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Sterilization of Anubias nana and Rotala macrandra for *In Vitro* Culture

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ABSTRACT

This study was conducted to establish a sterilization protocol for *Anubias nana* and *Rotala macrandra.* The plants from its originated area are highly exposed to microbial contamination such as bacteria and fungi. Those contaminants can be eliminated by using suitable sterilizing agents. The sterilization is a vital step in preparing a healthy and viable explant in tissue culture. In this study, three different sterilizing agents which are fungicide, silver nitrate ($AgNO_3$) and mercuric chloride ($HgCl_2$) were used. The percentages of survived and contaminated explants were observed after one week of culture. The results showed that 100% of *Anubias nana* explant survived when the explants were rapidly rinsed with 0.1% ($HgCl_2$). The explant of *Rotala macrandra* resulted in 56% of survival when it was rapid rinsed with 0.05% $HgCl_2$. However, treatment 0.05% $HgCl_2$ for *Rotala* macrandra also produced 33% of fungal contamination.

Keywords: *Rotala macrandra*, *Anubias nana*, sterilization, fungicide, silver nitrate, mercuric chloride

INTRODUCTION

Micropropagation is an excellent alternative method to produce a large scale of plants. Certain aquatic plants are responsive to *in vitro* propagation by proliferation from pre-existing buds or through adventitious shoot formation (Kane & Albert, 1989). *Anubias nana* belongs to Araceae family while *Rotala macrandra* belongs to Lythraceae family. *Anubias nana* and *Rotala macrandra* are ornamental aquatic plant which have a bright potential in aquarium trade. Aquarists demand for ornamental aquatic plant like *Anubias* sp. as well as *Rotala* sp. to embellish their aquarium. Aquarist would appreciate a quality of water plant that show bushy growth with more adventitious root (Christensen, 1996).Thus, the collection of those aquatic plant may lead to extinction. As

demand so high, most of the aquarium plants need to be imported. Problems such as short of supply, over collection, variable plant quality and frequent losses due to disease and destruction of endangered species can be solved by *in vitro* propagation of aquatic plants (Sarasan et al., 2006). Generally, micropropagation protocols of aquatic plants have lagged behind compared to land plants (Ea et al., 2015). The *Anubias* nana plant can be propagated vegetatively using rhizome. However, propagation of matured rhizome to form a shoot is inefficient as it occur at very slow rate compared to other organs.Therefore, to meet the demands of aquarium industry, rapid and efficient *in vitro* micropropagation were developed from the explants of *Anubias* sp. and *Rotala* sp.

In vitro propagation technique involves several steps which are selection of explant, sterilization and establishment and shoot proliferation, shoot regeneration and root regeneration of the explant. In this study, the sterilization protocol for *Anubias nana* and *Rotala macrandra* was established. This protocol can be used by researcher who obtain or collect the plant from the same source or similar environment for micropropagation purposes. The success of the culture depends on its aseptic condition. All materials, media and instruments used as well as explant must be sterilized (Badoni & Chauhan, 2010).- Three (3) factors have to be taken into account in order to optimize protocol for sterilization which are the type of sterilizing agents, its concentration and the time of exposure (Alam et al., 2016). In this study, *Anubias nana* and *Rotala macrandra* the sterilization protocol were demonstrated using fungicide (Thiram), silver nitrate ($AgNO_3$) and mercuric chloride ($HgCl_2$) for developing efficient sterilizing protocol with lower rate contamination and higher plant survival rate.

MATERIALS AND METHODS

Explant sources of *Anubias nana* and *Rotala macrandra*

The plants of *Anubias nana* and *Rotala macrandra* were obtained from Glami Lemi Fisheries Research Institute (FRI). The aquatic plants were grown under environmentally controlled fresh water aquariums at 28 ± 2 °C with sufficient light to allow the plants to acclimatize in new condition. Disease-free, young, healthy Anubias *nana* rhizome and *Rotala macrandra* stem internodes explants were selected for cultures.

In laminar air flow, rhizome explants were excised to remove undesirable portion after surface sterilization. The explants were cultured in vials on MS medium supplemented with 3 mg/L BAP. Each vial was labelled and covered with parafilm. All cultures were incubated under 16h light photoperiod using light emitting diode (LED) lights. The rate of survivability and contamination were observed after one week. For surface sterilization study, the rate of survivability and contamination of *Anubias nana* and *Rotala macrandra* were measured and the experiment were conducted in completely randomized design (CRD) using three replicates for each treatment. All parameters were analysed using One Way Analysis of Variance (ANOVA). The group means were analysed at significant level of $p < 0.05$. Statistical analysis were carried out using SPSS programme.

Media preparation

The macroelements, microelements, iron source, and vitamin of Murashige and Skoog basal medium were prepared in stock solution. The media was added with 30 g/L sucrose and 3 mg/L BAP. The pH was adjusted from 5.7 to 5.8 using sodium hydroxide (NaOH) or hydrochloric acid (HCl) before adding 0.25 % Phytagel. The media was autoclaved at 121 °C, 105 kPa for 15 minutes.

Effects of treatment with various concentration on Thiram of *Anubias nana* and *Rotala macrandra*

The explants of rhizome were washed under running tap water for 10 minutes to remove the dirt and other contaminants from the surface. The explants were then exposed to various concentration of sterilizing agent, Thiram in the range of 0 % (w/v) up to 2 % (w/v). Rhizome explants were washed under running tap water to remove the residue of Thiram on the explant. In laminar air flow, the explants were soaked in 15 % Clorox with two drops of Tween 20 for 15 minutes and washed thoroughly with sterile distilled water. The rhizome explants were excised and cultured on MS media supplemented with 3 mg/L BAP.

Effect of rapid rinse of various concentration of $HgCl_2$ on *Anubias nana* and *Rotala macrandra*

The explants of rhizome were washed under running tap water for 10 minutes to remove the dirt and other contaminants from the surface. The explants were then exposed to 2 % of Thiram. The explants were washed under running tap water. In laminar air flow, the explants were rinsed in 15 % Clorox with 2 drops of Tween 20 for 15 minutes followed by the treatment of various concentration of $HgCl_2$. (0.01% (w/v), 0.02%(w/v), 0.05% (w/v) and 0.1% (w/v)).The explants were excised and cultured on MS media supplemented with 3 mg/L BAP.

Effect of treatment with different exposure time of 0.1 %(w/v) $HgCl_2$ on *Anubias nana*

The explants of rhizome were washed under running tap water for 10 minutes to remove the dirt and other contaminants from the surface. The explants were then exposed to 2 %(w/v) Thiram. The rhizome explants were soaked in 5 %(v/v) Teepol for 10 minutes and washed thoroughly under running tap water to remove dirt. In laminar air flow, the explants were treated with 0.1 %(w/v) $HgCl_2$ with different exposure time followed by quick dip in 70 % (v/v)ethanol. The explants were washed thrice subjected to 70 %(v/v) ethanol. The rhizome explants were excised and cultured on MS media supplemented with 3 mg/L BAP.

RESULTS AND DISCUSSION

Effects of treatment with various concentrations of Thiram on *Anubias nana* and *Rotala macrandra* after one week.

The sterilization process was carried out in a series of step as the sample of both aquatic plants were highly contaminated with fungus and bacteria. The series of surface sterilization can reduce contamination (Ghoreyshi et al., 2010) and increase the number of aseptic culture as well. Fungicide also used as a surface sterilizing agent (Sen et al., 2013). Thiram react by inhibiting the pyruvic dehydrogenase system infungal cell levelwhere it hinders uptake of glucose and oxygen and as a result forming carbon dioxide by the fungal spores (Dias 2012).

Here the sterilization were carried out using Thiram sterilizing agent at different concentration. Figure 1 shows the percentage of contamination and survivability obtained from explants treated with various concentration of Thiram. There was no significant difference in bacterial and fungal contamination and survival rate as well due to high contamination by the explant The concentration of Thiram at 2%(w/v) showed the lowest bacterial contamination compared to other Thiram concentration. The high rate of contamination could be favoured to resistance or tolerance of microbes that present on the explant surface from its originated area especially in damp area. This finding is no significant to Alam et al., (2016) observation reported that the best sterilization was using 3 % Bavistin produced less contamination with 57.77% of explants of *Cucumis sativus* survived.. The difference of sterilizing agent effectiveness depends on a number of factors such as the nature of plants where plants exposed to soils or water excess and grown in tropical climate are more difficult to sterilize. (Knauss & Miller 1978; Duhem et al. 1988; Enjalric et al. 1988; De Fossard 1990; Leifert 1990).

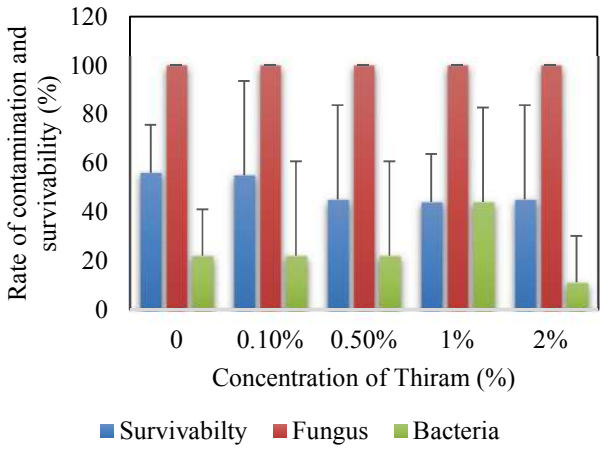

Fig. 1. The bar graph shown effects of treatment with various concentration of Thiram of *Anubias nana* after one week.

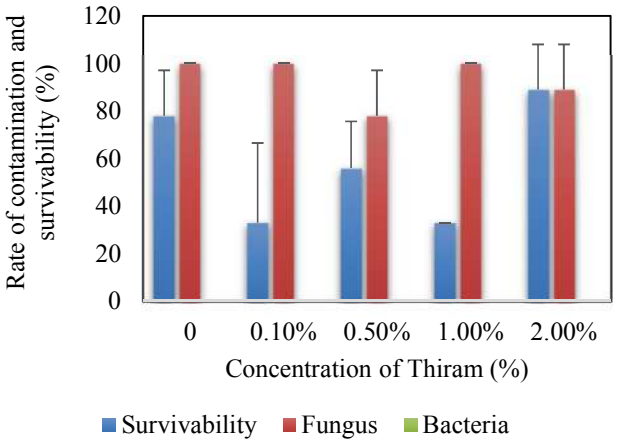

Fig. 2: The bar graph shown effects of treatment with various concentration of Thiram on *Rotala macrandra* after one week.

Treatment of Thiram at 2%(w/v) on *Rotala macrandra* stem internodes for 30 minutes showed maximum survival (89 %) of the explant and 89% were fungal contaminated. The lowest contamination was from treatment of 0.5%(w/v) of Thiram. Previously, Alam et al., (2016) reported that treatment of 1 %(w/v) Bavistin for *Cucumis sativus* explant showed contamination while treatment of 3 %(w/v) Bavistin for 12 minutes gave healthy explant. For *Rotala macrandra* sterilization process, treatment of Thiram was limited up to 2 % due to the stem tissue of *Rotala macrandra* were fragile We observed that the stem internode explant of *Rotala macrandra* produced browning after it was rinsed with Thiram fungicide. The fragility of *Rotala macrandra* compared to *Anubias nana* considered as one of the factor which cause the explant to turn brown when rinsed in Thiram. The browning might be due to damage of explants during the excision (Mahmoud & Al-Ani, 2016). Mahmoud and Al-Ani (2016) stated that, the more tender the tissue of an explant, the higher the damage to the explant when exposed to sterilizing agent.

Effect of rapid rinse of various concentration of $HgCl_2$ on *Anubias nana* and *Rotala macrandra*

We observed that no *Anubias nana* explants survived when rapid rinsed with $HgCl_2$ in the range of 0.01%(w/v) up to 0.1%(w/v) as shown in Figure. 3 as shown below. Solution of $HgCl_2$ is extremely toxic to plants and humans as well (Mahmoud & Al-Ani, 2016). This result showed deleterious effect of $HgCl_2$ at high concentration is in agreement with other researcher, Wesely et al., (2011) who works on *Alternanthera sessilis* using shoot tip and nodal segments. They obtained a rate of one hundred percent of contamination-free explants with high percentage of explants mortality when treated the explants with 0.1 %(w/v) $HgCl_2$ for 4 minutes and above and 0.15 %(w/v) for 3.5 minutes and above. Besides, the death of the explant might be due to ineffective surface sterilization such as the scalpel were over-flamed which damaged the explant tissue and also chemical traces such as Thiram and $HgCl_2$ were not fully removed. $HgCl_2$ traces need to be removed from the plant material by doing many washes (Mahmoud & Al-Ani, 2016) in order to get healthy decontaminated explant.

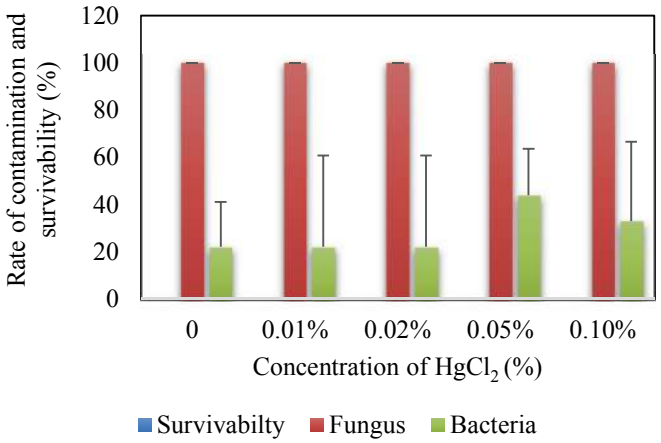

Fig. 3: The bar graph shown effect of rapid rinse of various concentration of $HgCl_2$ on *Anubias nana* after one week.

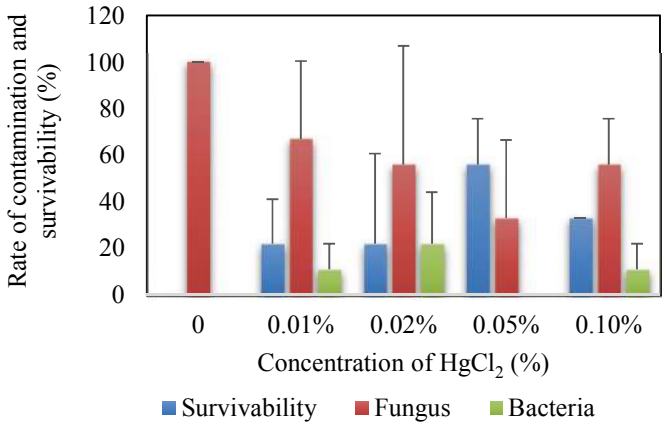

Fig. 4: The bar graph shown effects of rapid rinse of treatment with various concentration of $HgCl_2$ on *Rotala macrandra* after one week.

By removing $HgCl_2$ traces, healthy decontaminated explants can be produce due to no chemical exposed to the explant tissue. Based on Figure 4, bacterial and fungal contamination of the *Rotala macrandra* explants were

reduced compared to treatment with various concentration of Thiram. Since $HgCl_2$ would produce 2 Cl- and Hg^+, this response might be due to the bleaching action of two chloride atoms and also ions that strongly combines with proteins which cause death of the microorganism such as fungus (Alam et al., 2016). The stem internode explant showed blackening due to toxicity of the $HgCl_2$ where it results with the explants death after one week of culture. Here excessive treatment of $HgCl_2$ leads to blackening of the plant tissue on *Rotala macrandra* (Srivastra & Rajani, 2004).

Effect of treatment with different exposure time of 0.1 % $HgCl_2$ on *Anubias nana*

From the previous experiment, it was found that no *Anubias nana* explants was found to be survived when rinsed with various range of $HgCl_2$ concentrations. Therefore, the experiments were continued only using *Anubias nana* explants soaked in $HgCl_2$. Here we rinsed *Anubias nana* explants with the lowest concentration from the previous experiment but we vary the rinsing time. In this study, we obtained *Anubias nana* explants survival when it was rapid rinsed with 0.1% of $HgCl_2$ as shown in Figure.5. This result contrast to Ea et al., (2015) observation where shoot tip of *Anubias nana* were used instead and treated with 0.1% (w/v) $HgCl_2$ ranging up to 4 minutes rinse.

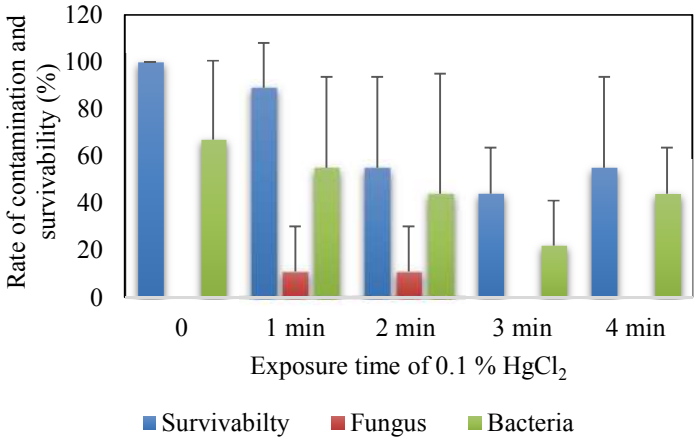

Fig. 5: The bar graph shown effect of treatment with different exposure time of 0.1% $HgCl_2$ on *Anubias nana* after one week.

Ea et al., (2015) reported that none of the shoot tip explant survived when treated with 0.1%(w/v) $HgCl_2$ for more than 1 minute. However, in this study *Anubias nana* explants shows a survival rate above 20% when rinsed with 0.1%(w/v) $HgCl_2$ more than 2 minutes with fungal contamination reduced to 0%. Present result also support the combination of 0.1%(w/v) $HgCl_2$ with 70% Ethanol as advocated by Yadav et al., (2017). Alcohols are rapidly bactericidal rather than bacteriostatic against vegetative form of bacteria (Sen et al., (2013).

Effects of treatment with various concentration of $AgNO_3$ on *Rotala macrandra*

From previous study on effect of $HgCl_2$ on *Rotala macrandra,* the treatment of $HgCl_2$ showed low survivability due to the toxicity of the $HgCl_2$. Here $AgNO_3$ was used to develop less damaging procedure due to $AgNO_3$ less stabilizing agent compared to $HgCl_2$. Here $AgNO_3$ to 0.1% reduced bacterial contamination on *Rotala macrandra* explant. This finding contrast to observation by Ambros et al., (2016) as different procedures using two sterilizing agents which are $HgCl_2$ and $AgNO_3$ on *Rosa canina*. The research team compared the effect of $AgNO_3$ which contrast to $HgCl_2$. The bud treated with 0.1% $HgCl_2$ gave 87% contamination-free while 98% of explant decontaminated when treated with 0.1% $AgNO_3$. In our study, $AgNO_3$ at concentration of 0.10% (w/v) showed no bacterial infections on *Rotala macrandra* explants. This is because that silver nitrate posses the ability to

interact with thiols group where the compound would react in neutralizing the silver activity. Neutralization plays a very important role where the release of its silver from $AgNO_3$ demonstrate its antibacterial experiments (Furr et al., 1994).

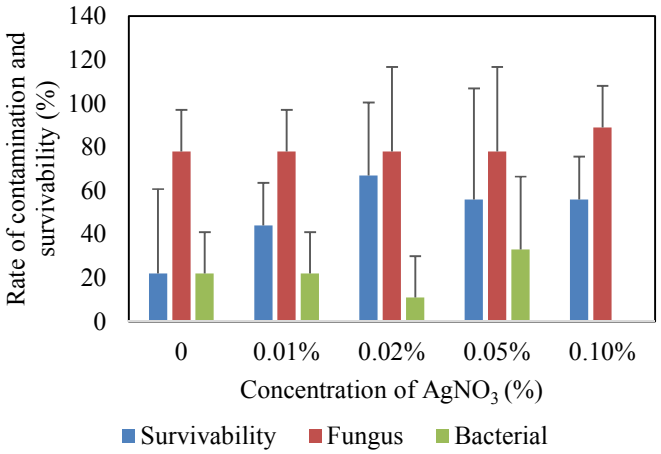

Fig. 6: The bar graph shown effects of treatment with various concentration of $AgNO_3$ on *Rotala macrandra* after one week.

CONCLUSION

The optimized in sterilizing protocol with lower rate contamination,sterilization have been developed for *Anubias nana* and *Rotala macrandra*. In sterilization of *Anubias nana* explant, 55% of survivability rate was achieved by 3 minute rinsed with the 0.1% $HgCl_2$. In this treatment, it was observed that the bacterial contamination rate was the lowest in comparison to other treatments (22%) with no fungal contamination. In sterilization of *Rotala macrandra,* treatments with Thiram, $HgCl_2$ and $AgNO_3$ still produced high contamination of fungus and bacterial contamination. *Rotala macrandra* explants also showed blackening of tissues where it resulted in the explants death after one week of culture using three (3) different sterilizing agents.

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