*International Journal of Plant Developmental Biology* ©2012 Global Science Books

# Sterilization Procedure for *in Vitro* Culture of Leatherleaf Fern (*Rumohra adiantiformis*)

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## ABSTRACT

When explants are introduced to the *in vitro* environment, sterilization is a fundamental process allowing contamination to be eliminated. A new sterilization procedure for a novel *in vitro* propagation of leatherleaf fern (*Rumohra adiantiformis*) was successfully devised using rhizomes – which are typically heavily contaminated – as the donor explant. The most effective sterilization procedure for *in vitro* micropropagation involved pretreatment of rhizomes in 80% alcohol for 3 min, washing them under tap water for 3 h then immersing them in 0.05% mercuric chloride for 10 min, followed by 96% alcohol for 1 min. Finally explants were rinsed 6 times (5 min each rinse) using sterile distilled water. This combination of treatments reduced the percentage of contamination to 33% and stimulated the percentage of rhizome regeneration to 73% with 2.3 rhizomes regenerated/replication (= 5 *ex vitro* rhizomes). This *in vitro* sterilization procedure will have an effective impact on the establishment of explants *in vitro* and on the micropropagation of leatherleaf fern.

**Keywords:** contamination, mercury chloride, regeneration, rhizome

## INTRODUCTION

Medium sterilization constitutes one of the most fundamental steps of plant tissue culture. Without effective sterilization of plant material, especially that which is introduced from the *ex vitro* environment, then there is no successful *in vitro* culture. Moreover, in *in vitro* culture, underground explant sources such as rhizomes, corms, bulbs and tubers are commonly used and for which aseptic culture establishment via sterilization is the most important and challenging step (Srivastava *et al*. 2010). Sterilization is the process of making explants free of contamination before the establishment of cultures. Various sterilization agents are used to decontaminate the tissues with not any adverse effect on explants (Badoni and Chauhan 2010; Mwirigi *et al*. 2010).

The successful sterilization of explants derived from underground plant parts in ornamental plants were reported for *Chlorophytum borivilliens* (Sharan *et al*. 2010), *Gladiolus* spp. (Priyakumari and Sheela 2005; Roy *et al*. 2006; Emek and Erdağ 2007), *Lilium* (Khawar *et al*. 2005; Nesi *et al*. 2009; Pandey *et al*. 2009; Kanchanapoom *et al*. 2011), *Muscari azureum* (Uranbey *et al*. 2011). In *C. borivilliens*, successful explant sterilization was possible by using 0.1% Bavistin + 0.25% Chloramphenicol for 30 min and 1% sodium hypochlorite solution (NaOCl) for 10 min and then rinsing with sterile distilled water (SDW) (Sharan *et al*. 2010). Treatment involving 0.4% Mancozeb (30 min), 'Labolene' solution (30 min), tap water (5 min), 0.08% mercuric chloride ($HgCl_2$) (10 min) and rinsing with SDW 4-5 times was suitable for *Gladiolus grandiflorus* (Priyakumari and Sheela 2005). $HgCl_2$ at 0.1% (w/v) for 7 min and a rinse with double SDW four times was sufficient for *Gladiolus* 'Paciflica' (Roy *et al*. 2006) while tap water (24 h), 70% EtOH (17 min) and 4.5% NaOCl (20 min) and three rinses with SDW was necessary for *Gladiolus anatolicus* (Emek and Erdağ 2007). Running tap water (30 min), 60% NaOCl (10 min) and one rinse in SDW was successfully applied to *Lilium candidum* (Khawar *et al*. 2005); 1.25% NaOCl (15 min) and two rinses with SDW was

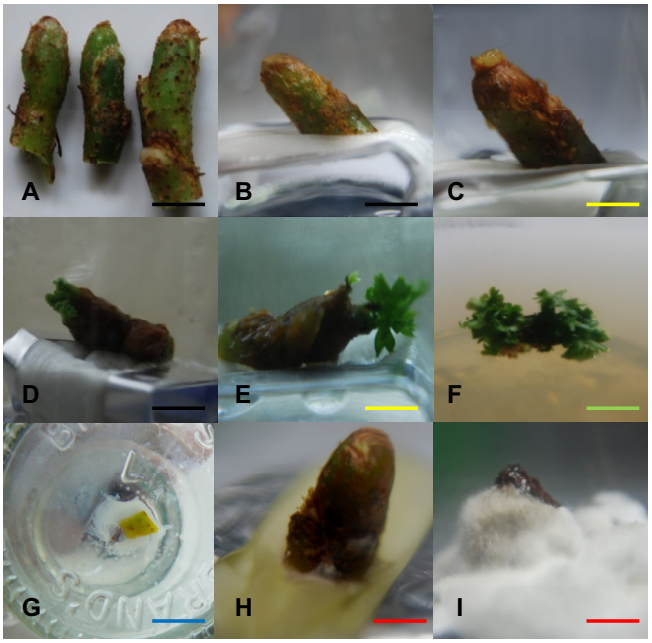


**Fig. 1 *In vitro* culture initiation of leather leaf fern.** (**A**) Rhizomes serve as donor explants; (**B**) Initial culture of rhizome on simple paper bridge containing select liquid medium (MS medium containing 0.25 mg/l 2,4-D, 0.2 mg/l NAA, 1.0 mg/l BA, and 0.5 mg/l TDZ with 30 g/l sucrose; IM-4); (**C**) Initial regenerated rhizome 10-15 days after culture in the dark; (**D**) Regenerated-rhizome one month after incubation in the light; (**E**) Regenerated rhizome 2 months after the first subculture (each subculture was 2 months) in semi-solid half-strength MS medium supplemented with 0.05 mg/l IAA, 0.25 mg/l BA, 0.5 mg/l Kin, 1 g/l activated charcoal, 20 g/l sucrose, and 1.8 g/l gelrite (according to Winarto and Teixeira da Silva 2012); (**F**) Regenerated and sliced tip rhizome 2 months after culture in the same selected medium; (**G**) Rhizome infected by bacteria; (**H**) Rhizome infected by bacteria as the first contaminant and fungi as the second contaminant; and (**I**) serious fungi contamination leading to the rhizome death. Black bars = 0.80 cm, yellow bar = 0.55 cm, green bar = 0.50 cm, blue bar = 0.95 cm, red bars = 0.45 cm.

Received: 26 February, 2012. Accepted: 15 March, 2012.

Original Research Paper

suitable for Asiatic hybrid lily (Nesi *et al*. 2009); tap water, 96% ethanol (2 min), 2.25% NaOCl + 0.1% Tween 80 (a wetting agent; 20 min) and four rinses in SDW for *Lilium candidum* (Altan *et al*. 2010); Tween-20 for 20 min, tap water for 30 min., 0.1% $HgCl_2$, 2% Bavistin and three rinses with SDW for *Lilium* sp. (Pandey *et al*. 2009); 70% ethanol for 1 min, 20% (v/v) NaOCl for 20 min, 10% (v/v) NaOCl for 10 min and three rinses with SDW for *Lilium longiflorum* 'Easter lily' (Kanchanapoom *et al*. 2011). For *M. azureum*, drying in the dark for 2 weeks, 3 min in 95% ethanol then in 100% NaOCl for 40 min and finally five rinses with sterile water was an effective procedure (Uranbey *et al*. 2011).

Leatherleaf fern (*Rumohra adiantiformis*) is considered to be the most popular cut foliage used in bouquets (Reid 2004). Ferns are conventionally propagated either sexually or asexually. In the latter route, or vegetative propagation, new plants can be produced from rhizomes, stolons, tubers, stipules, roots, buds, cuttings, and attached aerial stems (layering), as well as apospory and apogamy (Kottackal *et al*. 2006). Since frequent replanting is required through rhizome division for commercial propagation of *R. adiantiformis*, it takes time to produce the volume of fronds required at any particular time (Strandberg 2003). *In vitro* propagation is a viable solution for which a suitable protocol has been devised (Winarto and Teixeira da Silva 2012).

There are few publications on *in vitro* propagation protocols of fern via rhizomes as the donor explant. Successful *in vitro* propagation of ferns using this explant has only been reported so far in *Rumohra adiantiformis* (Chen and Read 1983) and *Matteuccia struthiopteris* (Zenkteler 2006). Chen and Read (1983) could successfully propagate *R adiantiformis in vitro* by successful explant sterilization. In their study, rhizome tips were first soaked in a 10% diluted commercial bleach (0.5% NaOCl) for 20 min, 95% ethanol for 5 min, 0.5% NaOCl for 20 min and three rinses with SDW for 5 min. For *M. struthiopteris*, excised long-creeping rhizomes were thoroughly washed with water and then soaked in a suspension of Clotrimazol for 24 h together with 2 mg/l kinetin (Kin), cut into 10-cm sections, starved of carbohydrates for 3 weeks in the dark, then disinfected with 0.1% $HgCl_2$ for 3 min and rinsed three times in SDW (Zenkteler 2006). Preliminary studies on Indonesian material and under Indonesian conditions, the use of NaOCl in combination with tap water, 96% alcohol and a rinse with SDW involved the application of a sterilization protocol derived from Chen and Read (1983), but had poor results in the aseptic establishment in *R. adiantiformis* rhizomes. In addition, the application of $HgCl_2$ has never been attempted. Thus, optimization of NaOCl concentration and the application of $HgCl_2$ in the establishment of an aseptic culture for *R. adiantiformis* rhizomes were addressed in this study. A suitable sterilization method as a precursor to a successful *in vitro* propagation protocol for *R. adiantiformis* was thus developed in this study.

## MATERIALS AND METHODS

### Plant material and explant preparation

*R. adiantiformis* mother stock plants (supplied by Tropika Flora Persada, Ltd., Sukoyoso village, Kajoran, Magelang District, Central Java Province, Indonesia) were repotted and grown in plastic bags (35 cm in diameter, 40 cm in height) with 38.5 $cm^3$ of potting medium (burned rice-husk, rice husk and bamboo peat; 1:1:1, v/v/v). Plants were placed in a glasshouse in the following conditions: 35-40°C during the day and 15-20°C at night; temperature assessed by a thermo-hygrometer (Haar-Synth-Hygro, Germany); 50-90% relative humidity during the day and 25-60% at night, assessed with a Haar-Shynth-Hygro; a 12-h photoperiod with 185-370 µmol/$m^2$/s light intensity during the dry season (April to October) and 37-111 µmol/$m^2$/s in the rainy season (November to March). Light intensity was measured using a Digital Lux Meter, Lutron LX 101 (Lutron Electronic Enterprise Co., Ltd., Taiwan). Measurement of data using the Lutron LX 101 was originally in lux but was then converted to µmol/$m^2$/s by multiplying each data point with a conversion factor for sunlight i.e. 0.0185 (Thimijan *et al*. 1982). The plants were watered with liquid fertilizer (1 g/l of N:P:K, 20:15:15; Nusa Tani, Ltd., Jakarta) at 3-day intervals.

New rhizomes about 2 cm in length were harvested from donor plants after repotting for 6-8 months (**Fig. 1A**). The rhizomes were cleared of remaining attached potting medium by running them under tap water for 30 min. Rhizome scales were gently removed by hand and after rinsing the explants several times using distilled water, they were ready for determining the ideal sterilization procedure used for *in vitro* culture (Winarto and Teixeira da Silva 2012).

### Effect of sterilization methods and initiation media on initiation culture of leather leaf fern

The sterilization methods applied in this experiment are summarized in **Table 1** while initiation media tested in the experiment are summarized in **Table 2**. Each sterilization procedure was carried out step by step involving all disinfectant components. The basic medium used in these tests was full-strength Murashige and Skoog (1962; MS). The combination of treatments between sterilization methods and initiation media is referred to as the first experiment.

### Improvement rhizome initiation

Culture conditions (CC) used in the experiment to improve rhizome initiation were prepared by culturing (CC-1) the sliced-shoot tip area of rhizomes on semi-solid initiation medium (MS medium containing 0.25 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), 0.2 mg/l α-naphthalene acetic acid (NAA), 1.0 mg/l $^6$N-benzyladenine (BA), and 0.5 mg/l thidiazuron (TDZ) with 30 g/l sucrose; IM-4), (CC-2) full rhizomes on semi-solid initiation medium (IM-4), (CC-3) sliced-shoot tip area of rhizomes on liquid initiation medium (IM-4), and (CC-4) full rhizomes on a simple paper bridge containing liquid IM-4 (**Fig. 1B**). The CC treatments are referred to as the second experiment.

Single and factorial experiments were arranged in a randomized complete block design (RCBD) with three replications each. Each treatment consisted of 5 bottles and each bottle contained one explant. A total of 350 *ex vitro* rhizomes harvested from mother stock plants were used in the initiation experiment, i.e., for all treatments in the study.

Parameters observed in the experiments were: (1) percentage contamination and total browning (%), (2) percentage rhizome regeneration and (3) number of regenerated rhizomes. Percentage rhizome regeneration and number of regenerated rhizomes were counted from the number of *ex vitro* rhizomes cultured from the point at which their tips broke and produced initial small fronds. Periodic observations were carried out to observe all alterations that might have occurred during incubation and all parameters were recorded 2 months after culture initiation.

### Data analysis

Quantitative data in all experiments were analyzed by two-way analysis of variance (ANOVA). Significant differences between means were assessed by Tukey's test at $P = 0.05$ (Westfall *et al*. 1999).

## RESULTS

The regeneration of rhizomes started when rhizome tips began to break and change from brown to pale yellow 10-15 days after culture initiation (**Fig. 1C**). The broken and pale yellow tips continued to grow and frond initials were obviously observed 25-40 days after incubation in the dark. New fronds continued to grow and changed from pale yellow to green after transferring to light (**Fig. 1D, 1E**).

One of the most important combinations of aspects in the *in vitro* propagation of fern in this study (personal observation) was the need to cut rhizome tips, which contain newly regenerated fronds, and culture them on semi-solid medium after 3.5-4.0 months of culture. This explant prepa-

**Table 1** Sterilization methods (SM) applied in this experiment.

| Disinfectant | Sterilization method (SM) | | | | |
|---|---|---|---|---|---|
| | SM-1 | SM-2 | SM-3 | SM-4 | SM-5 |
| Alcohol (80%) | - | - | - | 3 min | - |
| Tap water | 1 h | 2 h | 2.5 h | 2.5 h | 3 h |
| Detergent solution (1%) | 30 min | 30 min | 30 min | 30 min | - |
| Pesticide solution (1%) | 30 min | 1 h | 30 min | - | - |
| Streptomycin (150 mg/200 ml) | - | - | - | 10 min | - |
| NaOCl (0.5%) | - | - | 30 min | - | - |
| (1%) | 10 min | - | - | - | - |
| (2%) | 5 min | 10 min | - | - | - |
| $HgCl_2$ (0.05%) | - | - | - | - | 10 min |
| Alcohol (70%) | 30 sec | - | - | - | - |
| (96%) | - | 3 min | 3 min | 3 × 3 min | 1 min |

Detergent (Daia, PT. Sayap Mas Utama, Jakarta, Indonesia); pesticide (50% benomyl, Benlox® 50 WP, Dharma Guna Wibawa Ltd., Jakarta, Indonesia and 20% streptomycin sulphate, Agrept® 20WP, Mastalin Mandiri Ltd., Jakarta, Indonesia), NaOCl (Bayclin-Johnson Home Hygiene Products Ltd., Jakarta, Indonesia). All sterilization methods were completed by rinsing the explants with sterile distilled water sterile 6 times, 5 min each rinse.

**Table 2** Combinations and concentrations of plant growth regulators and sucrose in different initiation media (IM).

| IM | Combinations + concentrations (mg/l) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2,4-D | IAA | NAA | BA | 2-iP | Kin | TDZ | Zeatin |
| IM-1 | - | - | 0.1 | - | - | 2.0 | - | - |
| IM-2 | - | 0.5 | - | - | 2.0 | - | - | - |
| IM-3 | - | 0.25 | - | - | 2.0 | - | - | 0.5 |
| IM-4 | 0.25 | - | 0.2 | 1.0 | - | - | 0.5 | - |

Note: Full-strength Murashige and Skoog (MS, 1962) was used as basal medium. All media was supplemented with 20 g/l sucrose. Abbreviations: BA, 6-benzyl adenine; 2,4-D, 2,4-dichlorophenoxy acetic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; 2-iP, $N^6$-[2-isopentenyl]adenine; Kin, kinetin; NAA, α-naphthalene acetic acid; TDZ, thidiazuron.

**Table 3** Interaction effect of initiation medium (IM) and sterilization method (SM) on percentage contamination and total browning (%).

| SM | Percentage of contamination and total browning (%) | | | |
|---|---|---|---|---|
| | IM-1 | IM-2 | IM-3 | IM-4 |
| SM-1 | 100 a | 100 a | 100 a | 100 a |
| SM-2 | 96.7 ab | 96.7 a | 93.3 a | 96.7 ab |
| SM-3 | 83.3 bc | 86.7 ab | 83.3 a | 86.7 b |
| SM-4 | 73.3 c | 76.7 b | 63.3 b | 66.7 c |
| SM-5 | 43.3 d | 46.7 c | 33.3 c | 33.3 a |
| Coefficient of variation (%) | 6.51 | 8.49 | 9.15 | 6.91 |

Means followed by the same letter in the same column are not significantly different based on Tukey's test ($P = 0.05$).

**Table 4** Interaction effect of initiation medium (IM) and sterilization method (SM) on percentage of rhizome regeneration.

| SM | Percentage of rhizome regeneration (%) | | | |
|---|---|---|---|---|
| | IM-1 | IM-2 | IM-3 | IM-4 |
| SM-1 | 0.0 | 0.0 | 0.0 b | 0.0 b |
| SM-2 | 0.0 | 0.0 | 0.0 b | 0.0 b |
| SM-3 | 0.0 | 0.0 | 0.0 b | 13.3 b |
| SM-4 | 0.0 | 0.0 | 26.7 a | 53.3 a |
| SM-5 | 0.0 | 0.0 | 33.3 a | 73.3 a |
| Coefficient of variation (%) | - | - | 13.21 | 13.24 |

Means followed by the same letter in the same column are not significantly different based on Tukey's test ($P = 0.05$).

**Table 5** Interaction effect of initiation medium (IM) and sterilization method (SM) on average number of regenerated-rhizomes per replication.

| SM | Average number of regenerated-rhizomes per replication | | | |
|---|---|---|---|---|
| | IM-1 | IM-2 | IM-3 | IM-4 |
| SM-1 | 0.0 | 0.0 | 0.0 b | 0.0 b |
| SM-2 | 0.0 | 0.0 | 0.0 b | 0.0 b |
| SM-3 | 0.0 | 0.0 | 0.0 b | 0.7 b |
| SM-4 | 0.0 | 0.0 | 0.3 ab | 1.7 ab |
| SM-5 | 0.0 | 0.0 | 1.0 a | 2.3 a |
| Coefficient of variation (%) | - | - | 9.63 | 12.27 |

Means followed by the same letter in the same column are not significantly different based on Tukey's test ($P = 0.05$).

**Table 6** Effect of culture condition on percentage of contamination and total browning (%), percentage of rhizome regeneration (%) and average number of regenerated rhizomes per replication.

| Culture condition (CC) | Percentage of contamination and total browning (%) | Percentage of rhizome regeneration (%) | Average number of regenerated-rhizomes per replication |
|---|---|---|---|
| CC-1 | 96.0 a | 0.0 c | 0.0 c |
| CC-2 | 76.0 b | 14.0 b | 1.2 b |
| CC-3 | 98.0 a | 0.0 c | 0.0 c |
| CC-4 | 50.0 c | 36.0 a | 3.4 a |
| Coefficient of variation (%) | 7.39 | 10.19 | 8.03 |

Means followed by the same letter in the same column are not significantly different based on Tukey's test ($P = 0.05$).

ration is necessary since cultures can easily become contaminated by the presence of endogenous bacteria and since explants can brown (oxidize) easily due to the presence of phenolic compounds (visual assessment). Successful culture of rhizome tips with newly regenerated fronds resulted in an increase in the number of fronds as much as 257% more than the slowest rhizome tip growth (7 fronds resulted in 18 fronds) (**Fig. 1F**).

The choice of medium and sterilization method employed had a significant effect on culture initiation of fern rhizomes ($P = 0.05$; **Tables 1, 2**). Medium choice strongly affected the percentage rhizome regeneration (%) and the number of regenerated rhizomes. IM-4 was the most suitable IM during fern micropropagation, while SM-5 was the appropriate sterilization method for obtaining the highest number of uncontaminated rhizomes after culture initiation (**Tables 1, 2**). Both IM-4 and SM-5 showed significantly better results than other IM and SM media. SM-5 (**Table 1**) followed by IM-4 (**Table 2**) was the best medium combination that resulted in high levels of all parameters measured (**Tables 3-5**). This combination reduced the percentage contamination to 33%, stimulated rhizome regeneration up to 73% with 2.3 regenerated rhizomes/rhizome and was significantly different to all other combinations (**Tables 3-5**).

Contamination of explants in the first experiment was the main problem that significantly limited culture initiation, ranging widely from 20 to 100%, caused mainly by bacteria (observed visually), usually visible 24-72 h after culture initiation. The bacteria grew at the basal part of explants

that were in contact with the culture media forming a white layer (**Fig. 1G**). These bacteria spread rapidly in 3-7 days to a wide zone due to the movement of the film of water on the medium surface. Fungal contamination, which tended to follow bacterial contamination (**Fig. 1H**), was a serious problem in establishing aseptic cultures of leatherleaf fern, ranging from 10-75%, growing quickly and finally covering the entire explant surface (**Fig. 1I**). Although the percentage of fungal contamination was lower than bacterial contamination, in most cases, explant death was due to microbial contaminants.

Different CCs improved culture initiation significantly ($P = 0.05$). Using CC-4, i.e., liquid medium and culturing full rhizomes on a simple paper bridge (**Fig. 1B-E**) was the best CC resulting in high culture initiation, significantly more effective than other CCs. CC-4 reduced the percentage contamination to 50% while increasing rhizome regeneration to 36% with 3.4 regenerated rhizomes/rhizome (**Table 6**). CC-4 successfully improved culture initiation 400% more than the control (CC-2).

In the second experiment, browning of explants was a more important problem to fern propagation *in vitro* than bacterial contamination. The problem was compounded by slicing the rhizomes with a tissue culture blade. After 24-72 h of slicing, the edge of the cut area generally changed from green to brownish green, dark brown green, greenish dark brown and then totally dark brown after 72 h. In most cases, the browning of explants led to explant necrosis one month after incubation in the dark. In CC-1 and CC-3, 100% of explants died due to browning (**Table 6**).

## DISCUSSION

Bacterial contamination is a serious problem that causes the severe loss of *in vitro* cultures of a number of plants (Kulkarni *et al*. 2007), and this is no exception in leatherleaf fern. This problem becomes even more acute if the bacterial contamination is of endophytic origin (in *Musa sapientum*, *Piper* spp., *Taxus baccata* subsp. *wallichiana*, and *Withania somnifera*) (Habiba *et al*. 2002; Kulkarni *et al*. 2007) Species from the genera *Pseudomonas*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Staphylococcus*, *Lactobacillus*, etc. are frequently identified as bacterial contaminants in *in vitro* plant cultures (Leifert and Cassells 2001; Habiba *et al*. 2002; Marino *et al*. 2009). Instead of bacteria, fungal contaminants such as *Alternaria*, *Aspergillus*, *Cladosporium*, *Cylindrocarpon*, *Dictyostelium*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizopus* and *Saccharomyces* are also important contaminants in *in vitro* cultures (Susilowati and Lystiawati 2001; Altan *et al*. 2011). Both forms of contaminants, in fact, were also critical points in establishing *in vitro* cultures of leatherleaf fern, especially in (1) obtaining aseptic rhizomes with a high regeneration response and (2) cutting and culturing regenerated rhizome tips on semi-solid medium. Rhizomes died easily during culture initiation caused by bacterial and fungal infection (**Tables 3-5**), while, when cutting and culturing regenerated rhizome tips, explant death was mainly due to the existence of latent bacteria. All contaminated rhizomes showed reduced growth and most of them eventually (and expectedly) died.

Elimination and reduction of contaminants in *in vitro* culture caused by bacteria and fungi is an important key factor in obtaining aseptic explants (Moutia and Dookun 1999; Kulkarni *et al*. 2007; Morino *et al*. 2009). NaOCl, $HgCl_2$ and antibiotics (cefotaxime, gentamicin, rifampcin) are general disinfectants that are applied to eliminate contaminants individually or in combination with others (Habiba *et al*. 2002; Shukla *et al*. 2007; Yousef *et al*. 2007; Khaleghi *et al*. 2008; Naz *et al*. 2009; Dey *et al*. 2010). In this study, application of 0.05% $HgCl_2$ for 10 min in combination with a 3-min pre-treatment with 80% alcohol, then running rhizomes under tap water for 3 h and a final dip in 96% alcohol for 1 min was the best sterilization method for establishing an aseptic culture of rhizomes during the *in vitro* propagation of leatherleaf fern. The method successfully reduced the incidence of explant contamination and had a positive effect on rhizome regeneration (**Tables 3-6**). A similar result with 0.1% $HgCl_2$ for 3 min followed by a rinse with SDW was reported in ostrich fern (*Matteuccia struthiopteris*) (Zenkteler 2006). In several non ferns, $HgCl_2$ was also successfully applied to obtain aseptic cultures of rhizomes as the donor explant. The application of 0.1% $HgCl_2$ for 15 min followed by a rinse in SDW was successful for *Musa sapientum* (Habiba *et al*. 2002), *Curcuma longa* (Rahman *et al*. 2004), 70% ethanol for 10 min, 0.1% $HgCl_2$ for 15 min and a rinse with SDW three times for *Leucojum aestivum* (Kohut *et al*. 2007), 0.2% $HgCl_2$ for 15 min and 3-4 rinses in SDW for *Curcuma angustifolia* following a wash of rhizomes under running tap water (Sukla *et al*. 2007), or 0.1% $HgCl_2$ for 3-5 min followed by 3-4 rinses in double SDW for *Cymbopogon winterianus* (Dey *et al*. 2010). Application of a disinfectant also maintained rhizomes fresh leading to an increase in their regeneration.

In ferns, application of NaOCl in varied concentrations such as 5 g/l for 10 min and a rinse in SDW three times for *Polypodium cambricum* spores (Bertrand *et al*. 1999), soaking in water for 24 h and then surface sterilizing for 10 min in 0.5% NaOCl containing one drop of Tween 20 (polyoxyethylene sorbitan monolaurate) for *Woodwardia virginica* and *Dryopteris affinis* sp. *affinis* spores (Fernández *et al*. 1999), 20% (v/v) NaOCl along with a few drops of Tween-20 for 10 min, followed by a rinse in SDW for bird's nest fern (*Asplenium nidus*) spores (Khan *et al*. 2008), immersing in water for 24 h, sterilizing with 0.5% NaOCl containing 0.1% Tween 20 and rinsing in SDW for *Blechnum spicant* (a fern) spores (Menéndez *et al*. 2009) were successful methods used to obtain aseptic cultures. In non ferns, the application of NaOCl showed varied success (10-50%) in obtaining aseptic cultures and regenerating rhizomes as the donor explant, specifically for *Alstromeria* (Yousef *et al*. 2007; Khaleghi *et al*. 2008), *Curcuma longa* (Naz *et al*. 2009), *Curcuma*, *Kaempferia* and *Zingiber* (Ahmad *et al*. 2011), and *Alpinia zerumbet* (Rakkimuthu *et al*. 2011); bulbs in *Chlorophytum borivilliens* (Sharan *et al*. 2010), *Muscari azureum* (Uranbey *et al*. 2010), *Lilium candidum* (Altan *et al*. 2011); and corms in *Gladiolus anatolicus* (Emek and Erdağ 2007). However, these protocols, when tested in initial experiments, did not in fact have a positive effect on leatherleaf fern (data not shown). Use of NaOCl increased damage to the surface of rhizomes and phenolic compound production that led to death of explants (**Tables 3-6**). Application of NaOCl also reportedly caused phytotoxicity in sugarcane *in vitro* propagation, leading to 100% tissue necrosis and bud death (Moutia and Dookun 1999).

## ACKNOWLEDGEMENTS

We are grateful to Mr. Sapto Hadi from Tropika Flora Persada, Ltd., Sukoyoso village, Kajoran, Magelang District, Central Java Province, Indonesia for supplying leatherleaf fern rhizomes used in this research. We also express our deepest thank to Dedi Rusnandi for helping to maintain donor plants during the study.

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