugs, urgently need to find a solution, one of them is the use of natural ingredients that have herbal potential such as the Mango mistletoe. (*Dendrophthoe pentandra* (L.) Miq). Mango mistletoe is a type of parasitic plant that taxonomically belongs to the Loranthaceae family. Although it is a parasite, in the phytopharmaceutical study, the mango mistletoe has potential as a herbal because it has antioxidants. The part of the mango mistletoe usually used as a herb is the leaf part. The leaves of the mango mistletoe contain secondary metabolite compounds derived from flavonoids, they are quercetin and rutin, which reduce oxidative stress, so that the leaves of the mango mistletoe have the potential as an antihypertensive adjuvant herbal [1].

However, considering that nature as the main source of raw materials for medicines is limited, the continuous use of natural materials for pharmaceutical production sometimes creates new problems in the ecology, that is a decrease in the quantity and even the destruction of the biological resources of related plants. Therefore, needed to maintain the preservation of medicinal plants as phytopharmaca raw materials through the use of endophytic microorganisms found in plants, one of which is endophytic fungi [2]. Endophytic fungi are fungi that live in healthy plant tissues for a particular period without harming the host plant. Endophytic fungi can induce host plants to produce secondary metabolites. These secondary metabolites are beneficial for the host plant as; 1) communication and response to changes in habitat conditions, 2) defense efforts from viruses, bacteria, insects, nematodes, and other pathogens, and 3) increase the competitiveness of the host species against other species through the allelopathic effect [3].

Given the great potential, both possessed by the mango mistletoe and endophytic fungi and the absence of complete data or information on the isolation of endophytic fungi associated with Mango mistletoe. So, research on the isolation of endophytic fungi from Mango mistletoe needs to be done. But before that, as it was previously known that endophytic fungi live in plant tissues. Generally, the surface of plant organs are often exposed to various kinds of pollutant compounds or microorganisms from the environment (soil, air, and others) [4]. Therefore, before isolating endophytic fungi from the plant tissue, the preliminary research of surface sterilization to

remove various kinds on microorganisms that are not desired is needed. Thus, when the isolation process is carried out, the results obtained are true endophytic fungi that live in the host plant tissue not contaminant fungi that live on the outside or surface of the host plant.

Several chemicals are used to sterilize plant leaf surfaces, one of which is the most commonly used NaOCl (Sodium Hypochlorite) [5]. Sodium hypochlorite is a toxic compoundto several microorganisms such as bacteria, viruses, and fungi but does not damage plant tissues [6]. Sodium hypochlorite is generally used as a sterilizing agent in various plant surface with various concentrations and different immersion times [7]. The concentration of NaOCl and the length of immersion time used as a material for surface sterilization varied depending on the type of plant and the type of plant organ used [8]. This study aims to determine the surface sterilization method of the leaves of the Mango's mistletoe to isolate endophytic fungi.

RESEARCH METHODS

Research Design

This study used a two-factor completely randomized design. Sodium Hypochlorite (NaOCl) was 5% and 10% as the first factor, and the immersion time of 1, 2, 3, 4, and 5 minutes was the second factor. There are a total of ten treatments. In each treatment, there were three replications and one control.

Table 1. The Treatment of Surface Sterilization of Mango's Mistletoe Leaves

Treatment Code	Description	Treatment Code	Description
AP1	5% NaOCl for 1 minute	BP1	10% NaOCl for 1 minute
AP2	5% NaOCl for 2 minutes	BP2	10% NaOCl for 2 minutes
AP3	5% NaOCl for 3 minutes	BP3	10% NaOCl for 3 minutes
AP4	5% NaOCl for 4 minutes	BP4	10% NaOCl for 4 minutes
AP5	5% NaOCl for 5 minutes	BP5	10% NaOCl for 5 minutes

Materials

The materials used in this study were Mango's mistletoe leaves (*Dendrophthoe pentandra* (L.) Miq), PDA/Potatoes Dextrose Agar (Merck), antibacterial (chloramphenicol), NaOCl (Sodium Hypochlorite), distilled water, 96% alcohol, Bayclin, spirits, and detergents.

Sterilization of Tools and Materials

Sterilization of tools made of glass, such as Petri dishes, is done by immersing them in Bayclin solution for 24 hours. After that, the glassware then washed with detergent and dried. Sterilization of tools made of iron such as tweezers, spatulas, and scalpels is done by washing them with detergent (without immersing) and drying them. After drying, all the tools are wrapped in craft paper and then put in heat-resistant plastic. Before the sterilization process, tissue is placed in a glass and then covered with aluminium foil. The sterilization process of these tools was carried out using an autoclave at a temperature of 121^{o}C, and a pressure of 1 atm for 15 minutes. As for the ose needle, the sterilization process is done by dipping the ose needle into a 70% alcohol solution and then igniting it in a Bunsen flame until it smolders. Other tools such as dropper pipettes are sterilized by washing with detergent and then drying, and before being used, they must first be passed over a Bunsen fire [9].

Sterilizing materials such as distilled water is to put the water in a glass, and then cover it with aluminium foil. For PDA media inserted into the Erlenmeyer, then the Erlenmeyer is plugged with cotton and wrapped by craft paper and tied with wool. These materials were then sterilized using an autoclave at 121^{o}C and 1 atm pressure for 15 minutes. Materials such as alcohol and NaOCl (Sodium Hypochlorite) do not need to be sterilized [9].

Laminar Air Flow (LAF) Sterilization

LAF sterilization is done by spraying 70% alcohol onto the surface of the LAF, then drying with a tissue. Before being put into the LAF, all tools and materials are sprayed with 70% alcohol. Furthermore, LAF, along with tools and materials, are sterilized using an ultraviolet lamp for 30-60 minutes before the isolation process is carried out [10].

Preparation of Potatoes Dextrose Agar (PDA) Media

Weighed PDA media as much as 39 g/L and chloramphenicol 200 mg/L. The addition of chloramphenicol aims to prevent bacterial growth in the media [11]. After weighing, the two ingredients were suspended with 1000 ml of distilled water andboiledon a hot plate with a magnetic stirrer until homogeneous. After that, the media was sterilized using an autoclave at a temperature of 121^{o}C, and a pressure of 1 atm for 15 minutes [12].

After the sterilization process, the PDA media was poured into Petri dishes (each Petri dish contained 25 ml of PDA media) near the bunsen fire in the LAF. The Petri dish is then covered with plastic wrap and wrapped in craft paper. The media was then incubated at room temperature for 3-5 days. During the incubation

period, observations were still made on the media, if there was contamination of the media, then the media was then put into heat-resistant plastic and sterilized by autoclave at 121^{o}C, the pressure of 1 atm for 15 minutes, then the media was put into a dumpster, and Petri dishes were immersed in 5% NaOCl solution for 24 hours. The sterile media can be used for the isolation of endophytic fungi.

Sampling

Samples of the Mango mistletoe were obtained from the yard of one resident's house on Jl. Gajayana, Dinoyo Village, Lowokwaru District, Malang City, East Java. To ensure the suitability of the desired plant species, the Mango mistletoe that had been obtained was identified at Balai Materia Medica, Batu City, East Java.

The part of the Mango mistletoe used was the leaf part. The parts of the plant selected as the research sample were intact parts, without spots or signs of disease. The sample is then put in a plastic bag, then put into an icebox that has been filled with some ice tube and tightly closed to retain the freshness. After arriving at the laboratory, the samples were then transferred to the freezer at a temperature of ± 4^{o}C [13].

Sterilization of Plant Materials

Mango's mistletoe leaves are cleaned by washing using detergent and rinsing with running water for 5 minutes to remove dust or other dirt attached to the leaf surface. After that, the leaves are dried on a tissue [14]. The sterilization process of planting material was then carried out in the LAF. The leaves were then sterilized using several solutions such as 70% alcohol for 1 minute, then put into a NaOCl solution with a concentration and immersing time according to the treatment table (Table 1), and then the leaves were put into 70% alcohol for 1 minute, after that the leaves are rinsed using sterile distilled water for 3 minutes, rinses and each rinse were carried out for 30 seconds [15]. The leaves that have been sterilized with some of these solutions are then drained on sterile Petri dishes that have been lined with sterile tissue. Then, the leaves were cut with a sterile scalpel into a size of 1x1 cm.

Isolation of Endophytic Fungi

Isolation of endophytic fungi was carried out by direct seed planting technique [14]. The cut plant samples were then placed in a Petri dish containing sterile PDA media. The planting of samples was carried out with 3 replications, and each Petri dish contained 2 sample pieces. The last rinsed water was used as a control by taking it using a dropper and leveling it on the surface of the media. If the controlled media grows fungi, the fungi that grow on the media are not endophytic [16]. The process of isolation of endophytic fungi was carried out aseptically in the LAF. After the isolation process, incubation was carried out for 14 days in an incubator at 30^{o}C. Observations were made every day during the incubation process to see the growth of fungal colonies. The observational variables include; the percentage of live explants, the percentage of explant contamination, the source of contamination in the explants, the time of contamination in the explants, and the percentage of explants covered with endophytic fungi [17].

Data Analysis

Data obtained from observations in qualitative data, such as sources of contamination in explants, are presented in tables and figures and analyzed descriptively. Quantitative data, such as the percentage of live explants, the percentage of explant contamination, the time of contamination in the explants, and the percentage of explants with endophytic fungi were presented in tables and diagrams and analyzed descriptively [17].

RESULTS AND DISCUSSIONS

Isolation of endophytic fungi can be defined as a process of growing endophytic fungi derived from plant tissue on an artificial medium in a laboratory under aseptic conditions. Generally, endophytic fungi can be isolated from various plant organs, ranging from roots, stems, bark, leaves, flowers, fruit to seeds [18]. The general purpose of the process of isolating endophytic fungi from plant tissue is to obtain biologically active secondary metabolite compounds [19].

One of the important steps in the process of isolating endophytic fungi is the surface sterilization of the plant parts used. Surface sterilization is carried out to prevent the growth of unwanted surface microorganisms on the isolation media. Surface sterilization can be done by immersing plant explants in several solutions such as; alcohol with a concentration between 70%-96%, and Sodium Hypochlorite (NaOCl) with a concentration of 1%-10%, or other sterile materials at certain concentrations and within a certain period. The concentration of the sterilant and the immersion time varies depending on the type of tissue or plant part that is used [20].

The combination of the use of alcohol-NaOCl-alcohol has been proven to be effective in killing microorganisms found on the surface [20]. Sodium hypochlorite (NaOCl) is a sterilizing agent that is commonly used for surface sterilization of planting material., This is because the material has an effective ability to affect the activity of microorganisms so that microorganisms can die. In addition, these

materials are also relatively safe for humans and plant tissues, easy to obtain, and have a selling price that tends to be cheaper [4, 21].

Based on the observations that have been made to the five observation variables; the percentage of live explants, the percentage of explant contamination, the source of contamination in the explants, the time of contamination in the explants, and the percentage of explants covered with endophytic fungi, the results obtained are presented in the form of tables, diagrams, and pictures, and will be explained in the following description.

Percentage of Living Explants

The percentage of live explants indicated the number of surviving Mango's mistletoe leaf explants in each treatment expressed as a percent (%). The indicator to determine whether an explant is alive or dead is to see whether the explant can still be overgrown with fungi or not. If the explants can still be overgrown with fungi, both endophytic fungi and contaminant fungi, it means that the explants are still alive because the fungi can still grow, live, and take nutrients from plants both on the surface and plant tissues.

Figure 1 showedthat in the sterilization treatment using 5% NaOCl, the highest percentage levels of live explants were immersed for 1 minute (AP1), 2 minutes (AP2), and 3 minutes (AP3) with a value of 1 minute (AP1), the percentage of live explants was 100%. The lowest was at 5 minutes of immersion (AP5) with a percentage value of 0% of live explants. Meanwhile, in the sterilization treatment using 10% NaOCl, the highest percentage of live explants was immersed for 1 minute (BP1), 2 minutes (BP2), and 3 minutes (BP3), with the percentage value of live explants being 100%, and the lowest was at 5 minutes of immersion (BP5) with a percentage value of 0% live explants.

Based on these results, the lower concentration and the length of immersion time can make the percentage of live explants higher, and conversely, the higher concentration and the length of immersion time can make the percentage of live explants lower. Too high a concentration of NaOCl and length of immersion time can cause browning of explants. Browning (browning) is a condition where the explants that have been initiated on the media change their state from green to brownish. The change in the color pigment of the explants to brown was due to the explants releasing phenolic compounds [22]. In general, these phenolic compounds were released by explants when the explants were injured so that they could increase the activity of the Polyphenol oxidase (PPO) enzyme [23]. Apart from injury, the phenol released by the explant is also a signal or response that the explant is in an unstable condition (stress) due to too high a concentration of the sterilant used or due to too long exposure time in the sterilant. This means that too high a concentration of sterile material and exposure of desk plant to sterile material for a long period will cause the explant to brown (browning) and even cause the death of the explant. In general, browning can cause stunted growth of explants, and if the level of browning in explants is very high, it can cause explants to die [23]. If the explant dies, then the microorganisms that live in the explant's tissue and on the outer surface of the explant also cannot live because they do not have a place to live or a place to take nutrients to maintain their sustainability.

Percentage of Explant Contamination

The percentage of contamination of explants indicated the number of contamination occurrences in mango's mistletoe leaves explants in each treatment which was expressed in the form of a percent (%). The indicator to determine if the explant was contaminated is by looking at the growth of microorganisms from both fungal and bacterial groups on the part of the explant that is not injured as on the surface.

Figure 2 showed that in the sterilization treatment using 5% NaOCl, the highest level of contamination was at 1 minute immersion time (AP1) with a percentage value of 100% contamination of explants, and the lowest was at the 5 minutes immersion time (AP5) with a percentage value of 0% explant contamination. Meanwhile, in the sterilization treatment using 10% NaOCl, the highest level of contamination was at 1 minute immersion time (BP1) with a percentage value of 100% explant contamination. The lowest was at 3 minutes (BP3), 4 minutes (BP4), and 5 minutes (BP5) with the percentage value of explant contamination was 0%.

However, as it was known in the previous discussion that in the sterilization treatment of the surface of the mango's mistletoe leaf using 5% NaOCl and 10% NaOCl for 5 minutes of immersion time, the explants had died because the explants had browned so that no microorganism can grow on explants. Therefore, the 5% and 10% NaOCl concentrations for 5 minutes of immersion time were considered too high for the sterilization of explants of mango's mistletoe leaves. In general, based on the percentage level of contamination of explants, it was known that the surface sterilization treatment of Mango's mistletoe leaves using a NaOCl concentration of 10% with a soaking time of 3 to 4 minutes was the best result because the concentration and length of immersing time could help reduce the number of

contamination events as evidenced by the percentage value of explant contamination was 0%, lower than the other treatments. Based on observations, it is known that in all treatments, there was no contamination in the control media, except for the surface sterilization treatment of mango's mistletoe leaves using NaOCl with a concentration of 5% with a soaking time of 1 minute (AP1) which was contaminated by fungi on the control media.

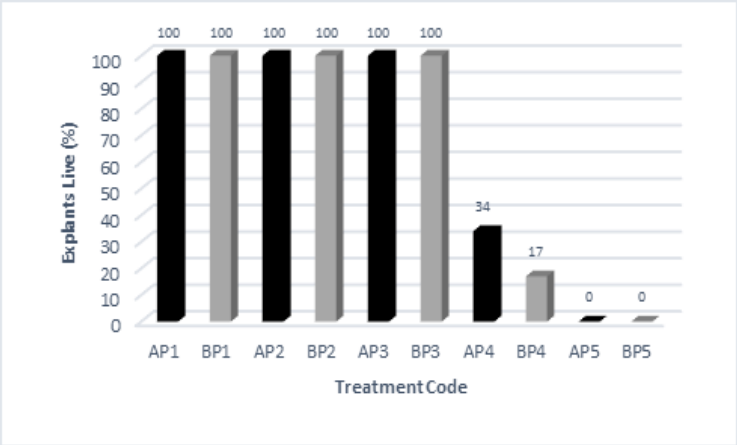


Figure 1. Diagram of the percentage of live explants. [AP1= Immersion with 5% NaOCl 1 minute; AP2= Immersion with 5% NaOCl 2 minutes; AP3= Immersion with 5% NaOCl 3 minutes; AP4= Immersion with 5% NaOCl 4 minutes; AP5= Immersion with 5% NaOCl 5 minutes; BP1= Immersion with 10% NaOCl 1 minute; BP2= Immersion with 10% NaOCl 2 minutes; BP3= Immersion with 10% NaOCl 3 minutes; BP4=Soaking with 10% NaOCl 4 minutes; BP5= Immersion with 10% NaOCl 1 minute].

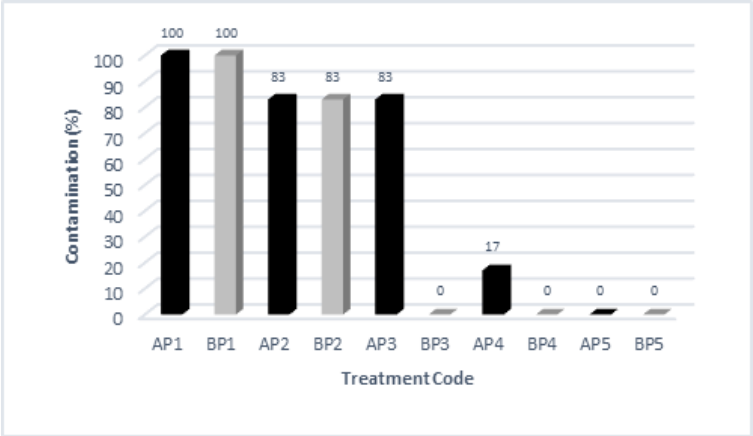


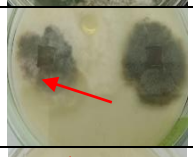
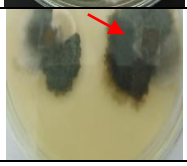
Figure 2. Diagram of percentage contamination of explants. [AP1= Immersion with 5% NaOCl 1 minute; AP2= Immersion with 5% NaOCl 2 minutes; AP3= Immersion with 5% NaOCl 3 minutes; AP4= Immersion with 5% NaOCl 4 minutes; AP5= Immersion with 5% NaOCl 5 minutes; BP1= Immersion with 10% NaOCl 1 minute; BP2= Immersion with 10% NaOCl 2 minutes; BP3= Immersion with 10% NaOCl 3 minutes; BP4=Soaking with 10% NaOCl 4 minutes; BP5= Immersion with 10% NaOCl 1 minute].

The source of contamination in explants explains the type of contaminant source that causes contamination of explants. In this study, observations regarding the source of contamination in explants were limited to microorganisms, such as fungi and bacteria. As for the results of observations on the source of contamination in explants, it can be seen that the main source of contamination is derived from fungi. Contamination by fungi in the above results occurred on the surface of the leaf explants. The contaminants in the form of fungi found on the surface of the explants were because the leaves are the plant organs that have the most direct contact with the environment, so fungal spores are carried by the wind and may stick a lot to the leaf surface [17]. The dominance of contaminant fungi that grow on the surface can inhibit the growth of endophytic fungi in the leaves so that endophytic fungi are still able to grow, but their growth is inhibited or stopped and even cannot grow at all on the isolation medium. In general, if the source of contamination is still found on the surface of the explant, then this can be an indication of the ineffectiveness of the surface sterilization method being carried out.

Based on the results of observations of the source of contamination in the explants, it was also known that there was no source of contamination in the explants from bacteria. This

has occurred because the media was added with antibacterial (200 mg/L of chloramphenicol). The addition of antibacterial to endophytic fungi isolation media can help suppress bacterial growth [18] so that the isolation media will only be able to grow fungal colonies according to the purpose of isolation. Chloramphenicol is a broad-spectrum antibacterial that can inhibit the growth of bacteria from both gram-positive and gram-negative groups. Chloramphenicol is a type of antibacterial that works by inhibiting protein synthesis on bacterial ribosomes [24].

Table 2. Source of contamination and the first time of contamination in explants

Treatment Code	Source of Contamination	First Time Contamination	Picture
AP1	Mold	3 DAP	
AP2	Mold	4 DAP	
AP3	Mold	5 DAP	
AP4	Mold	5 DAP	
BP1	Mold	4 DAP	
BP2	Mold	4 DAP	

*DAP (*Days After Planting*)

The time of contamination in explants showed the average time of the first occurrence of contamination in each treatment expressed in DAP (Days After Planting). Meanwhile, based on the results of observations of the time of occurrence of contamination in explants, it was found that the surface sterilization treatment of mango's mistletoe leaves using 5% NaOCl with an immersing time of 3 to 4 minutes had the longest time value of contamination occurrence (5 DAP) when compared to other treatments. As for the results of the observation of the time of the occurrence of contamination in the sterilization treatment using 5% NaOCl with an immersing time of 5 minutes and sterilization treatment using 10% NaOCl with an immersing time of 3 minutes, 4 minutes, and 5 minutes, it did not have a source of contamination and did not have a time value of the occurrence of contamination. This is because in this treatment, there was no contamination from the first day to the last day of observation.

The incidence of contamination during isolation activities can be influenced by several factors such as the surface of the explant that is still not perfectly sterile, microorganisms that enter the media during the isolation process, microorganisms that stick to the equipment and have not died during the sterilization process in the environment in the LAF, the environment around the LAF, as well as the carelessness of researchers [25]. The level of contamination was influenced by several factors, including; working environment conditions, equipment used during the isolation process, media used, and the origin of taking explants. In general, explants that are taken from the field have a much higher risk of contamination than those from laboratory or greenhouse collections [26]. For this reason, the use of sterilants such as NaOCl and alcohol at a certain concentration and duration of immersion is needed to support the success of surface sterilization of explants of Mango's mistletoe leaves.

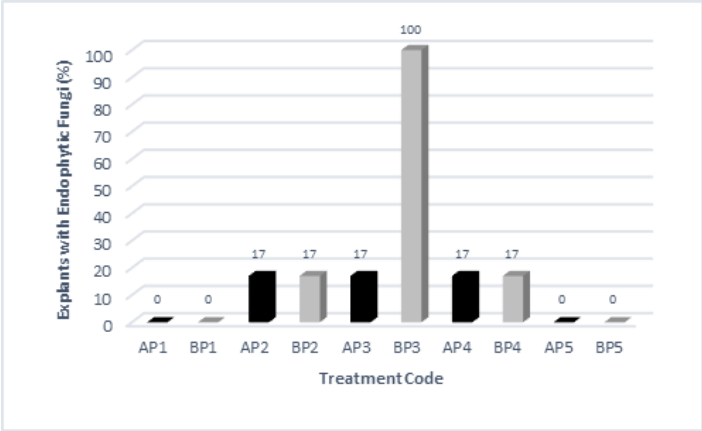


Figure 3. Diagram of percentage contamination of explants. [AP1= Immersion with 5% NaOCl 1 minute; AP2= Immersion with 5% NaOCl 2 minutes; AP3= Immersion with 5% NaOCl 3 minutes; AP4= Immersion with 5% NaOCl 4 minutes; AP5= Immersion with 5% NaOCl 5 minutes; BP1= Immersion with 10% NaOCl 1 minute; BP2= Immersion with 10% NaOCl 2 minutes; BP3= Immersion with 10% NaOCl 3 minutes; BP4=Soaking with 10% NaOCl 4 minutes; BP5= Immersion with 10% NaOCl 1 minute]

The combination of the use of alcohol-NaOCl-alcohol has been proven to be effective in killing microorganisms found on the surface [20]. Sodium hypochlorite (NaOCl) is a sterilizing agent that is commonly used for surface sterilization of planting material. This is because the material has an effective ability to affect the activity of microorganisms so that microorganisms can die. In addition, these materials are also relatively safe for use by humans and plant tissues, easy to obtain, and have a selling price that tends to be cheaper [4, 21]. Based on the results of research which showed that the use of a combination of 70% alcohol and 5% NaOCl with a 10-minute immersion was able to suppress the incidence of contamination on the surface of breadfruit (*Artocarpus altilis*) leaves, as evidenced by the percentage value of explant contamination 33% lower than the other treatments, and the time of contamination incident was 28 DAP which was longer when compared to other treatments [26]. The time of the occurrence of contamination will generally take place from a few days after planting until one month after planting [17]. Furthermore, another study showed that the use of a combination of 70% alcohol and 0.5% NaOCl with an immersion time of 5 minutes was able to reduce the contamination rate on the surface of Andalas (*Morus macroura* Miq.) leaves [27].

Percentage of Explants with Endophytic Fungi

The percentage of explants covered with endophytic fungi showed the number of explants of Mango's mistletoe leaves that could be grown with endophytic fungi in each treatment expressed as a percent (%). The indicator to determine an explant is overgrown with endophytic fungi is to look at the growth point of the fungus. If the fungus grows from the part that was injured during the isolation process, it means that the fungus that grows on the explant is endophytic.

Based on the diagram of the percentage of explants covered with endophytic fungi presented in Figure 3, it can be seen that the surface sterilization treatment using 10% NaOCl with a 3-minute immersion was the best result because it had the highest percentage rate of explants with endophytic fungi growing at 100% when compared to another treatment.

What must be a concern in seeing the best results is to pay attention to the results of observations on the three main variables, which include; The first was to find out which treatment had the highest percentage of live explants, the second was to find out which treatment had the lowest percentage of explant contamination, and the third was to find out which treatment had the highest percentage of explants overgrown with endophytic fungi. Because even though the percentage of live explants is high, if the growth is contaminant fungi, the explant sterilization method is considered not optimal or not effective. Thus, according to the description of the results above, it is known that 10% NaOCl concentration with a long soaking time of 3 minutes is the best result when compared to other treatments because at the concentration and soaking time, the percentage of live explants is high (100%), low contamination explants (0%), and the high percentage of explants with endophytic fungi (100%). The percentage of contamination is 0%, which means that there is no contamination in the treatment and the source of contamination and the time of contamination cannot be known.

CONCLUSION

The results of surface sterilization of mango's mistletoe leave using NaOCl with a concentration of 10% and soaking time for 3 minutes is the best and recommended because it has a 100% live explant percentage value, 0% explant contamination percentage, and the percentage of explants covered with endophytic fungi was 100%.

ACKNOWLEDGEMENT

Thanks to The Deputy for Strengthening Research and Development of the Ministry of Education, Culture, Research and Technology Republic of Indonesia, Single Research Contract for the Fiscal Year 2021 Number: 567/G164/U.LPPM/K/B.07/IX/2021, who has supported grant funding for research Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) with the title "Exploration and Domestication of Tea and Mango Mistletoe as Raw Materials for Antihypertensive Herbal Medicines".

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