

Gametophyte Propagation and Sporophyte Formation of Asian Chain Fern [*Woodwardia japonica* (L. f.) Sm.] Under Various Medium Conditions *In Vitro* and *Ex Vitro*

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Abstract - This study investigated a suitable method that could be applied for Asian chain fern [*Woodwardia japonica* (L. f.) Sm.] to propagate gametophytes and promote sporophyte formation. The gametophytes used in all experiments were obtained from germinated spores *in vitro* and were subcultured at 8-week intervals. The most appropriate media for gametophyte propagation was identified by culturing 300 mg of gametophyte in Murashige and Skoog (MS) basal medium (1/8, 1/4, 1/2, 1, 2), and Knop medium for 8 weeks. As a result, fresh weight of the gametophyte was increased by 56.7-fold on MS medium. Moreover, antheridium formation as well as gametophyte growth was improved on MS medium, especially. To improve the sporophyte formation *ex vitro*, 1.0 g of gametophyte was ground with distilled water and spread on eight combinations onto four different culture mediums, such as bed soil, peat moss, perlite and decomposed granite. Then generation and growth of sporophytes were investigated after cultivation for 10 weeks. As a result of this experiment, peat moss had a promotive effect of sporophyte formation at single-use and mixed culture soils. In particular, a mixture of bed soil, peat moss and perlite in a 1:1:1 ratio (v/v/v) led to the accelerated formation (782.5 ea/pot) and the frond growth of sporophytes. This included increases in length and width of fronds. However, promotive effect of gametophyte growth and sporophyte formation was not found at single-use and treatment with high ratio of bed soil.

Key words – Antheridium, Blechnaceae, MS medium, Peat moss, Pteridophyte

Introduction

The Asian chain fern [*Woodwardia japonica* (L. f.) Sm.], native to Korea, is an evergreen perennial that is classified as a large type species among pteridophytes. It is present only in Asia, in countries such as China, Japan, and Vietnam, and a few are found in Jeju and Jeollanam-do in Korea. The rhizome is thick and stands at an angle, with dense brown lanceolate scales. The leaves are radical and the petioles are thick, hard, and 30-50 cm long. The leaf blade is elliptical, ovate, 40-80 cm long, 20-35 cm wide, and split. Split leaves appear in 10-15 pairs, and are linear, lanceolate, feather shaped, split to the center, pointed, and green. The leaf backside is light green, with brown scales on the veins. The

sorus is 2-5-mm long, close to the central leaf vein, and spore formation occurs from July to September (KBIS, 2019).

Habitats of the Asian chain fern are rich in litter layers in forests in the mountains; thus, the fern can grow in shade. Recently, owing to its ornamental value the collection of Asian chain fern has been indiscriminate. As a result, only young individual plants are found in their natural habitats (KBIS, 2019); in 2017, it was categorized under endangered wildlife class II and evaluated as a vulnerable species (VU), which is an endangered category in the Korean Red List (BK, 2019). Therefore, a preservation plan for this species is urgently required.

Ferns are increasing in demand due to their high utilization. Some species, such as *Microlepia strigose* (Cho and Lee, 2017), *Cheilanthes argentea*, *Athyrium sheareri* (Jang *et al.*, 2019a; 2019b), *Arachniodes aristata* (Cho *et al.*, 2017), *Polystichum braunii* (Kwon *et al.*, 2017), and

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Leptogramma pozoi (Lee *et al.*, 2019) have a mass production system. However, most of these systems are not well established. In addition, because pteridophytes are unlike spermatophytes, suitable proliferation methods must be determined at each of the two growth stages, namely, the gametophyte and sporophyte. Tissue culture techniques and container propagation methods must be performed in parallel to achieve effective growth.

The purpose of this study was to investigate suitable conditions for gametophyte proliferation and sporophyte production that produce a large number of individuals in order to preserve the species and enhance the utilization of the ornamental value of the Asian chain fern.

Materials and Methods

Plant material

Plants were collected from Seogwipo-si, Jeju-do, South Korea (lat. 33°18'0.59"N, long. 126°34'31.9"E) and transplanted into a plastic-film greenhouse under 70% shading at Chungbuk National University, Cheongju-si, Korea (lat. 36°37'29.0"N, long. 127°27'18.4"E). Sporophylls were harvested on August 18, 2014 and spores were selected using the method of Cho and Lee (2017). Collected spores were sealed in a vial bottle and stored at a low temperature ($4 \pm 1^\circ\text{C}$).

Spore germination *in vitro*

To obtain gametophytes for the experiment, spore germination was performed according to methods described by Cho *et al.* (2017). The supernatant was removed by centrifugation (HA-12, Hanil Science Industrial, Incheon, Korea) for 3 min in a suspension of 50 mg of selected spores immersed in 15 mL of distilled water for 24 h. This was sterilized for 13 min by adding 1.4% sodium hypochlorite solution, and then this was washed five times with sterile water. The spore solution was prepared by adding 15 mL of sterile water to the sterilized spores.

For spore germination, 30 mL of Knop medium, supplemented with 3.0% (w/v) sucrose and 0.8% (w/v) agar and adjusted to pH 5.8, was dispensed in 8.9 mm Petri dishes. After the mixture solidified, 1 mL of sterile water and 1 mL of prepared spore solution were inoculated together. The spores

were germinated at $25 \pm 1^\circ\text{C}$ under a light intensity of $30 \pm 1.0 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (16/8 h photoperiod). Gametophytes obtained from germinated spores were subcultured in MS medium at 2-week intervals.

Gametophyte proliferation

MS medium, adjusted to concentrations of 1/8, 1/4, 1/2, 1, and $2 \times$ the original concentration, and Knop medium (0.5% sucrose, 0.8% agar, and pH 5.8) were applied. Gametophytes in a uniform state and secured by subculture were chopped into 300 mg pieces using a scalpel and inoculated into prepared media, followed by the addition of sterile water. The inoculated media bottles were placed in a culture room at $25 \pm 1.0^\circ\text{C}$ under a light intensity of $43 \pm 2.0 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (16/8 h photoperiod). All treatments were performed in four replicates, and after 8 weeks of incubation, the fresh weight, development, and morphogenesis of each gametophyte were observed using an electronic scale and a stereomicroscope.

Sporophyte formation

To establish suitable substrate conditions, methods using blender (Cho and Lee, 2017) were referred to for sporophyte formation. Eight soil mixtures of the same composition used in Cho *et al.* (2017) study were prepared using horticultural substrates, peat moss, perlite, and decomposed granite (Table 1), and the chemical properties of each soil were the same as in the results of Cho and Lee (2017). The physical properties of each soil are: commercial bed soil (horticultural substrates, Hanareum no. 2; Shinsung Mineral Co., Ltd., Korea), 60-80% water holding capacity; peat moss (SunShine, Sun Gro Horticulture, Canada), 94.2-95.2% porosity; perlite (Newpershine no. 2; GFC. Co., Ltd., Korea), 20% available water, pH 6.0-7.0, over $1000 \text{ mm}\cdot\text{Hr}^{-1}$ coefficient of permeability; decomposed granite (Samgye Masato, Korea), 2 mm particle size.

Square pots ($75 \times 75 \times 75 \text{ mm}$) were filled with the prepared soil mixtures and placed in a plastic box ($503 \times 335 \times 195 \text{ mm}$). *In vitro*-cultured gametophytes were sterilized for 1 h with a $1000 \times$ fungicide solution (Hymexazol 30%; Tachigaren, Dongbu Agrotech, Korea) and washed five times with distilled water. Sterilized gametophytes (1 g) were added to 25 mL of distilled water in a plastic beaker and were ground

Table 1. Soil compositions used for sporophyte production

Treatment	Soil composition
B1	Bed soil only
Pt1	Peat moss only
B2-Pr1	Bed soil : Perlite = 2:1 (v:v)
B2-D1	Bed soil : Decomposed granite = 2:1 (v:v)
Pt2-Pr1	Peat moss : Perlite = 2:1 (v:v)
Pt2-D1	Peat moss : Decomposed granite = 2:1 (v:v)
B1-Pt1-Pr1	Bed soil : Peat moss : Perlite = 1:1:1 (v:v:v)
B1-Pt1-D1	Bed soil : Peat moss : Decomposed granite = 1:1:1 (v:v:v)

for 10 s using a hand blender (V-8000, Boowon, Korea). Then, ground gametophytes were spread uniformly on top of the soil mixtures and the boxes were covered with glass plate. These plastic boxes were incubated for 10 weeks at $25 \pm 1^\circ\text{C}$ under a light intensity of $43 \pm 2.0 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (16/8 h photoperiod).

During incubation, subirrigation was performed to a height of 1 cm in each plastic box, and a relative humidity of $72 \pm 2\%$ was maintained. After a young gametophyte formation was observed, water was sprayed daily on the surface of the gametophyte to facilitate the formation and development of sporophytes. All treatments were performed in four replicates and, after incubation for 10 weeks, the number of sporophytes generated by each pot was counted. Finally, ten sporophytes were selected from each pot and examined.

Statistical analysis

SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) was used to calculate the mean \pm standard error for each treatment, and a factorial analysis was performed using Duncan's multiple range test, with a significance level of $p < 0.05$.

Results and Discussion

Effect of medium on gametophyte proliferation

Gametophytes in a uniform state that were obtained from germinated spores were inoculated on several media and incubated for 8 weeks. Gametophyte proliferation was the highest in $1 \times$ MS medium as the fresh weight increased 56.7-fold (Fig. 1). Gametophyte proliferation decreased

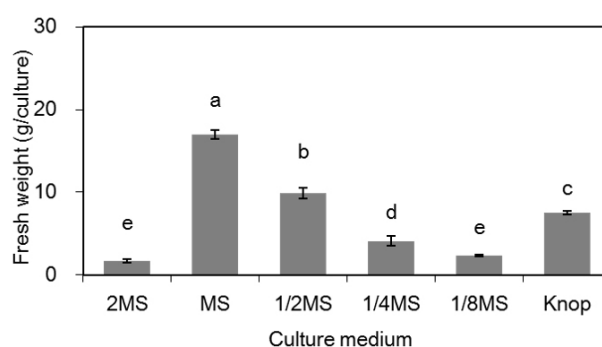


Fig. 1. Effects of culture medium on gametophyte fresh weight in *Woodwardia japonica* (L.f.) Sm. cultured for 8 weeks. Bars represent standard error (n=4). Different letters indicate significant difference based on Duncan's multiple range tests at $p < 0.05$.

significantly as the total concentration of MS medium decreased. However, $2 \times$ MS medium, with the highest nutrient content, showed the lowest biomass increase of 5.7-fold. The fresh weight of gametophytes in the Knop medium was 7.5 g, which was greater than that in the 1/4 MS medium.

The gametophytes of ferns, classified as leptosporangiate, grow well in low-nutrient media (Fernández and Revilla, 2003). Among ferns native to Korea, gametophytes of *Pteridium aquilinum* var. *latiusculum* show excellent growth and proliferation in medium containing high nutrient content such as $2 \times$ MS medium (Shin *et al.*, 2009). *A. aristata* shows remarkable proliferation in MS media (Cho *et al.*, 2017). Gametophytes of *Microlepia strigosa* proliferate actively in $2 \times$ MS and $1 \times$ MS media; however, the proliferation decreases as the total concentration of medium decreases (Cho and Lee,

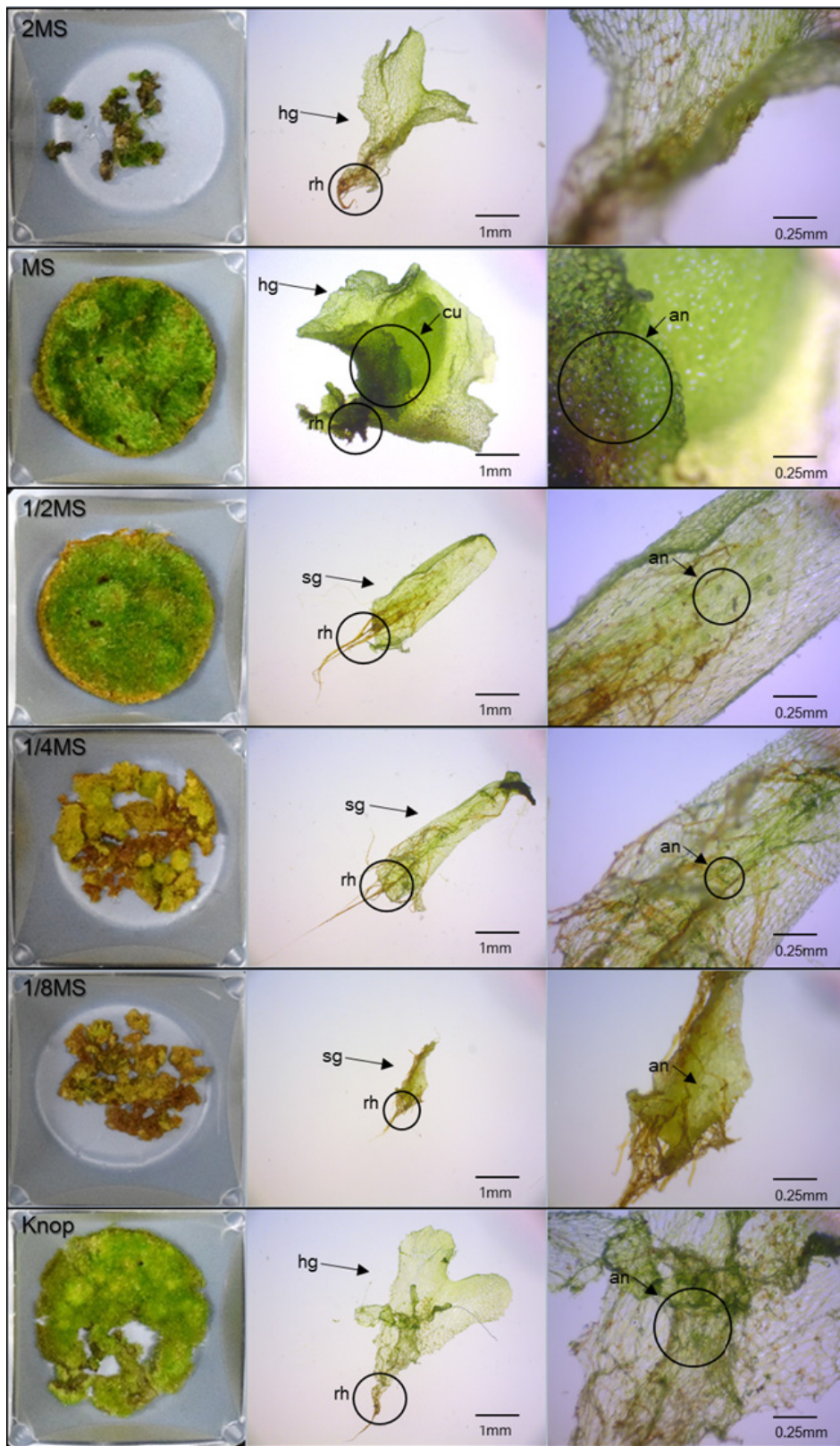


Fig. 2. Cultural response and organ formation of gametophyte of *Woodwardia japonica* (L. f.) Sm. cultured for 8 weeks in different media. Abbreviations: an, antheridium; cu, cushion; hg, heart shaped gametophyte; rh, part of rhizoid; sg, spatulate gametophyte.

2017), which is similar to the proliferation of the Asian chain fern in this study. In *Osmunda japonica*, the growth of gametophytes is active in Knop and 1/8 MS media (Shin and Lee, 2009), which is in contrast to this study.

Therefore, the demand for nutrients for the proliferation of gametophytes does not show a constant trend according to taxa (Shin *et al.*, 2009). For the development of a mass proliferation method for ferns using gametophytes, the selection of an appropriate medium according to species should be considered.

Difference in the development of gametophytes according to medium

In the observation of the morphogenesis of gametophytes according to medium, the mature heart shaped gametophyte with wings developed in 2 × MS, 1 × MS, and Knop media, and cushion was observed only in the 1 × MS medium (Fig. 2). Cushion is a multicellular layer that develops in the center of the gametophyte. It is expressed in dark green plants and is observed in mature gametophytes in which the reproductive organ develops normally (Shorina, 2001). Therefore, the presence of a cushion can be regarded as a standard of healthy gametophytes. In the 1/2, 1/4, and 1/8 MS media, spatula shaped gametophytes, which is the stage preceding heart shaped gametophytes, were observed. In 1/2, 1/4, 1/8 MS, and knop media, gametophytes were thin and some browned areas were observed; thus, this state was considered to be a sign of aging caused by the low content of minerals and carbon in the media (Shin and Lee, 2009).

The gametophyte develops by zygosis of the antheridia and archegonia, which are reproductive organs and develops in the form of hermaphrodite. The *in vitro* fragmenting and chopping methods that were used in this study enabled the mass propagation of uniform gametophytes, even with a small amount of gametophyte tissue (Miller, 1968). Therefore, to increase the number of normal gametophytes, the development of the reproductive organs, antheridia and archegonia, should be induced. Antheridiogen, a hormone with a skeletal structure similar to that of gibberellin, induces male organ (antheridium) development in juvenile gametophytes (Menéndez *et al.* 2006). Yamane (1998) reported that addition of exogenous gibberellin induces the formation of

antheridium. In a study by Carafa (1990), temperature did not affect the pattern of development of gametophytes on *Woodwardia radicans* (L.) Sm.; however the sowing density, presence of antheridiogen in the culture medium, and nutritional conditions affected sex expression in the gametophyte.

The concentration of nutrient in medium appears to have a direct effect on ferns gametophyte weight, indicative of its proliferation. The *Dryopteris affinis* sp. *affinis* L. exhibited greater prothallus growth when cultured on MS medium (Fernández *et al.*, 1996). *Arachniodes aristata* (G. Forst.) Tindale prothalli grew most actively in MS medium, but poor growth was showed in rich (2 ×) or lacking (1/2, 1/4, and 1/8 ×) nutrients medium than MS medium (Cho *et al.*, 2017).

In this study, cushion, the developmental background of the reproductive organs, and antheridium were identified in the 1 × MS medium. Therefore, the appropriate medium for the induction of normal development and proliferation of gametophytes in Asian chain ferns was determined to be the 1 × MS medium.

Effect of soil types on sporophyte production

To generate a large number of sporophytes of the Asian chain fern, ground gametophytes were spread on top of the soil mixtures and cultivated for 10 weeks. Sporophyte formation was the greatest in a mixture containing a 1:1:1 (v/v/v) ratio of bed soil, peat moss, and perlite (B1-Pt1-Pr1) or decomposed granite (B1-Pt1-D1), at 782.5 and 708.8 ea/pot, respectively (Table 2). No sporophyte formation occurred with the single use of horticultural soils (B1) and sporophyte formation was achieved at 18.0-68.8 ea/pot in a mixture containing a 2:1 ratio of bed soil and perlite (B2-Pr1) or decomposed granite (B2-D1). The sporophyte formation was at 223.5 ea/pot with the single use of peat moss (Pt1), and the number of sporophytes did not increase when perlite (Pt2-Pr1) or decomposed granite (Pt2-D1) was added to peat moss (v/v), which was different from the trend in bed soil.

The fresh weight of the sporophyte formed on the aerial part was high in B2-D1 and B1-Pt1-Pr1, and on the underground part was high in B1-Pt1-D1, which was similar to the dry weight analysis (Table 3). The shoots and leaves of sporophytes in B1-Pt1-Pr1 were significantly longer than those in other treatments (Fig. 3). In contrast, sporophyte

Table 2. Effect of soil types on sporophyte formation and biomass of *Woodwardia japonica* (L.f.) Sm. cultivated for 10 weeks *ex vitro*

Medium ^z	No. of sporophyte / pot ^y (ea)	Fresh weight (mg/sporophyte)		Dry weight (mg/sporophyte)	
		Aerial part	Underground part	Aerial part	Underground part
B1	0.0 ± 0.00 c ^x	-	-	-	-
Pt1	223.5 ± 15.71 b	3.8 ± 0.71 cd	3.2 ± 0.88 c-e	0.7 ± 0.07 c	0.4 ± 0.09 b
B2-Pr1	18.0 ± 14.76 c	5.0 ± 0.85 bc	1.1 ± 0.45 de	0.8 ± 0.14 bc	0.2 ± 0.06 bc
B2-D1	68.8 ± 50.31 c	7.1 ± 0.71 a	4.7 ± 1.64 cd	1.2 ± 0.13 a	0.4 ± 0.11 b
Pt2-Pr1	180.0 ± 31.28 b	2.3 ± 0.53 de	1.8 ± 0.35 c-e	0.5 ± 0.08 c	0.2 ± 0.02 bc
Pt2-D1	77.8 ± 16.64 c	2.0 ± 0.14 e	9.7 ± 2.11 b	0.7 ± 0.10 bc	0.8 ± 0.16 a
B1-Pt1-Pr1	782.5 ± 27.91 a	5.6 ± 0.83 ab	5.3 ± 1.84 c	1.0 ± 0.20 ab	0.4 ± 0.10 b
B1-Pt1-D1	708.8 ± 33.50 a	3.0 ± 0.13 de	15.6 ± 1.32 a	0.8 ± 0.04 bc	0.8 ± 0.13 a

^zRefer to Table 1.

^yPot (75 × 75 × 75 mm) containing 0.3 L culture medium was used for this experiment.

^xMean ± S.E. (n=10) separation within columns by Duncan's multiple range test at *P* < 0.05.

Table 3. Effect of soil types on sporophyte growth of *Woodwardia japonica* (L.f.) Sm. cultivated for 10 weeks *ex vitro*

Medium ^z	Shoot length (mm)	Shoot width (mm)	No. of leaves (ea)	Leaves length (mm)	Leaves width (mm)	SPAD
B1	-	-	0.0 ± 0.00 c	-	-	-
Pt1	10.3 ± 0.75 de ^y	10.5 ± 1.18 cd	2.2 ± 0.13 ab	11.9 ± 0.80 c	5.8 ± 0.51 bc	8.0 ± 1.43 bc
B2-Pr1	11.4 ± 1.14 cd	10.8 ± 1.32 c	2.1 ± 0.10 ab	13.0 ± 1.07 bc	6.6 ± 0.63 b	8.7 ± 0.94 bc
B2-D1	14.2 ± 0.87 b	18.8 ± 1.04 a	2.3 ± 0.15 a	18.8 ± 0.80 a	8.4 ± 0.62 a	13.1 ± 1.41 a
Pt2-Pr1	8.2 ± 0.77 e	8.6 ± 1.16 cd	1.8 ± 0.13 b	9.6 ± 0.58 d	4.9 ± 0.35 c	7.7 ± 1.06 bc
Pt2-D1	9.8 ± 0.56 de	7.5 ± 0.49 d	1.9 ± 0.10 b	11.1 ± 0.42 cd	5.4 ± 0.30 bc	6.8 ± 0.59 c
B1-Pt1-Pr1	18.1 ± 0.95 a	15.6 ± 1.26 b	2.1 ± 0.10 ab	19.4 ± 0.66 a	8.6 ± 0.47 a	10.3 ± 0.76 b
B1-Pt1-D1	12.8 ± 0.53 bc	9.4 ± 0.48 cd	1.8 ± 0.20 b	13.9 ± 0.42 b	8.4 ± 0.23 a	6.5 ± 0.25 c

^zRefer to Table 1.

^yMean ± S.E. (n=10) separation within columns by Duncan's multiple range test at *P* < 0.05.

development did not progress at all because the growth of gametophyte was not confirmed in B1. In addition, a large number of browned sporophytes was observed in B2-Pr1 and B2-D1, which had relatively high ratios of bed soil.

A favorable soil environment for the growth of crops can be created by controlling porosity, air permeability, and water holding capacity (Choi *et al.*, 1997). In native ferns, terrestrial species generally show vigorous sporophyte formation in commercial bed soil, and the mixture soil, which has improved air permeability by adding vermiculite or perlite, is favorable to sporophyte formation in epiphytic species (Lee, 2001). *M. strigosa*, an epiphytic species, shows excellent sporophyte formation and growth in mixture soil containing at a 1:1:1 (v/v/v) ratio of bed soil, peat moss, and decomposed

granite (Cho and Lee, 2017). However, in *C. argentea*, a small epiphytic fern that grows in rock and stone cracks, the formation of sporophytes is promoted in soils mixed with bed soils and perlite at a 2:1 (v/v) ratio (Jang *et al.*, 2019a); thus, there is a difference in the composition of soils that can promote the formation of sporophytes among species of epiphytic fern. In Cho *et al.* (2017), gametophytes of *A. aristata* do not develop to the sporophyte in the peat moss. In *P. braunii*, the most sporophytes were formed in mixture soil with bed soil and granite at a 2:1 (v/v) ratio (Kwon *et al.*, 2017).

The Asian chain fern is an epiphytic species that grows on rocks and trees (BK, 2019). Therefore, the mixture of bed soil, peat moss, and perlite in a 1:1:1 ratio (v/v/v) led to the

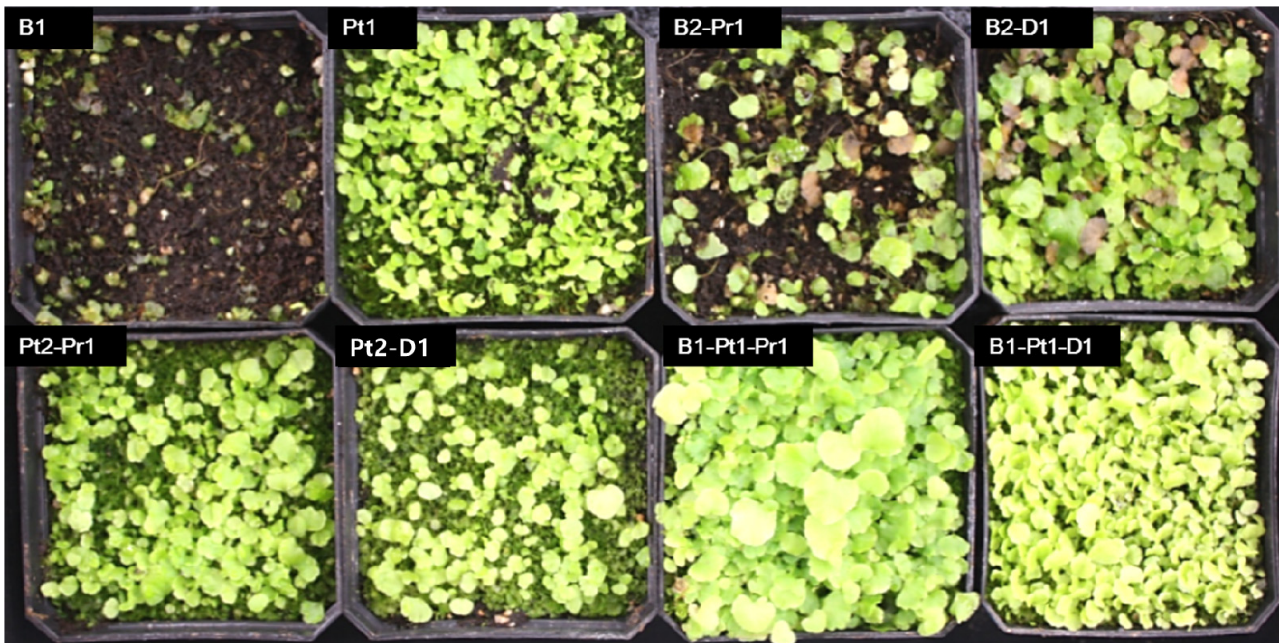


Fig. 3. Gametophyte proliferation and sporophyte formation of *Woodwardia japonica* (L. f.) Sm. cultivated for 10 weeks *ex vitro*. B, bed soil; Pt, peat moss; Pr, perlite; D, decomposed granite, and the number next to each alphabet means the weight ratio.

accelerated formation and growth of sporophytes in this study. According to an analysis by Jang *et al.* (2019a), in the same bed soil and peat moss as used in this study accounts for more than 80% of the water phase. Therefore, the water phase content must be adjusted for the production of Asian chain fern using bed soil and peat moss. However, perlite is standardized and cheaper than the same volume of decomposed granite; thus, perlite is more suitable for the economical production of sporophytes.

In optimal soils, sporophytes of the Asian chain fern had a greater production (782.5 ea/pot) from the same input of gametophytes than that in *M. strigosa* (44.1 ea/pot, Cho and Lee, 2017) and *C. argentea* (74.3 ea/pot, Jang *et al.*, 2019a); thus, the production efficiency was high in the Asian chain fern. Consequently, the soil mixture containing a 1:1:1 (v/v/v) ratio of bed soil, peat moss, and perlite (B1-Pt1-Pr1) was suitable for the proliferation and development of gametophytes and sporophytes by the maintenance of adequate water holding capacity and air permeability. The results of this study will contribute to farm household income by enabling the mass production of uniform seedlings of the Asian chain fern, which are valuable as ornamental horticultural crops.

Acknowledgments

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