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Short Communication

Spore Germination of *Diplazium simplicivenium* Holtt. (Athyriaceae) in Peninsular Malaysia

Nurul Nadhirah[1], Haja Maideen[1]*, Ab Rahman Zuraida[2] and Othman Ayu Nazreena[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, Persiaran MARDI-UPM, 43400 Serdang, Selangor, Malaysia*

ABSTRACT

Diplazium simplicivenium Holtt. a species of fern in Peninsular Malaysia. This study reports an efficient method *for D. simplicivenium* spore sterilisation and the effect of plant growth regulators (PGRs) via green globular bodies (GGB). Sterilisation with 0.1% mercuric chloride effectively allowed spore germination. The result showed that the culture media supplemented with 0.5 mg/L gibberellin positively affects the weight of GGB and the number of shoots with significant differences (ANOVA, $p < 0.05$). The soaking technique established in this study for spore sterilisation is an efficient approach, and the optimal plant and concentration of plant hormones were identified. This procedure can be applied to other indigenous ferns and closely similar species.

Keywords: *Diplazium*, fern, *in vitro*, micropropagation, spore

INTRODUCTION

Fern is a vascular plant widely distributed worldwide with approximately 12,000 species (Sharpe et al., 2010). Malaysia has 1,165 fern taxa, 647 of which are found in Peninsular Malaysia (Maideen et al., 2019, 2020). However, the diversity of the plant decreases due to many factors, such as habitat loss and climate change. *Diplazium simplicivenium* is a fern species known as “pokok paku” among Malaysians. The species belongs to the Athyriaceae and can potentially be used as an ornamental plant due to the uniqueness of its frond and daily life as a rope for tying goods and vegetables (Abrori et al., 2022). This species had been found in

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E-mail addresses:
p112492@siswa.ukm.edu.my (Nurul Nadhirah)
deen@ukm.edu.my (Haja Maideen)
azuraida@mardi.gov.my (Ab Rahman Zuraida)
ayureena@mardi.gov.my (Othman Ayu Nazreena)
*Corresponding author

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shady mountain valleys and exposed areas near roadside drains. In the last few decades, anthropogenic disturbances and climate change have resulted in the loss of habitat for plant species such as ferns in the Cameron Highlands, making it more challenging for fern species to develop. In Asia, particularly in Peninsular Malaysia, more studies are needed on *in vitro* spore propagation of ferns. For instance, two studies *in vitro* of fern *Platycerium coronarium* by Taha et al. (2011) and *Cyathea latebrosa* by Nadhirah et al. (2022). The current research deals with the *in vitro* spore propagation of *D. simplicivenium*, which will provide insight into conservation strategies.

MATERIAL AND METHODS

Culture Media

Spores had been used to germinate in a Murashige and Skoog (1962) (Duchefa Biochemie BV, Netherlands) medium with macronutrients at half strength. Each treatment's medium is pH-adjusted to 5.8 and contains 3% (w/v) sucrose (Duchefa Biochemie BV, Netherlands) and 0.4% (w/v) gelrite (Duchefa Biochemie BV, Netherlands) before being autoclaved at 121°C for 15 min.

Spore Collection

Mature pinnae bearing *D. simplicivenium* spores were freshly collected from Parit Fall, Cameron Highland, Pahang, Malaysia (Figures 1a and b). The mature pinnae were then rinsed in a conical flask for 10 min with running tap water.

Sterilisation of Spores

The sterilising procedure was essential before beginning the germination procedure. Mature pinnae were treated with a fungicidal solution consisting of 1 g of benomyl (Benex 500 WP, Imaspro Corporation Berhad, Malaysia) with 3–5 drops of Tween-20 (Sigma-Aldrich, Germany) and 200 ml of distilled water before being shaken for an hour on an orbital shaker. The pinnae were then rinsed with sterile distilled water three times. Then, the soak method (SM) for treatment is used.

Soak Method

The pinnae were soaked in mercuric (II) chloride ($HgCl_2$) (Sigma-Aldrich, Germany) for 10 min in each treatment (0.05, 0.1, 0.5, and 1.0%). The pinnae were then air-dried on filter paper after being rinsed with three distilled waters. After drying, the spores were scraped off the pinnae and cultured on Murashige and Skoog (1962) medium with ½ strength of macronutrients (Figure 1c). The spores were incubated in the growth chamber with a photoperiod of 16 hr at 25°C and white fluorescent light at a light level of 3,000 lux. All experiments were done in aseptic conditions.

GGB Induction and Multiplication

After four weeks in culture, spores began to germinate, and after three months, several gametophytes appeared. Each GGB induced throughout all treatments was fragmented into four segments, each measuring 1.0 cm in diameter and 1.0 g in weight. In Figure 1 (e, f, g, h, i, j, k, l, m, n, o, p, q, and r) order

to study the effect of cytokinin on GGB induction, GGB was cultivated with 0.5, 1.0, 3.0, and 5.0 mg/L on various culture medium such 2,4-dichlorophenoxyacetic acid (2,4-D) (Duchefa Biochemie BV, Netherlands), gibberellin (GA3) (Duchefa Biochemie BV, Netherlands) and a combination of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6 benzylaminopurine (BAP) (0.5 mg/L 2,4-D + 2.0 mg/L BAP; 1.0 mg/L 2,4-D + 4.0 mg/L BAP; 3.0 mg/L 2,4-D + 12.0 mg/L BAP; 5.0 mg/L 2,4-D + 20.0 mg/L BAP). Half strength of Murashige and Skoog (MS) culture media without cytokinin was used as a control. After 12 weeks of culture, the final fresh weight and the quantity of GGB shoots were recorded.

Data Analysis

Using IBM SPSS Statistics software (version 27), data on the final fresh weight and the number of shoots from GGB were analysed using analysis of variance (ANOVA), and the differences between means were analysed by pairwise comparisons test with a 5% probability. Accordingly, the study's findings were provided.

RESULTS AND DISCUSSION

Ferns are commonly cultivated *in vitro* via spores. The serious issue of spore loss and contamination remains, although several techniques have been discovered for spore surface sterilisation and sowing (Wu et al., 2009). A simple and highly efficient approach has been developed for the first study's *in vitro* micropropagation of *D. simplicivenium*.

The *in vitro* spore production technique includes several life cycle stages, from spores through gametophytes and sporophytes. The growth of gametophytes begins with the outgrowth of the protonemal cell, or both, and develops through several morphological appearances, such as filamentous, spatulate, and heart-shaped. Surface sterilisation is the crucial first phase in the aseptic culture of the spore culture (Dyer, 1979).

The soak method, used in a recent study by Nadhirah et al. (2022), proved to be the most effective way to propagate *C. latebrosa* because it can minimise fungal contamination and spore loss when using 0.1% $HgCl_2$ and 30% NaOCl, while achieving high germination rates of 90 and 80%, respectively. Both studies utilised the same type of explant (pinnae); however, compared with ours, which mainly used $HgCl_2$, their study used two types of disinfectant, $HgCl_2$ and NaOCl. However, the findings showed that 0.1% $HgCl_2$ is an efficient disinfectant in both studies.

Golamaully et al. (2015) demonstrated that spore surface sterilisation of *D. proliferium* was efficient at lower concentrations of $HgCl_2$ (0.05%) without using fungicide. However, this study's higher concentrations of $HgCl_2$ (0.1%) using fungicide were efficient. Since the spore surface sterilisation in this study used the entire pinnae, whereas Goullamaully et al. (2015) only used the scarpered spore that dropped during air drying, the area of the sterilisation surface may influence the concentration needed for surface sterilisation. The highest percentage of

spore germination (87.5±2.20) was observed in the 0.1% $HgCl_2$ treatment.

Our studies show that 0.1% of $HgCl_2$ plus fungicide was efficient in spore sterilisation, resulting in a high germination rate (87%). However, the study by Golamaully et al. (2015) in *D. proliferium* showed that 0.05% of $HgCl_2$ without fungicide was efficient in spore sterilisation. Spores provide all the nutrients required for early growth and are a common beginning material for fern development. Regarding the species, it might take a few days to a few months for fern spores to germinate (Chou et al., 2007; Fernández & Revilla, 2003). Thus, during the early stages of germination, the low-nutrient medium of the Murashige and Skoog (1962) medium is commonly used and can be modified. However, this study supports the use of half MS for spore germination and the development of a tiny filament known as protonema after 30-40 days of cultivation (Figure 1a).

Tables 1 and 2 represent the mean percentage of succession toward weight and the number of shoots in GGB for three months. The medium GA_3 supplement (0.5 mg/L) generated the best results in terms of GGB weight (3.46±0.08) and shoot number (20±2.38). This treatment resulted in the growth of greenish GGB with rhizomes (Figure 1f). The treatment was subsequently added with GA_3 (1.0 mg/L), which had similar effects in terms of weight (2.55±0.10) and the number of shoots (11.25±0.97). However, this treatment resulted in brownish GGB and the formation of rhizomes (Figure 1g).

In this study, GGB cultivated on GA_3-containing media resulted in varied success in shoot initiation and weight, which was influenced by the concentration (Table 2). Similar results were also observed on *P. coronarium* (Taha et al., 2011) *and Pteris tripatita* (Ravi et al., 2014). *Lygodium japonicum* (Takeno & Furuya, 1977), *Blechnum spicant* (Fernández et al., 1997), *Anemia phyllitidis* (Kaźmierczak, 1998, 2003), and *Osmundastrum cinnamomeum* (Babenko et al., 2018).

In the previous study, the plant growth regulator had proven varied effects on gametophyte growth. For instance, adding GA_3 to the media at concentrations of 0.5, 5.0, and 50 M dramatically slowed the formation of gametophytes in *B. spicant* at all phases of ontogenesis. They inhibited sporophytes from growing (Fernández et al., 1997). Then, in the *A. phyllitidis* thallium, GA_3 affected cell division, which resulted in a decline in cell division and the appearance of smaller thalli. Prothallium enlarged because of the expansion of individual cells (Kaźmierczak, 1998, 2003). Similar outcomes were shown in *O. cinnamomeum* (L.) C. Presl, where reduced hormone concentration significantly increased the size of the prothallium, while a slight rise in concentration significantly decreased the size of the thallus (Hollingsworth et al., 2012). In MS media supplemented with 1.0 and 1.5 mg/L GA_3, Taha et al. (2011) reported that *P. coronarium* successfully regenerated sporophytes from gametophyte explants. The results reported by Ravi et al. (2014) in their study on *P. tripatita* at

lower concentrations of this hormone also influence the overall morphological growth of gametophytes.

Regenerated plantlets were placed on a medium containing 0.5 mg/L GA_3 to sustain this species. Through a series of acclimatisation stages, the *in vitro*-grown full plantlets (Figure 1r) were transplanted outdoors. Plantlets were transplanted into pots made of a 3:1 mixture of peat moss and vermiculite (Figure 1s). In the glasshouse, they were kept at a relative humidity of around 70 with 75% shade. After six weeks, a 95% survival rate resulted (Figure 1s, 1t, and 1u).

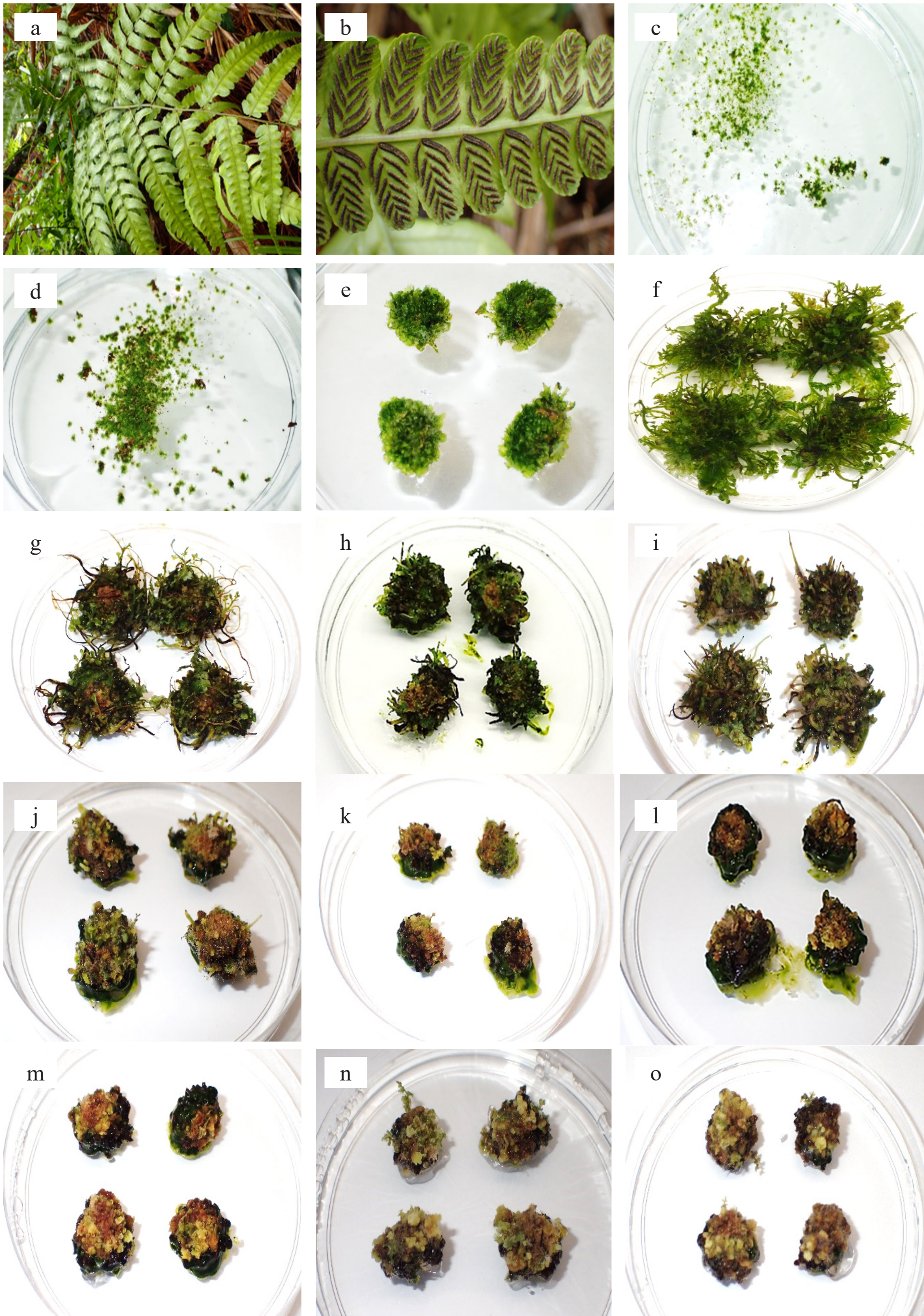
Table 1
Percentage of germination and contamination from sterilisation method with different concentrations of mercuric (II) chloride ($HgCl_2$)

Disinfectant	Concentration (%)	Contamination	Germination	Remarks
$HgCl_2$	0.05	94.8 ± 2.00	0.00 ± 0.00	Fungus growth
	0.10	45.0 ± 3.37	87.5 ± 2.20	Germinate
	0.50	14.0 ± 1.60	24.5 ± 5.90	Germinate
	1.00	3.80 ± 1.10	0.00 ± 0.00	Over sterile

Note. Results represent mean±standard error mean (SEM)

Table 2
Effect of 2,4-dichlorophenoxyacetic acid (2,4-D), gibberellin (GA_3), and combination of 2,4-D and 6-benzylaminopurine (BAP) on weight and shoot from green globular bodies (GGB) Diplazium simplicivenium after 12 weeks of culture

Type of media	Weight of GGB	Number of shoots
Half Murashige and Skoog (MS)	0.73 ± 0.03^{b}	6.25 ± 0.32^{d}
0.5 mg/L 2,4-D	1.39 ± 0.05^{cd}	5.00 ± 0.49^{cd}
1.0 mg/L 2,4-D	0.83 ± 0.04^{b}	1.58 ± 0.25^{ab}
3.0 mg/L 2,4-D	1.27 ± 0.04^{cd}	0.00 ± 0.00^{a}
5.0 mg/L 2,4-D	1.28 ± 0.42^{cd}	0.00 ± 0.00^{a}
0.5 mg/L GA_3	3.46 ± 0.08^{f}	20.0 ± 2.38^{f}
1.0 mg/LGA_3	2.55 ± 0.10^{e}	11.25 ± 0.97^{e}
3.0 mg/L GA_3	1.44 ± 0.08^{d}	1.75 ± 0.37^{ab}
5.0 mg/L GA_3	2.38 ± 0.08^{e}	3.33 ± 0.432^{bc}
0.5 mg/L 2,4-D + 2.0 mg/L BAP	1.20 ± 0.05^{c}	1.75 ± 0.32^{ab}
1.0 mg/L 2,4-D + 4.0 mg/L BAP	1.34 ± 0.04^{cd}	1.50 ± 0.31^{ab}
3.0 mg/L 2,4-D + 12 mg/L BAP	0.73 ± 0.03^{b}	0.00 ± 0.00^{a}
5.0 mg/L 2,4-D + 20 mg/L BAP	0.41 ± 0.02^{a}	0.00 ± 0.00^{a}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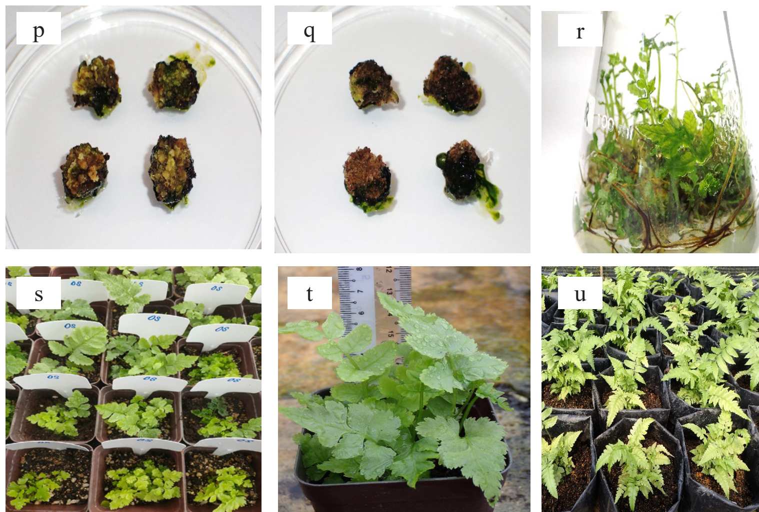

Figure 1. *In vitro* regeneration of *Diplazium simplicivenium*. (a) Mature pinnae of *D. simplicivenium*, (b) sporangium, (c) spore germination on ½ Murashige and Skoog (MS) for 4 weeks of sowing, (d) spore germination on ½ MS for 8 weeks of sowing, (e) green globular bodies (GGB) multiplication on ½ MS (control) for three months, (f) GGB growth on 0.5 mg/L gibberellin (GA_3), (g) GGB growth on 1.0 mg/L GA_3, (h) GGB growth on 3.0 mg/L GA_3, (i) GGB growth on 5.0 mg/L GA_3, (j) GGB growth on 0.5 mg/L 2,4-D, (k) GGB growth on 1.0 mg/L 2,4-D, (l) GGB growth on 3.0 mg/L 2,4-D, (m) GGB growth on 5.0 mg/L 2,4-D, (n) GGB growth on 0.5 mg/L 2,4-D + 2.0 mg/L BAP, (o) GGB growth on 1.0 mg/L 2,4-D + 4.0 mg/L BAP, (p) GGB growth on 3.0 mg/L 2,4-D + 12.0 mg/L BAP, (q) GGB growth on 5.0 mg/L 2,4-D + 20.0 mg/L BAP, (r) plantlets growth on 0.5 mg/L GA_3 for 3 months, (s) Plantlets planted in 72-hole plug for 1 month, (t) plantlets planted in 72-hole plug for 2 months, and (u) Acclimatised plant cultivated in the glasshouse for 4 months

CONCLUSION

This article reports on *in vitro* spore culture for *D. simplicivenium* via GGB. Based on the result, the mean germination was significantly higher in 0.1 and 0.5% $HgCl_2$, respectively. It has been observed that 0.5 mg/L GA_3 culture medium followed by 1.0 mg/L GA_3 medium is best for GGB of *D. simplicivenium* toward efficient weight and quantity of shoots. An efficient spore culture approach would allow for large-scale production of *D. simplicivenium*, which might contribute to conserving species on the verge of extinction and be applied to propagate other fern species.

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