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Research Article

The inhibition of bacterial metalloenzymes and fungal protein synthesis on explants surfaces by sterilizing agents

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ABSTRACT

Surface sterilization is a very important key for reaching a successful explants introduction to the in vitro environment. However, to get the best protocol of explants surface sterilization is not easy. Each explants has its characteristics and the contaminants (which usually are bacteria and fungus) are vary depending on its source. In order to know the best sterilization protocol of Phaleria macrocarpa leaves, some sterilizing agents were tested. This experiment suggests that ethylene bis dithiocarbamate may eradicate fungal infection by metalloenzymes inhibition, which is showed by 91.7% survivals. In the other hand, streptomycin sulfate may eliminate bacterial infection by inhibition of protein synthesis, which is showed by 95.6% to 98.3% survivals depending on the exposure time.

Keywords: ethylene bis dithiocarbamate, metalloenzymes, streptomycin sulfate, protein synthesis, sterilization

INTRODUCTION

Phaleria macrocarpa has been known as a multi-benefit plants in medical sector (Harmanto, 2003). The scientists have explored all of this plant's parts and the results showed that every part of this plant represents an essential resource. In light of this, there is a need to multiply this plant to cover the large demand in medical sector and also for hindering this plant from extinction.

Plant tissue culture technique is the most effective way to multiply plants in a short time. There are a lot of methods that can be used in the plant tissue culture technique depending on the aim of propagation. For the purpose to multiply a plant, scientist may use direct shoot organogenesis method. This method can be reached by using various explants source. However the leaves explants are rarely used. The reason is that to grow an adventives shoot from a leaf is really difficult.

Surface sterilization is a key to obtain sterile explants. Surface sterilization of plants leaves is not easy because the contaminants are many. The contaminants can be from bacteria or fungus. They can disrupt the viability of explants in in vitro environment. Some antibacterial and antifungal substances that often used are NaOCl, Alcohol, Bavistin, Dithane M-45, Agrept 20WP, 8-HQC-8-hydroxy quinoline citrate, HgCl2, H2O2 etc. This experiment intends to acquire the best protocol for Phaleria macrocarpa leaves sterilization.
MATERIALS AND METHODS

Sample collection

Leaves explants were taken from a vigorous *Phaleria microcarpa* tree which was aged between 2-3 years old. The young green leaves (1, 2 or 3 from the branches' apex) were collected in a beaker glass containing water. Those leaves were then transported to the laboratory for sterilization process.

Explants sterilization

First, the leaves were rinsed under running water. After that, a series of sterilization methods were done inside the Laminar Air Flow (LAF), including dipping the leaves in a bactericide solution (Agrept 20WP), fungicide solution (Dithane-45), 70% EtOH, and or NaOCl (1%, 1.3%, 2.6%) with various time exposures. The explants were rinsed using sterile distilled water after their dipping inside the sterilizing agents solution. The leaves were then left to dry in a Petri dish by using a normal wind and temperature in the LAF.

Media preparation and sterilization

Murashige and Skoog (1962) medium were used to provide nutrition for explants. Sucrose was used as the sole source of carbon. The pH was adjusted at 5.8 while HCl or NaOH were used to reach that point. Agar was used to solidify the medium. The solution was then boiled and dispensed to the culture bottles. Each bottle was covered with aluminum foil and then prepared for autoclaving. The autoclave was set at 121°C and 1.5atm and the sterilization process was lasted for 20 minutes.

Equipments sterilization

All of glassware's (Petri dishes, beaker glasses, Erlenmeyer flasks, etc.) and other equipment such as dissecting set were well covered with aluminum foil. Those equipments were then autoclaved at 121°C and 1.5atm for 20 minutes to 1 hour. After the sterilization process, they were transferred to an oven which was set at 60°C for drying.

Explants implantation

The sterile leaves explants were cut into small squares ± (0.7 x 0.7) cm² after the vascular vessels and the edges were removed. They were then planted onto the culture bottles containing sterile MS medium and then incubated for 4 weeks. The explants positions followed the normal condition where the adaxial part was facing the bottles cover and the abaxial part was attached to the medium. The data were collected at 4th week.

Incubator condition

The incubator was set at 23-25°C with 80-90% humidity. Light intensity was set at 10,000-25,000 lux by using white fluorescent lamps. The explants were incubated under 24 hours illumination.

RESULTS

Explants sterilization is an important key in plant tissue culture technique. The sterile explants are very expected in this process. By attempting the production of sterile explants, the probability to obtain a success work of plant tissue culture are increasing. This table shows the sterile explants obtained after series of sterilization procedures.
**Table 1. Effect of sterilization on survival of culture**

<table>
<thead>
<tr>
<th>No.</th>
<th>Sterilizing agents*</th>
<th>Exposure Time</th>
<th>No. of explants</th>
<th>Infected explants</th>
<th>Died explants</th>
<th>Healthy explants</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>a. 70% EtOH</td>
<td>1 minute</td>
<td>200</td>
<td>81</td>
<td>-</td>
<td>59.5%</td>
</tr>
<tr>
<td></td>
<td>b. 1% NaOCl + 2 drops of Tween-20</td>
<td>15 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>a. 2.3% NaOCl</td>
<td>10 minutes</td>
<td>39</td>
<td>7</td>
<td>3</td>
<td>74.4%</td>
</tr>
<tr>
<td></td>
<td>b. 1.3% NaOCl</td>
<td>20 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>a. Dithane M-45 (6g/L)</td>
<td>24 hours</td>
<td>36</td>
<td>3</td>
<td>-</td>
<td>91.7%</td>
</tr>
<tr>
<td></td>
<td>b. 2.6% NaOCl</td>
<td>10 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c. 1.3% NaOCl</td>
<td>20 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>a. Dithane M-45 (6g/L) + Agrept 20WP (2g/L)</td>
<td>19 hours</td>
<td>105</td>
<td>31</td>
<td>-</td>
<td>70.5%</td>
</tr>
<tr>
<td></td>
<td>b. 2.6% NaOCl</td>
<td>10 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c. 1.3% NaOCl</td>
<td>20 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T5</td>
<td>a. Dithane M-45 (6g/L) + Agrept 20WP (2g/L)</td>
<td>24 hours</td>
<td>120</td>
<td>44</td>
<td>1</td>
<td>62.5%</td>
</tr>
<tr>
<td></td>
<td>b. 2.6% NaOCl</td>
<td>10 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c. 1.3% NaOCl</td>
<td>20 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T6</td>
<td>a. Agrept 20WP (2g/L)</td>
<td>2 hours</td>
<td>48</td>
<td>2</td>
<td>-</td>
<td>95.8%</td>
</tr>
<tr>
<td></td>
<td>b. 2.6% NaOCl</td>
<td>10 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c. 1.3% NaOCl</td>
<td>20 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T7</td>
<td>a. Agrept 20WP (2g/L)</td>
<td>16 hours</td>
<td>120</td>
<td>3</td>
<td>-</td>
<td>97.5%</td>
</tr>
<tr>
<td></td>
<td>b. 2.6% NaOCl</td>
<td>10 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c. 1.3% NaOCl</td>
<td>20 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T8</td>
<td>a. Agrept 20WP (2g/L)</td>
<td>26 hours</td>
<td>176</td>
<td>2</td>
<td>1</td>
<td>98.3%</td>
</tr>
<tr>
<td></td>
<td>b. 2.6% NaOCl</td>
<td>10 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c. 1.3% NaOCl</td>
<td>20 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Sterilization process was started from 'a' solution to 'b' and so on, respectively.

**Figure 1.** Explants condition after 7 days of incubation. A, Healthy explants; B, Infected explants

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DISCUSSION

Table 1 shows that treatment number 7 and 8 (T7 & T8) are the best among others with the survival 97.5% and 98.3% respectively. However, T7 is favorable because the exposure time in the Agrept 20WP is shorter (16 hours) and also no sign of died explants which means that the concentration is not strong enough to kill the tissues. Furthermore, if T6, T7 and T8 are compared, it can be concluded that the dipping of explants in Agrept 20WP for 16 hours generates the best result among others. In the other hand, treatment number 3 (T3) with the exposure in Dithane M-45 solution for 24 hours showed 91.7% healthy explants. Even though Dithane M-45 inhibition is not as good as Agrept 20WP, this sterilizing agent is still favorable because the healthy explants obtained are high.

Dithane M-45 contains 80% ethylene bisdithiocarbamate. The inhibition mechanism of this active substance is by the inhibition of metalloenzyme and sulhydryl enzyme in fungus and bacteria (Miller, 1982). Whereas Agrept 20WP contains 20% streptomycin sulfate. Streptomycin sulfate is an antibiotic extracted from Streptomyces griseus, a bacterium. This antibiotic works by binding to 30S subunit so that it can inhibit bacterial protein synthesis and results in the death of bacteria. This substance is very active to inhibit Gram-negative bacteria but also can inhibit some Gram-positive bacteria (www.lifetechnologies.com).

The previous study showed that the combination of Agrept 20WP and Dithane M-45 solution was effective to kill fungus and bacteria. In that experiment, the researcher mentioned that the dipping of leaves explants in the combination solution for 24 hours followed by the soaking of them in the 2.6% NaOCl (10 minutes) and 1.3% NaOCl for 20 minutes respectively gave the best result for obtaining healthy explants of Phaleria macrocarpa (Hananto, 2010). This experiment showed the opposite result, Dithane M-45 or Agrept 20WP alone gave a good result, while their combination leads to more infected explants. From this data, this experiment suggests that the combination of Dithane M-45 and Agrept 20WP is not recommended for leaves explants sterilization of Phaleria macrocarpa. Alternatively, those solutions may be used respectively.

In many experiments, surface sterilization by using 70% EtOH for 30 seconds to 1 minute followed by the soaking in the 0.3%-1.5% NaOCl with 2 drops of Tween-20 for 5-15 minutes is almost always used for sterilization procedures. For example, to get a sterile Valerina officinalis leaves explants, Abd i and Kho Kris (2007) dipped them in the 1.5% NaOCl for 10 minutes. Hedayat et al (2009) used 70% EtOH (1 minutes) followed with the dipping in the 1.5% NaOCl (20 minutes) for Tanacetum cinerariifolium leaves sterilization. Meanwhile, for Pulsatilla korandula leaves sterilization, Lin et al (2011) applied 70% EtOH for 10 seconds followed by 2% NaOCl with 2 drops of Tween-20 for 15 minutes. In this experiment, the application of EtOH 70% for 1 minute followed by the soaking in the 1% NaOCl with 2 drops of Tween-20 for 15 minutes (Treatment 1), unfortunately did not give a good result on the attempt to get a sterile leaves explants of Phaleria macrocarpa. This maybe because of the short time exposure or the low concentration of NaOCl.

Although NaOCl alone did not give a good result for contaminants elimination, the application of NaOCl in this experiment is also important. Treatment 3, 7 and 8 are successfully obtained high percentage of healthy explants probably because followed by the application of NaOCl. NaOCl is widely used as sterilizing agents and usually obtained from commercial bleach. This substance is effective to kill bacteria, but the inhibition mechanism has not well understood (Oyebanji, 2009).

CONCLUSION
The use of Agrept 20WP followed by 2.6% and 13% NaOCl (respectively) gives a successful surface sterilization for explants leaves of *Phaleria macrocarpa* showed by 95.8% to 98.3% healthy explants depending on the exposure time. Dithane M-45, although not as good as Agrept 20WP can also be an alternative if applied with the same procedure showed by 91.7% survivals.

REFERENCES


Streptomycin sulfate.