Malaysian Applied Biology (2022) 51(5): 159-163
https://doi.org/10.55230/mabjournal.v51i5.2422

Research Article

Optimizing *In Vitro* Surface Sterilization of *Cyathea latebrosa* Spore

Nurul Nadhirah[1], Haja Maideen[1]*, Zuraida Ab Rahman[2] and Ayu Nazreena Othman[2]

[1]Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
[2]Biotechnology and Nanotechnology Research Centre, Malaysia Agricultural Research and Development Institute MARDI HQ, Persiaran MARDI-UPM, 43400 Serdang, Malaysia
*Corresponding author: deen@ukm.edu.my

ABSTRACT

Cyathea latebrosa is one of the lowland tree fern species found in Peninsular Malaysia. This fern species is highly demanded in ornamental landscaping. The *in vitro* cultures are an important tool for propagation which may contribute toward the reduction of over-exploitation. To overcome these problems, an effective spore surface disinfection protocol is crucial to allow the germination stage to be carried out. This studied had carried out three types of methods which are the packet method (PM), centrifuged method (CM), and soak method (SM) with difference percent of concentration (0, 0.1, 0.5, 1.0, 10 & 30) Mercury Chloride ($HgCl_2$) and Sodium hypochlorite (NaOCl). In contrast, the method and concentration of disinfection affect germination. Our results showed that the soak method in both types of disinfection is a significant difference due to statistical analysis (MANOVA) which gives a positive effect on the germination of the spore. This method is efficient for sterilizing which spore loss is kept to a minimum and has a higher rate of germination ($HgCl_2$-90% & NaOCl-80%). The optimum concentration of $HgCl_2$ was 0.1%, then followed by 0.5% and 1.0%, while for NaOCl was 30%, 20%, and 10%.

Key words: Fern, spore, *in vitro* culture, surface sterilization

Article History
Accepted: 28 November 2022
First version online: 26 December 2022

Cite This Article:
Nadhirah, N., Maideen, H., Ab Rahman, Z. & Othman, A.N. 2022. Optimizing in vitro surface sterilization of *Cyathea latebrosa* spore. Malaysian Applied Biology, 51(5): 159-163. https://doi.org/10.55230/mabjournal.v51i5.2422

INTRODUCTION

Ferns are the second-largest vascular plant group, with 12,000 species in 319 genera (Sharpe *et al*., 2010). Approximately 1165 taxa of Malaysia's ferns have been identified, with 647 species found in Peninsular Malaysia (Parris & Latiff, 1997; Maideen *et al*., 2019; 2020). *Cyathea* is a tree fern genus with more than 400 species all over the world (Maideen *et al*., 2020). According to Parris & Latiff (1997), there are 39 species of *Cyathea* recorded in Malaysia. However, only 22 species were recorded for Peninsular Malaysia and 29 species for Sabah and Sarawak. Only three species namely *Cyathea moluccana*, *Cyathea latebrosa,* and *Cyathea glabra* have been found in lowland forests, the rest were reported in highland areas such as in Fraser's Hill and Cameron Highland (Maideen *et al*., 2001). Tree ferns have long been used for many socio-economic purposes such as construction, horticulture, food, and medicine resulting in their heavy exploitation as a source of income (Large & Braggins, 2004; Rout *et al*., 2009).

Spore culture is an alternative approach for *in situ* conservation to meet the rising demand for tree ferns in Malaysia. For *Cyathea* species in Malaysia, no study has been conducted on fern spore culture optimization. According to Tomaszewicz *et al*. (2022), *in vitro* cultures have been used to grow 27 different kinds of tree fern, for example, *C. atrovirens* (Moura *et al*., 2012; de Vargas & Droste, 2014), *C. cooperi* (Moura *et al*., 2012), *C. corcovadensis* (Marcon *et al*., 2014), *C. cunninghamii* (Moura *et al*., 2012), *C. delgadii*

(Tomaszewicz *et al*., 2022), *C. gigantea* (Supriya *et al*., 2013) and *C. spinulosa* (Shukla & Khare, 2012).

Cyathea latebrosa , habitat expands in Indochina, Cambodia, Thailand, Malaysia, and Indonesia. It is widely distributed in Peninsular Malaysia, probably in all states except Perlis and Kelantan. The present study seeks to improve the method of sterilizing *C. latebrosa* spore, which will aid in the micropropagation of tree ferns and perhaps other closely related species. Various sterilization techniques including the soak method (SM), packet method (PM), and centrifugation method (CM) will be tested. Two disinfectant solutions of variable concentrations will be determined for the ability to reduce contamination but not reduce germination of this species.

MATERIALS AND METHODS

Culture media

Murashige & Skoog (1962) medium with ½ strength of macronutrients were used for the germination of spores. The medium in each treatment contains 3% (w/v) sucrose and 0.4% (w/v) plant agar and is adjusted to pH 5.8 before being autoclaved at 121 °C for 15 min.

Spore collection

Mature pinnae bearing *C. latebrosa* spores were freshly collected from the Bangi Botanical Garden at Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia (Figure 1). Then, the mature pinnae were washed in running tap water for 10 min in a conical flask and divided into three groups according to different methods of sterilization.

Sterilisation of spores

Surface sterilization of the spores embedded in the mature pinnae is essential before the germination step can be performed. Mature pinnae were soaked in fungicidal solution (1 g of fungicide (Benex), 3-5 drops of Tween-20 in 200 mL of distilled water) and shaken on an orbital shaker for one hr. This is followed by treatment either by the soak method (SM), packet method (PM), or centrifugation method (CM). All treatments were performed in the laminar air chamber with three mature pinnae per method.

Centrifugation method (CM)

The centrifugation method by Fernandez *et al*. (1993) was followed. The spores were separated from the pinnae by scraping using a sterile blade and collected to fill half of the 1.5 mL centrifuge tube. The spores were soaked into either mercury chloride ($HgCl_2$) or sodium hypochlorite (NaOCl) solutions at different concentrations for 30 min (Table 1 & Table 2). The spores were centrifuged at 2000 r.p.m. for 3 min (Figure 1b) and the pellet was distributed onto 10 culture medium (Figure 1c).

Packet method (PM)

The packet method by Ford and Fay (1999) was followed. The spores were separated from the pinnae by scraping using a sterile blade and collected into the paper packet. The collected spores were transferred to the middle of the filter paper and were folded twice to form a packet (Figure 1d & 1e). This packet is soaked into either $HgCl_2$ or sodium hypochlorite NaOCl solutions at different concentrations for 30 min (Table 1 & Table 2). The packet with the spores was cut at four sides and cultured onto 10 culture media.

Soak method (SM),

For SM, the mature pinnae were directly soaked into either $HgCl_2$ or NaOCl solutions at different concentrations for 30 min (Table 1 & Table 2). The pinnae were air-dried and spores were scraped off into 10 culture media per treatment (Figure 1f). This method is newly developed for spore culture sterilization.

Germination capability and contamination

The spores were incubated in the culture room under white fluorescent light with a light intensity of 3000 lux and a photoperiod of 16 h at 25 ± 2 °C. After 4 weeks of culture, the percentage of germination (number of visible germinations from plates out of 10 plates) (Figure 1g & 1h) and signs of contamination (number of visible contaminations from plates out of 10 plates) were recorded. Over-sterile is indicated when there is no visible germination or contamination (Figure 1c).

Statistical analysis

Data of spore contamination and germination were analyzed using Multivariate Analysis of Variance (MANOVA) and the differences between means were tested by Pairwise Comparisons test at 5% probability, using IBM SPSS Statistics version 27 software (IBM Corp., Armonk, NY). Results were presented accordingly.

RESULTS AND DISCUSSION

Surface sterilization of spores is the first step in the aseptic culture of ferns, and it is essential before germination takes place. We observed that the soak method was an effective way to sterilize spores since this method achieved the highest mean percent of germination with 90% when using 0.1% of $HgCl_2$ and 80% when using 30% of NaOCl (Table 1). SM was the only sterilization method with a significant effect on spore germination (p<0.05). However, a comparison between methods revealed that SM is not effective in reducing

contamination, $p>0.05$ but significant only in promoting germination $p<0.05$. By using pairwise comparison, the mean score of SM (25.86 ± 0.43) was significantly higher than CM (0.00 ± 0.43) and PM (0.00 ± 0.43) on the percentage of germination (Table 2).

In the packet method, the spore surface is not totally in contact with the disinfectant solution. This explains why a higher percentage of contamination was noted in this study. The centrifugation method showed low contamination, but this method had bleached out most of the spore, leaving only a

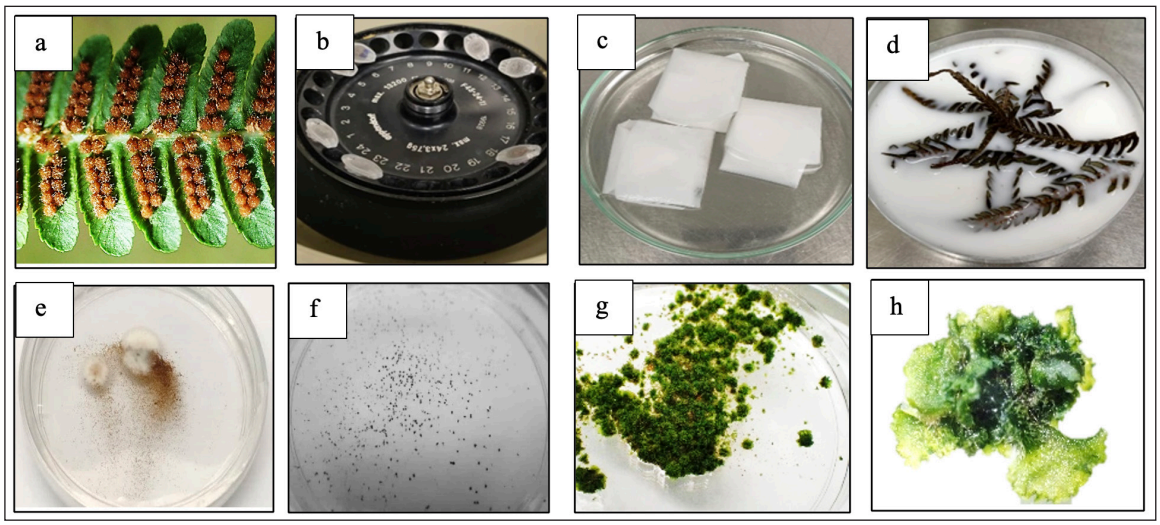

Fig. 1. Material and sterilization method of *C. latebrosa* (a) spores, (b) centrifugation method, (c)packet method, (d) soak method, (e) result of centrifugation method, (f) result of packet method, (g-h) result of soak method.

Table 1. The descriptive result represents as mean ± SD of percentage germination and contamination from three sterilization methods with different concentrations of $HgCl_2$ or NaOCl

	Sterilization method					
Concentration	PM		CM		SM	
($HgCl_2$)	C	G	C	G	C	G
0	98.0 ± 2.0	0.0 ± 0.0	99.33 ± 0.57	0.0 ± 0.0	99.66 ± 0.57	0.0 ± 0.0
0.1	93.3 ± 7.6	0.0 ± 0.0	85 ± 5.0	0.0 ± 0.0	25.0 ± 5.0	90.0 ± 5.0
0.5	81.6 ± 10.4	0.0 ± 0.0	65.33 ± 5.0	0.0 ± 0.0	13.33 ± 7.63	75.0 ± 5.0
1	61.6 ± 7.63	0.0 ± 0.0	2.6 ± 2.51	0.0 ± 0.0	2.66 ± 2.51	35.0 ± 5.0
10	20.0 ± 5.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.67 ± 2.88
20	1.6 ± 1.56	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 2.0
30	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Concentration	PM		CM		SM	
(NaOCl)	C	G	C	G	C	G
0	95.0 ± 5.0	0.0 ± 0.0	100 ± 0.0	0.0 ± 0.0	100 ± 0.0	0.0 ± 0.0
0.1	86.67 ± 7.63	0.0 ± 0.0	90.33 ± 5.5	0.0 ± 0.0	85.0 ± 5.0	0.0 ± 0.0
0.5	80.0 ± 10.0	0.0 ± 0.0	78.33 ± 7.63	0.0 ± 0.0	75.0 ± 5.0	0.0 ± 0.0
1	66.66 ± 7.63	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	55.0 ± 5.0	2.66 ± 2.0
10	30.0 ± 10.0	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	15.0 ± 5.0	15.0 ± 5.0
20	10.0 ± 5.0	0.0 ± 0.0	0.0 ± 0.2	0.0 ± 0.0	15.0 ± 5.0	45.0 ± 5.0
30	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.3	0.0 ± 0.0	5.67 ± 4.04	80.0 ± 5.0

Notes: C: percentage of contamination G: percentage of germination

Table 2. The average value of contamination and germination between three sterilization methods with two types of disinfectant solutions of $HgCl_2$ and NaOCl towards *C. latebrosa*

Disinfectant	Sterilization Method	Contamination (Mean ± SE)	Germination Mean ± SE	Remarks
$HgCl_2$ & NaOCl	CM	37.21 ± 1.06	0.00 ± 0.43	Over Sterile
	PM	51.76 ± 1.06	0.00 ± 0.43	Fungus Growth
	SM	35.10 ± 1.06	25.86 ± 0.43	Germinate

small amount of spore remaining after the entire sterilization procedure. No significant statistical difference in germination and contamination for both methods. Unfortunately, no spore germination occurs due to fungus overgrowth in the packet method and over-sterilization in the centrifuge method (Table 1). SM provides the most economical use of solvents and only required a small amount of spore for propagation. Development of this method allows spore sterilization that substantially impacts successful fern micropropagation that will be beneficial in fulfilling increasing tree fern demand.

CONCLUSION

The soak method is the most efficient method for *C. latebrosa* propagation from spores compared to the CM and PM methods. The mean percentage germination in this method was recorded as 90% using 0.1% $HgCl_2$ and 80% when using 30% NaOCl respectively. Fungal contamination was kept to a minimum of 25% when using $HgCl_2$ and 5.67% in NaOCl. This study provides the most economical use of solvents with small amounts of spores required for producing fern cultures. This method could be further tested and applied to other fern species' propagation.

ACKNOWLEDGEMENTS

The authors would like to thank Universiti Kebangsaan Malaysia (UKM) and the Ministry of Higher Education for funding this research through the FRGS/1/2020/WAB11/UKM/02/1 grant. The first and second authors would like to thank Malaysia Agricultural Research and Development Institute (MARDI) for their assistance and for providing infrastructure for this research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

de Vargas, I.B. & Droste, A. 2014. In vitro propagation of *Cyathea atrovirens* (Cyatheaceae): Spore storage and sterilization conditions. Revista de Biologia Tropical, 62(1): 299–308. https://doi.org/10.15517/rbt.v62i1.3661

Fernandez, H., Bertrand, A. & Sanchez-Tames, R. 1993. In vitro regeneration of *Asplenium nidus* L. from gametophytic and sporophytic tissue. Scientia Horticulturae, 56(1): 71–77. https://doi.org/10.1016/0304-4238(93)90103-W

Ford, M.V. and Fay, M.F. 1999. Spore-derived axenic cultures of ferns as a method of propagation. In: Plant Cell Culture Protocols, R.D. Hall (Eds.). Human Press Inc, Totowa. pp. 159–168. https://doi.org/10.1385/1-59259-583-9:159

Large, M.F. & Braggins, J.E. 2004. Tree ferns. Timber Press, United States of America. pp. 15-312.

Maideen, H., Farhana, N., Nadirah, N., Khaduwi, N.A. & Norhazrina, N. 2019. Ferns and lycophytes of Pulau Pangkor and its vicinity. The Malaysian Forester, 82(1): 77–86.

Maideen, H., Jaman, R., Bidin, A.A., Ibrahim, A.Z. and Latiff, A. 2001. Pteridophytes of Fraser's Hill. In : Bukit Fraser Persekitaran Fizikal, Biologi dan Sosio-ekonomi. A. Latiff, Z. Zakaria, Z. Isa, M. Salleh, M. Hashim, N. Md. and L.Y. Din (Eds.). Pusat Pengurusan Penyelidikan Universiti Kebangsaan Malaysia, Bangi. pp. 224-280.

Maideen, H.M.K, Nur Iwani, M.S & Nur Aliah, M.K. 2020. Paku Pohon Cyatheaceae. Penerbit Universiti Kebangsaan Malaysia, Bangi. pp. 11-24.

Marcon, C., Silveira, T. & Droste, A. 2014. Germination and gametophyte development of *Cyathea corcovadensis* (Raddi) Domin (Cyatheaceae) from spores stored at low temperatures. Acta Scientiarum Biological Sciences, 36(4): 403–410. https://doi.org/10.4025/actascibiolsci.v36i4.24318

Moura, I.R., Simões-Costa, M.C., Garcia, J., Silva, M.J. & Duarte, M.C. 2012. In vitro culture of tree fern spores from Cyatheaceae and Dicksoniaceae families. Acta Horticulturae, 937: 455-461. https://doi.org/10.17660/actahortic.2012.937.55

Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15(3): 473-497.

Parris, B.S. & Latiff, A. 1997. Towards a pteridophyte flora of Malaysia: A provisional checklist of taxa. Malayan Nature Journal, 50(4): 235–280.

Rout, S.D., Panda, T. & Mishra, N. 2009. Ethnomedicinal studies on some pteridophytes of Similipal Biosphere Reserve, Orissa, India. International Journal of Medicine and Medical Sciences, 1(5): 192–197.

Sharpe, J.M., Mehltreter, K. & Walker, L.R. 2010. Ecological importance of ferns. In: Fern Ecology. K. Mehltreter, L.R. Walker and J.M. Sharpe (Eds.). Cambridge University Press, New York. pp. 1-18.

Shukla, S.P. & Khare, P.B. 2012. *In-vitro* mass multiplication of a threatened tree fern, *Cyathea spinulosa* Wall. ex Hook. International Journal of Genetic Engineering and Biotechnology, 3(1) : 15-23. https://doi.org/10.13140/2.1.2282.8168

Supriya, D., Choudhury, M.D. & Mazumder, P.B. 2013. *In vitro* propagation of *Cyathea gigantea* (Wall ex . Hook) - A tree fern. International Journal of Recent Scientific Research, 4(3): 221–224.

Tomaszewicz, W., Cioć, M., Szewczyk, K.D.S., Grzyb, M., Pietrzak, W., Pawłowska, B. & Mikuła, A. 2022. Enhancing *in vitro* production of the tree fern *Cyathea delgadii* and modifying secondary metabolite profiles by LED lighting. Cells, 11(3): 1–19. https://doi.org/10.3390/cells11030486