Malaysian Applied Biology
https://doi.org/10.55230/mabjournal.v54i3.3480

Research Article

In Vitro Shoot-Tip Propagation of Blue Peacock Fern *Selaginella willdenowii* P.Beauv. (Selaginellaceae) an Ornamental Fern

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ABSTRACT

Selaginella willdenowii, an ornamental fern with iridescent bluish green fronds, is found naturally on forest floors. Many Asian countries consumed it as a traditional medicine and food. It is also suitable for decorative plants since it requires low light and moist conditions, making it perfect for ground covers and house plants. There are currently no efficient methods for propagating this species. Therefore, the goal of this research was to establish an effective *in vitro* culture propagation method for this species. The shoot-tips were sown in half-strength Murashige & Skoog (MS) as a control, and we observed the most effective culture conditions for shoot-tip proliferation and growth on agar-cultured media at varied concentrations of plant growth regulator (PGR). The findings demonstrated that the culture media supplemented with 0.5 mgL $^{-1}$ GA_3 has a positive effect on the shoot tips, forming a maximum number, lengths of the shoots, and numbers of rhizomes with significant difference (ANOVA, ά<0.05). By using this method, it will be possible to speed up the process for growing a large number of *S. willdenowii*, and it may also be used to propagate other related plants.

Key words: Medicinal fern, micropropagation, ornamental fern, Selaginellaceae

INTRODUCTION

Selaginella is a heterosporous ferns that produce megaspores and microspores in two distinct sizes. One of the 700 species of vascular plants in the Selaginellaceae is *Selaginella willdenowii* P. Beauv (Smith *et al*., 2006). This species is native to Thailand, Peninsular Malaysia, and Malesia. Due to its iridescent blue leaves, this spike moss is often referred to as peacock fern in Malaysia. Traditional use and modern pharmacological studies support the renowned medicinal potential of this species. However, to ensure sustainable use and conservation, effective propagation strategies are needed, such as micropropagation or spore culture. This will enhance the quality and consistency of plant materials, essential for clinical applications and commercialization. These strategies also support conservation efforts by reducing pressure on wild populations.

In Malaysia, decoction of *S. willdenowii* leaves is used to treat backaches, high fevers, and wounds (Hanum & Hamzah, 1999). It is also used as a tonic (Eswani *et al*., 2010). It is used in Brunei to alleviate gastrointestinal discomfort and urinary tract infections (Mohiddin *et al*., 1991). In Indonesia, it is used to cure wounds, menstrual problems, and skin ailments, as well as being consumed as a vegetable (de Winter & Jansen, 2003; Setyawan, 2009). *S. willdenowii* infusion is used to cure high fever in India, and its ashes are used as a backache liniment (Khare, 2007). Apart from *S. willdenowii*'s usage in traditional medicine, several other *Selaginella* species are commonly used as medicinal herbs in a number of cultures. According to Chai and Wong (2012), *S. willdenowii* is a potential medicinal plant and dietary antioxidant source.

Numerous fern species have been successfully propagated *in vitro*. This method has been used to propagate for species conservation, medicinal, and ornamental purposes. There are various culture methods that have successfully developed for other *Selaginella* species, such as *S. microphylla* (Jha *et al*., 2013), *S. martensii* (Park *et al*., 2020), *S. pulvinata* (Yu *et al*., 2021), and *S. tamariscina* (Park *et al*., 2021). However, no *in vitro* culture for the reproduction of *S. willdenowii* has been conducted. In the present research, a newly developed method was used, which could be applied to other *Selaginella* species to improve the success and effectiveness of propagation.

Article History
Accepted: 11 September 2025
First version online: 30 September 2025
Cite This Article:
Nadhirah, N., Maideen, H., Zuraida, A.R., Aliah, N. Nazreena, O.A. 2025. In vitro shoot-tip propagation of blue peacock fern *Selaginella willdenowii* P.Beauv. (Selaginellaceae) an ornamental fern. Malaysian Applied Biology, 54(3): 130-134. https://doi.org/10.55230/mabjournal.v54i3.3480
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MATERIALS AND METHODS

Basal medium

Explant had been used to germinate in a Murashige and Skoog (1962) medium with macronutrients at half strength. All treatments' medium is pH-adjusted to 5.8 and consists of 3% (w/v) sucrose and 0.4% (w/v) plant agar before autoclaving at 121°C for 15 min.

Explant collection

Selaginella willdenowii plants were collected from Taman Paku Pakis, Universiti Kebangsaan Malaysia (UKM). The plant's shoot tip (0.5 cm in length) was used as an explant to induce shoots and stimulate adventitious shoot growth (Figure 2-a).

Effective sterilization of explant

Before the sterilization procedure, the shoots were rinsed for a few min under running tap water. Next, the shoots were soaked in fungicide (Benex) with one to three drops of Tween-20 as a wetting agent in 20 mL of distilled water for 5 min. After that, the shoots were immersed in Mercury chloride ($HgCl_2$) for 10 min in the four treatments with different concentrations of $HgCl_2$ (0.05%, 0.1%, 0.1% & 0.5%). The shoots were then air-dried on filter paper after washing three times with sterile distilled water. Finally, the shoot tips were inoculated on ½ MS media (Murashige and Skoog, 1962) with 3% sucrose as basal medium (BM). The pH of the media was adjusted to 5.8±0.1 before being autoclaved at 121°C for 15 min. The shoot culture was incubated in the culture room for 16 hr at 25±2°C with a photoperiod of 16 hr and a light intensity of 3000 lux. To sustain all cultures, subculturing was done every four to six weeks.

Effect of various PGRs on *In Vitro* propagation

After four weeks of culturing, the young shoots were observed on agar-cultured media. Then the young shoots were cultured on with 0.5 mL^{-l} of PGR such as a-Naphthaleneacetic acid (NAA), Benzyl aminopurine (BAP), 2,4-Dichlorophenoxyacetic acid (2,4-D), Kinetin (K), and Gibberellins (GA_3). Half-strength of MS medium without PGR was used as a control. After 12 weeks of cultivation, the number of shoots, their final lengths, and the number of rhizomes were measured (Figure 1- a, b, c, d, e & f).

Data analysis

All the data were analysed using Analysis of Variance (ANOVA). The means differing significantly were compared using Duncan's Multiple Range Test (DMRT) at 5% probability. The experiments were carried out three times with ten replicates each. Results were expressed as mean ± standard error (SE) for sterilization and mean ± standard deviation (SD) for PGR effects on shoot-tip proliferation and organ formation.

RESULTS AND DISCUSSION

The current study is the first study of *S. willdenowii* shoot propagation *in vitro*. Even ferns can cultivate and propagate *in vitro* using various explant forms. Similar explants (shoot tips) were used in both the current research and Park *et al*. (2020) study of *S. martinsii*. However, according to Yu *et al*. (2021), their study used frond tips as an explant for *S. pulvinata*.

The young shoot was produced within 4-5 weeks of culture. Sterilization is the first step for aseptic culture in these studies. Treatment with 0.05% results in maximum contamination (>90%), and no growth has been observed. Treatments with 0.1% and 0.5% $HgCl_2$ resulted in minimal contamination (<40%), while growth was very high (>90%) for 0.1 %$HgCl_2$ but lower growth rates (<10%) with 0.5% $HgCl_2$. Treatments with 1.0 $HgCl_2$ result in minimal contamination (<10%), and no growth was recorded (Table 1). Hence, 0.1 % $HgCl_2$ was selected as the optimum concentration for the sterilization procedure, influencing germination.

Table 1. Descriptive result that represents the mean ± SE of the percentage shoot growth and contamination from the sterilization method with different concentrations of $HgCl_2$

Disinfectant	Concentration (mgL)	Contamination (%)	Growth (%)	Remarks
		Mean ±SE	Mean ±SE	
$HgCl_2$	0.05	97.3±1.25^{d}	0.00±0.00^{a}	Fungus growth
	0.1	38.0±1.69^{c}	93.4±1.44^{c}	Germinate
	0.5	14.0±1.63^{b}	10.5±2.16^{b}	Germinate
	1.0	2.60±0.87^{a}	0.00±0.00^{a}	Over sterile

#The mean number of explants is shown as mean ± SE, and different letters indicate significant differences at α=0.05.

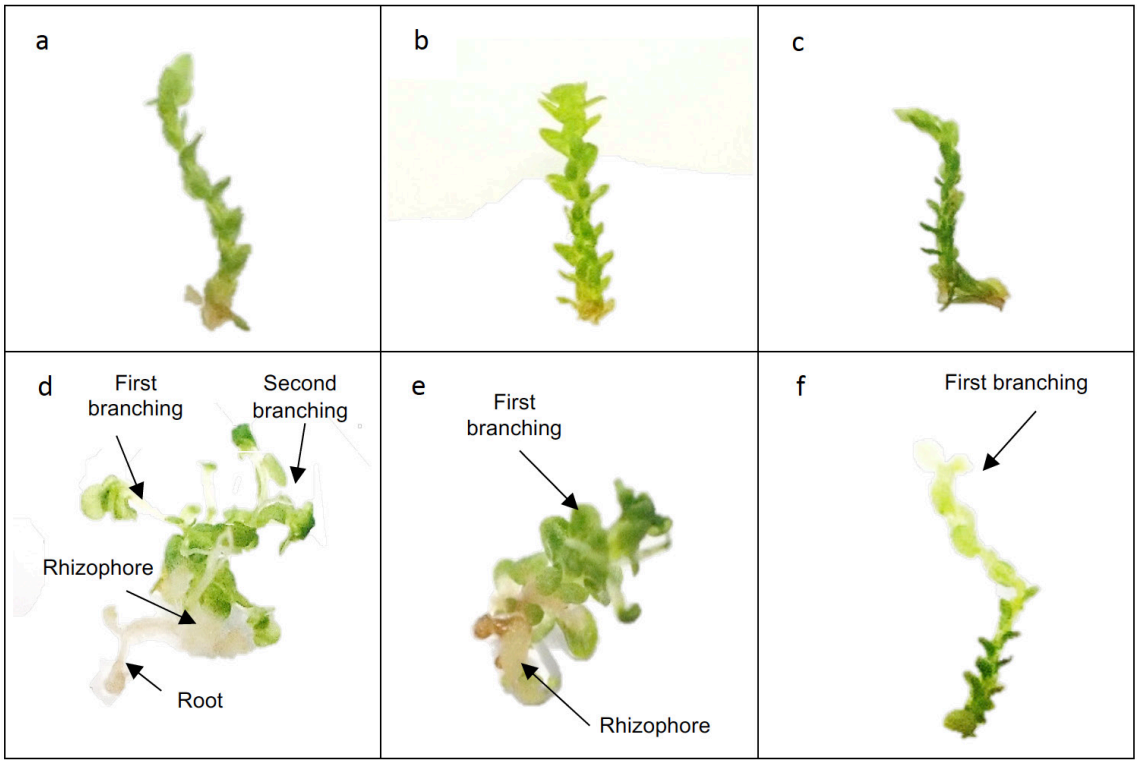

Fig. 1. Shoot tips grow in a medium containing various types of PGR for three months: (a) media 1/2 MS, (b) 0.5mg/L BAP, (c) 0.5mg/L K, (d) 0.5mg/L GA_3, (e) 0.5 mg/L NAA, and (f) 0.5 mg/L 2,4 D.

Table 2 represents the mean percentages of shoot length, number of shoots, and number of rhizomes after three months of culture. Shoot tips grow in a medium containing various types of PGR (Figure 1). The medium GA_3 supplement generated the most significant results for the length of shoots (1.74±0.054), number of shoots (5.50±0.60), and number of rhizomes (3.20±0.32), respectively. This treatment resulted in the formation of the first branching and rhizophore, followed by the second branching and root. The second significant medium was NAA, which promoted branching and rhizophore development, resulting in shoot length (0.95±0.04), number of shoots (1.90±0.23), and rhizome formation (1.70±0.15). However, 2,4 D supplement only led to rhizophore formation (1.00±0.00) and no shoot formation (0.00±0.00). No new or first branching of shoots and rhizomes were developed in the 1/2 MS, 0.5 mgL^{-1} BAP, and 0.5 mgL^{-1} K media, and the mean length of shoot tips was 0.41±0.02, 0.67±0.05, and 0.75±0.03, respectively. Therefore, further optimization of the medium was based on the medium GA_3 supplement.

Since *Selaginella* is a heterosporous ferns that produce both microspores and megaspores, the timing of the spores should match to produce gametophytes. In this study, shoot tips cultivated on GA_3-containing media influenced the success in the length of the shoot, number of shoots, and number of rhizomes (Table 2). However, previous studies of other species in this genus had resulted in successful culture in different PGR. For example, *S. martensii* was successfully cultivated in MS medium, producing a maximum of 6.77 nodes per explant, and developed two new shoot tips (Park *et al*., 2021). While in *S. pulvinata*, half-strength (1/2) MS medium supplemented with 0.1 mg L^{-1} and 1.0 mgL^{-1} N6-benzyl amino purine (BAP) resulted in the highest induction rate of original shoots and the most effective medium for the proliferation of adventitious shoots, while quarter-strength (1/4) MS containing 0.1% (w/v) active charcoal (AC) was ideal for plantlets proliferated from adventitious shoots and plantlet growth (Yu *et al*., 2021). Next, in *S. tamascarina*, the highest number of sporophytes (65.7) was obtained with ¼ MS medium (Park *et al*., 2021).

Table 2. Effect of different types of PGR on shoot-tip proliferation response and organ formation of *Selaginella willdenowi*

Concentration PGRs (mgL^{-1})	Organ Formed (After 3 months)	Length of shoots (Mean±SD)	Number of shoots (Mean±SD)	Number of rhizomes (Mean±SD)	Survival rate (%)
½ MS (Control)	-	0.41±0.02^a	0.00±0.00^a	0.00±0.00^a	70
0.5 mgL^{-1} BAP	-	0.67±0.05^b	0.00±0.00^a	0.00±0.00^a	80
0.5 mgL^{-1} K	-	0.75±0.03^b	0.00±0.00^a	0.00±0.00^a	80
0.5 mgL^{-1} GA_3	First branching shoot, Second branching Rhizophore, Root	1.74±0.054^d	5.50±0.60^c	3.20±0.32^d	100
0.5 mgL^{-1} NAA	First branching shoot, Rhizophore,	0.95±0.04^c	1.90±0.23^b	1.70±0.15^c	100
0.5 mgL^{-1} 2,4 D	Rhizophore	1.06±0.05^c	0.00±0.00^a	1.00±0.00^b	70

Results represent mean±standard deviation (SD)

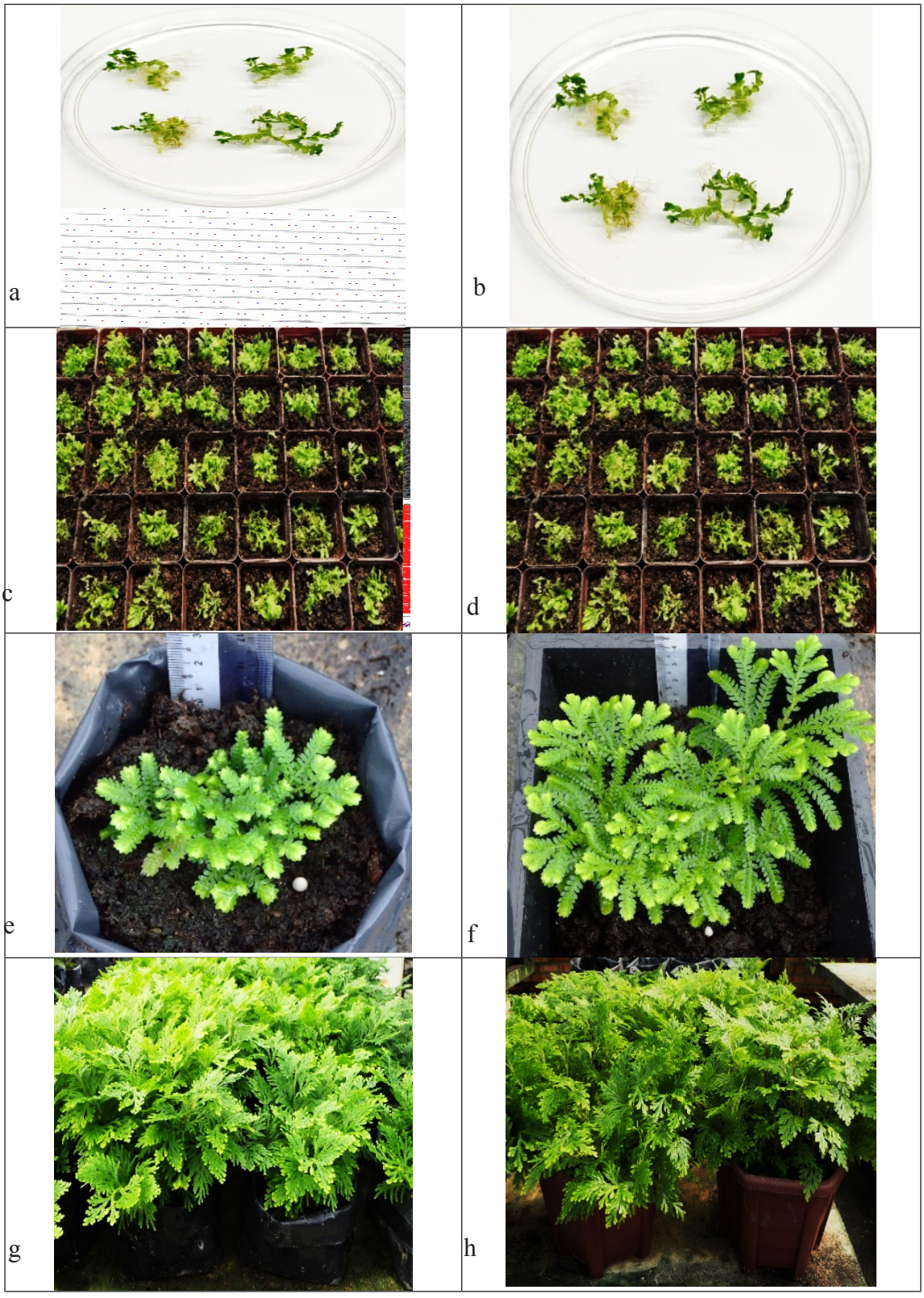

Fig. 2. The effect of efficient PGR (0.5 mg/L GA_3) on the growth and weight of *S. willdenowii*. (a) Explant, (b) Shoot growth after eight weeks of culture, (c) Growth of shoot and rhizophore after three months of treatment with PGRs, (d) Acclimatized plantlet planted in 72-hole plug, (e-h) Plantlets were transferred into polybags and placed in the glasshouse.

Regenerated plantlets were transferred onto the most efficient agar media (0.5 mg/L GA_3) to maintain this species culture. Then, the plantlet will be planted in a glasshouse onto a combination of peat moss and vermiculite (3:1) for 12 weeks (Figure 2-f) after the acclimatization phase (Figure 2-b & c) and maintained at about 75% shading in the glasshouse (Figure 2-d, e & f).

CONCLUSION

In summary, this study developed an efficient in vitro shoot-tip propagation protocol for *S. willdenowii*, an ornamental fern known for its medicinal properties. The study found that the optimal sterilization method was 0.1% $HgCl_2$, followed by 0.5% $HgCl_2$ in the initial culture MS medium. The most successful culture medium for this species was 0.5 mg/L GA_3 medium, followed by 0.5 mg/L NAA medium, in terms of succession towards length, number of shoots, and number of rhizomes. The protocol was optimized for reliable and scalable shoot multiplication, advancing the field of fern propagation. This methodology makes it possible to produce large-scale production consistently and without contaminants. It encourages further research into this species' medicinal components, along with potential medicinal uses.

ACKNOWLEDGEMENTS

This research was funded by the Universiti Kebangsaan Malaysia (UKM), and the Ministry of Higher Education provided the grant number FRGS/1/2020/WAB11/UKM/02/1. We appreciate the collaboration and involvement of the herbaria curators and the Bangi Botanical Garden.

ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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