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MICROPROPAGATION

Practical methodology for gametophyte proliferation and sporophyte production in green penny fern (*Lemmaphyllum microphyllum* C. Presl) using mechanical fragmentation

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Abstract

Propagation of gametophytes and sporophytes using mechanical fragmentation has been considered a suitable method for mass production of ferns. This study aimed to develop a practical propagation method for *Lemmaphyllum microphyllum* C. Presl, which is a fern of significant ornamental and medicinal value. Gametophytes were obtained through *in vitro* spore germination and used for propagation experiments. The gametophyte was mechanically fragmented using a scalpel into small fragments, which were then used to investigate gametophyte proliferation. In addition, the gametophyte was fragmented using a blender and then used to study sporophyte formation. Optimal proliferation conditions of the gametophyte were determined using Murashige and Skoog (MS) basal medium (double-, full-, half-, quarter-strength), Knop medium, and medium components (sucrose, nitrogen sources, activated charcoal), at various concentrations. The fresh weight of the gametophyte was 14-fold higher than that of gametophytes (300 mg) used as culture material, when cultured on double-strength MS. Moreover, 1 g of the gametophyte fragmented in 25 mL of distilled water formed more than 430 sporophytes in a soil mixture in an area of 7.5 cm^2. The sporophytes were successfully cultivated in the greenhouse after acclimation. A large-scale production method for *L. microphyllum* that can be easily implemented in a fern production farm is outlined.

Keywords Gametophyte mechanical fragmentation · Green bean fern (green penny fern) · Homogenization · Mass propagation · Polypodiaceae

Introduction

Lemmaphyllum microphyllum C. Presl, from the family Polypodiaceae, is a warm and temperate evergreen perennial, which is epiphytic, and grows on rocks and tree trunks in mountains of China, Taiwan, Japan, South Korea, Vietnam, and NE India. Mature plants are very short, and the rhizome is long, and trails on the surface. There are two types of leaves, the trophophylls are orbicular or oval, and the sporophylls are broadly linear or narrowly oblanceolate, and longer than trophophylls. In South Korea, the spores mature between July and September. The sori are formed on both sides of the midrib of the sporophyll, and the spores mature without any membranes (Lee and Lee 2018). The sporophylls with mature sori (referred to as "green bean fern" in Korea), resemble a split bean and are referred to in the Korean language as "Kong-jja-gae-deong-gul." In the English language, the species is known as green penny fern, and named after its small leaves. The species is used as bonsai and in landscaping because of its high ornamental value (Kawano 2015). In oriental medicine, whole plants are used to treat rheumatoid arthritis, sores, and cough (Zheng and Xing 2009).

The propagation process of ferns starts with spores that germinate into a prothallus (gametophyte), and the formation of the reproductive organs known as antheridium and archegonium (Dyer 1979; Raghavan 1989; Banks 1999; Moran 2004). The sperm is released from the antheridium and transported to the egg in the archegonium, where it fertilizes the egg and forms a zygote (Gabriel y Galán and Migliaro 2011). This zygote divides and develops into a young

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sporophyte, which matures and forms spores, and completes the propagation process. Gametophyte cells have a very high regenerative capacity; new, identical gametophytes can be regenerated from fragments of gametophyte tissues (Ito 1962; Miller 1968). Due to this regeneration ability of the gametophyte, it is possible to mass-proliferate gametophytes in a short time, and induce sporophyte production from the fragmented gametophyte (Knauss 1976; Cooke 1979; Maeda and Ito 1981; Fernández *et al.* 1997). In South Korea, ferns (bracken), are grown by dividing 2- to 3-y-old roots. However, root colonization is difficult, time consuming, and labor intensive. The propagation method that uses the gametophyte is effective, because young sporophytes can be obtained in 10 to 14 wk from the fragmented gametophyte. Unlike the rhizome division method, the planned production of a sporophyte seedling is possible. In the present study, a mechanical fragmentation method is described, which uses a scalpel and hand blender to propagate gametophytes and produce sporophytes of *L. microphyllum* on a large scale. This method will enable production of this species throughout the year.

Materials and methods

Plant material Mature leaves of *Lemmaphyllum microphyllum* C. Presl were collected from Hogeun-dong, Seogwipo-si, Jeju-do, Korea (33° 17′ 30.1″ N, 126° 31′ 47.1″ E), in November 2016. The leaves were dried in a paper box at 25 ± 1°C for 1 wk, and the spores were removed and sieved through a 75 μm sieve (Chunggye Sieve, Gunpo, Korea), to remove impurities.

Spore germination Spore germination was conducted according to the methods described by Jang *et al.* (2019a). Spores (10 mg), were placed in a conical tube filled with 15 mL distilled water, and the tube was incubated at 4°C for 24 h. The spore solution was centrifuged (3 min, 1811×*g*), to remove the supernatant, sterilized with 1.4% (v/v) sodium hypochlorite (Yuhanrox; Yuhanclorox Co., Ltd., Hwaseong, Korea) for 13 min, and washed three times with sterilized water. Finally, the spore solution was diluted to 10 mg of spores per 30 mL of sterilized water. The spore solution (1 mL), was inoculated in Knop medium (Knop 1865), and spores were germinated at 25 ± 1°C, under a 16/8 h photoperiod, and light intensity of 30 ± 1.0 μmol m^{-2} s^{-1}. Prothalli obtained from the spores were subcultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962), at 8-wk intervals, and used for prothallus proliferation and sporophyte formation.

Prothallus proliferation For prothallus proliferation, the prothalli were chopped with a scalpel and cultured in various media (Jang *et al.* 2019b, 2019c). To identify the optimal media for prothallus proliferation, prothallus fragments were cultured on quarter-, half-, full-, and double-strength MS, and in Knop medium. Next, the selected optimal culture medium (Fig. 2*A*, double-strength MS medium), was amended with various concentrations of sucrose (0%, 1%, 2%, 3%, and 4%; w/v), a total nitrogen source [NH_4Cl (CAS 12125-02-9, Samchun Chemicals, Pyeongtaek, Korea) and KNO_3 (CAS 7757-79-1, Wako Pure Chemical Industries, Ltd., Osaka, Japan), 1:2; 20:40 (60), 40:80 (120), and 80:160 (240) mM], and activated charcoal (CAS 7440-44-0, Junsei Chemical Co. Ltd., Tokyo Japan) (0%, 0.2%, 0.4%, and 0.8%, w/v), to confirm the prothallus proliferation. Next, the cultures were incubated at 25 ± 1°C, under a light intensity of 30 ± 1.0 μmol m^{-2} s^{-1}, and 16/8 h photoperiod. Morphogenesis and the proliferation rate of the prothallus tissues were examined after incubation for 8 wk.

Sporophyte formation For sporophyte formation, 1.0 g of prothallus was ground with a hand blender for 10 s in 25 mL distilled water in a beaker. The fragments were cultured in various soil substrates (Jang *et al.* 2019b, 2019c). Five soil mixtures were prepared using horticultural substrate (Hs, Hanareum no. 2; Shinsung Mineral Co., Ltd., Goesan, Korea), peat moss (Pt, Sunshine; Sun Gro Horticulture, Vancouver, BC, Canada), perlite (Pr, Newpershine no. 2; GFC. Co., Ltd., Hongseong, Korea), and decomposed granite (D, 2 mm; Samgye Masato, Gimhae, Korea); pots (75 × 75 × 75 mm), were filled with this prepared soil and placed in a plastic box. The volume of each soil was measured at a 1:1 (v/v) ratio, and mixed according to five soil combinations. Hs alone, 2:1 mixtures of Hs and Pr, 2:1 mixtures of Hs and D, 1:1:1 mixtures of Hs, Pt and Pr, and a 1:1:1 mixture of Hs, Pt, and D. After sowing the ground prothalli over the soil, the box with the cultures was covered with a glass plate, and incubated at 25 ± 1°C, light intensity of 43 ± 2.0 μmol m^{-2} s^{-1}, and 16/8 h photoperiod for 14 wk. The pots were sub-irrigated, and the humidity was maintained at 84 ± 1.4%. After prothallus formation was observed, water was sprayed on the surface of the prothallus to induce fertilization.

Seedlings obtained from the sporophyte formation experiment were transplanted into a plastic pot, which contained horticultural substrates, and acclimated for 3 wk at 25 ± 1°C, light intensity of 43 ± 2.0 μmol m^{-2} s^{-1}, and 16/8 h photoperiod. Next, the seedlings were cultivated in the greenhouse located at the Chungbuk National University, Cheongju-si, Korea.

Data collection and statistical analysis Germinated spores and prothallus development were observed using a microscope (CKX53, SZ61; Olympus, Tokyo, Japan). Spore and prothallus images were captured using a CMOS camera (eXcope F630; Dixi Sci., Daejeon, Korea), and eXcope 3.7.12277 software. The success of sporophyte production was assessed by determining the number of sporophytes, the fresh weight, leaf

length, leaf width, number of leaves, root length, and number of roots. All experiments were performed in a completely randomized design. Four replicates were used to investigate prothallus proliferation, and four replicates with eight plants per replicate were used to assess sporophyte production. SAS version 9.4 (SAS Institute Inc., Cary, NC), was used to calculate the mean ± standard error for each treatment, and a factorial analysis was performed using Tukey's honestly significant difference (HSD) test, with a significance level of $p < 0.05$.

Results

Spore germination and prothallus development Spores obtained from the *Lemmaphyllum microphyllum* sporophyll collected in their natural habitat were germinated into gametophytes (Fig. 1*A*–*D*). The sowed spores were considered to be germinated after rhizoids and chloroplasts were observed. Spores germinated 9 d after sowing (Fig. 1*B*), and rhizoid elongation and primary filament development were observed 12 d after sowing (Fig. 1*C*). It also developed as a mature gametophyte 4 wk after sowing (Fig. 1*D*). The gametophytes that developed in spores (Fig. 1*D*), were morphologically identical to the subsequently cultured gametophytes, which were fragmented by scalpel (Fig. 1*E*). Afterwards, they were transferred to a full-strength MS medium rich in nutrients for rapid growth of the gametophytes and used as experimental material after subculture.

Effect of medium components on prothallus proliferation The *L. microphyllum* gametophyte was cut with a scalpel into fragments that were cultured on MS media of various strengths and Knop medium, to observe the changes in fresh weight of the regenerated prothallus. The fragments were able to effectively develop into many gametophyte clones in a short time (Fig. 1*F*). The fresh weight of the regenerating gametophytes increased with increasing concentrations of the MS medium; the highest fresh weight (4.3 g), was observed for gametophytes cultured on double-strength MS medium (Fig. 2*A*). Unlike spore germination, gametophyte proliferation required a high-concentration medium. The effect of the medium components was evaluated based on the effects on the control, which was double-strength MS medium. Nitrogen in the double-strength medium was present at a concentration of 120 mM. However, the fresh weight of the prothallus was higher when cultured in medium supplemented with 60 mM nitrogen than in medium with 120 mM nitrogen (Fig. 2*B*). The addition of sucrose increased the fresh weight of the gametophyte significantly compared with the medium without sucrose (Fig. 2*C*). Activated charcoal did not affect the fresh weight of regenerated gametophytes regardless of the concentration (Fig. 2*D*). The gametophyte cells, as observed with the naked eye, started browning or aging as the concentration of the MS medium decreased (Fig. 3*A*–*E*). Gametophyte cells cultured in the Knop medium also browned (Fig. 3*A* and *F*). As a result, *L. microphyllum* gametophyte proliferation was most effective in double-strength MS medium that contained 2% sucrose, 0% activated charcoal, and 60 mM of total nitrogen source.

Production of sporophyte seedlings The mixture containing a 2:1 (v/v) ratio of horticultural substrate and decomposed granite, increased sporophyte formation to 430.0 sporophytes per pot, which corresponded to 57.3 sporophytes per cm^2 (Table 1). Horticultural substrate alone and a 2:1 mixture of horticultural substrate and perlite, induced the development of 424.5 and 382.5 sporophytes per pot, respectively. No significant difference was observed for all three soil types. The number of roots was also 2.2 to 2.5, without a significant difference (Table 1). Growth of the formed sporophytes (Fig. 1*G*) was similar to that of natural habitat plants (Fig. 1*A*) in three soil types, and after 3 wk of acclimation, the sporophytes developed into mature plants (Fig. 1*H*). In contrast, unlike other soil substrates, peat moss-added substrates inhibited the regeneration of fragmented gametophytes, and no sporophytes were formed.

Discussion

This study examined gametophyte proliferation and sporophyte formation of *Lemmaphyllum microphyllum* cultured on *in vitro* and soil *ex vitro* conditions, to identify optimal conditions for the growth of regenerated prothallus, and to develop a practical method for mass production of this species throughout the year. Gametophytes or sporophytes can be easily propagated using homogenization culture methods (Knauss 1976; Cooke 1979; Fernández *et al.* 1999; Fernández and Revilla 2003). In the present study, the gametophyte was mechanically fragmented with a scalpel and hand blender, and gametophyte proliferation and sporophyte formation were induced effectively by exploiting the high regenerative capacity of the fragmented gametophyte (Jang *et al.* 2019a, b, c).

Ferns may have different nutritional requirements depending on the species (Menéndez *et al.* 2010; Wu *et al.* 2010), and the concentration of the medium appears to have a direct effect on gametophyte weight, which is indicative of its proliferation. Medium components, particularly nitrogen and sucrose, are essential for gametophyte growth and development (Miller 1968). Nitrogen supply affected spore germination and gametophyte development of *Botrychium dissectum* Spreng. and *Psilotum nudum* (L.) P. Beauv (Melan and Whittier 1990; Whittier 1990), and the reduction in total nitrogen concentration by 25%, produced the highest number of *Adiantum capillus-veneris* L. sporophytes (Kuriyama *et al.* 2004). In

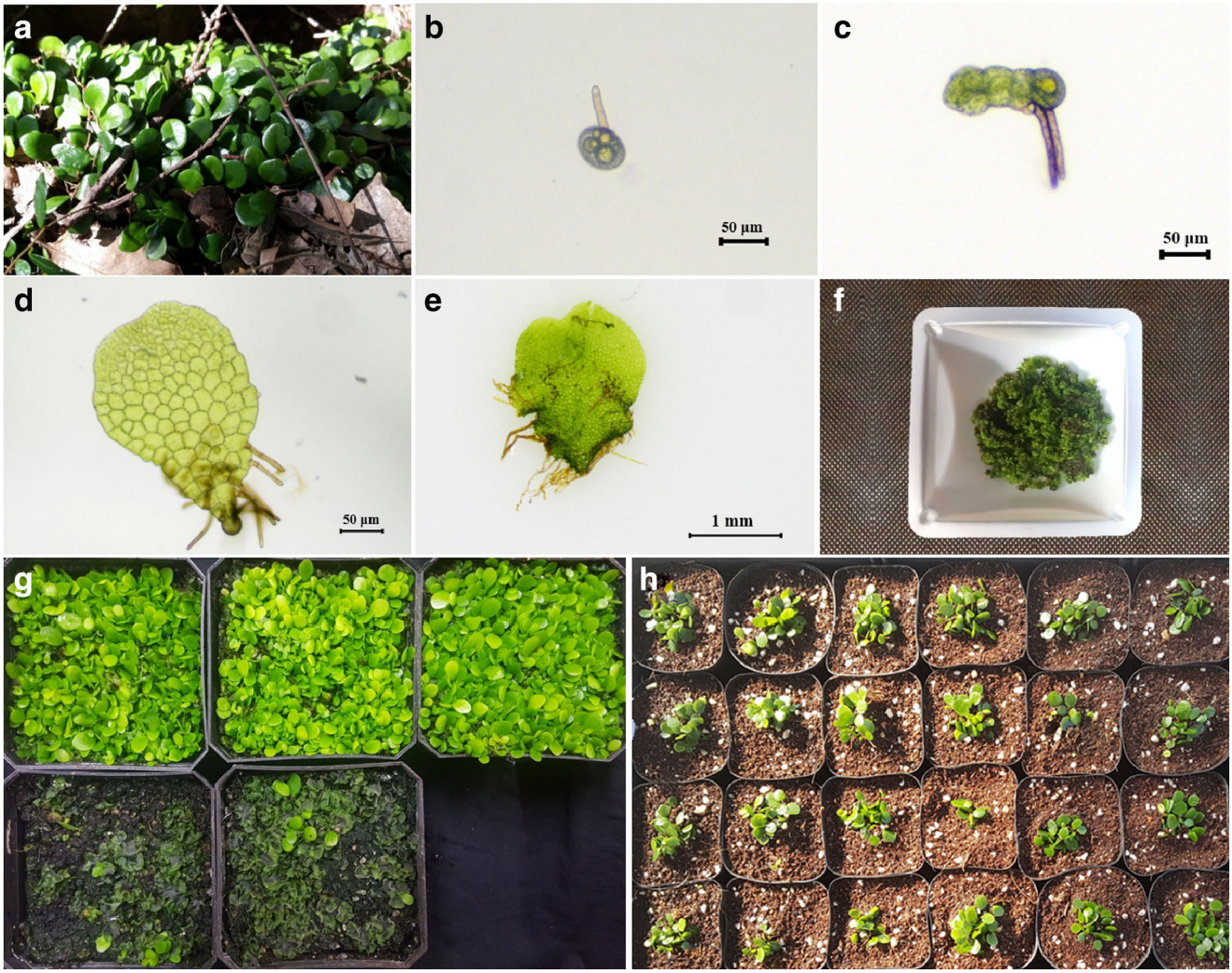

Fig. 1. Life cycle of *Lemmaphyllum microphyllum* C. Presl through spore germination and propagation method: (*A*) mature plant found in natural habitat; (*B*) germinated spore; (*C*) filamentous gametophyte; (*D*), normal gametophyte; (*E*, *F*), gametophytes cultured for 8 wk, and obtained from gametophyte fragments with using scalpel; (*G*) young sporophytes cultured for 14 wk, and obtained from gametophyte fragments prepared using a hand blender; (*H*) sporophytes growing in greenhouse after 3 wk of acclimation.

the present study, the changes in gametophyte weight were caused by the concentration of the nitrogen source in the medium (Fig. 2*B*), and the optimal gametophyte growth was observed at low total nitrogen concentrations. In the early stages of tissue culture, due to the limited photosynthesis, an external energy source is required for healthy growth, proliferation, and differentiation (Kozai 1991; Waman *et al.* 2014). Sucrose is the most commonly used energy source for tissue culture, as it promotes gametophyte growth, and is deeply involved in sex determination (Menéndez *et al.* 2010). The fresh weight of the *Dicksonia sellowiana* Hook. gametophyte increased in MS medium supplemented with sucrose (Renner and Randi 2004), whereas the formation of the *Microgramma vacciniifolia* (Langsd. & Fisch.) Copel. sporophyte was progressively promoted with increasing sucrose concentrations (Hirsch 1975). In this study, sucrose increased the fresh weight of the sporophyte by promoting gametophyte proliferation and division (Fig. 2*C*). Sucrose is an essential factor for tissue culture regardless of its concentration. In contrast, activated charcoal is not essential for tissue culture, although it has often been used in tissue culture to promote cell growth and development (Pan and van Staden 1998). Activated charcoal is used in both liquid and solid media, and serves to adsorb substances that inhibit growth (Thomas 2008). Although the beneficial effects of activated charcoal were expected in the present study, there was no significant difference in fresh weight and development of the gametophyte cultured at different charcoal concentrations (Fig. 2*D*). In another study, activated charcoal did not affect the proliferation of *Dryopteris erythrosora* (D.C. Eaton) Kuntze gametophytes (Jang *et al.* 2019a). In this study, mechanical fragmentation of the gametophyte with scalpels generated a large number of gametophytes. However, the fresh weight of the fragmented gametophyte was affected by medium

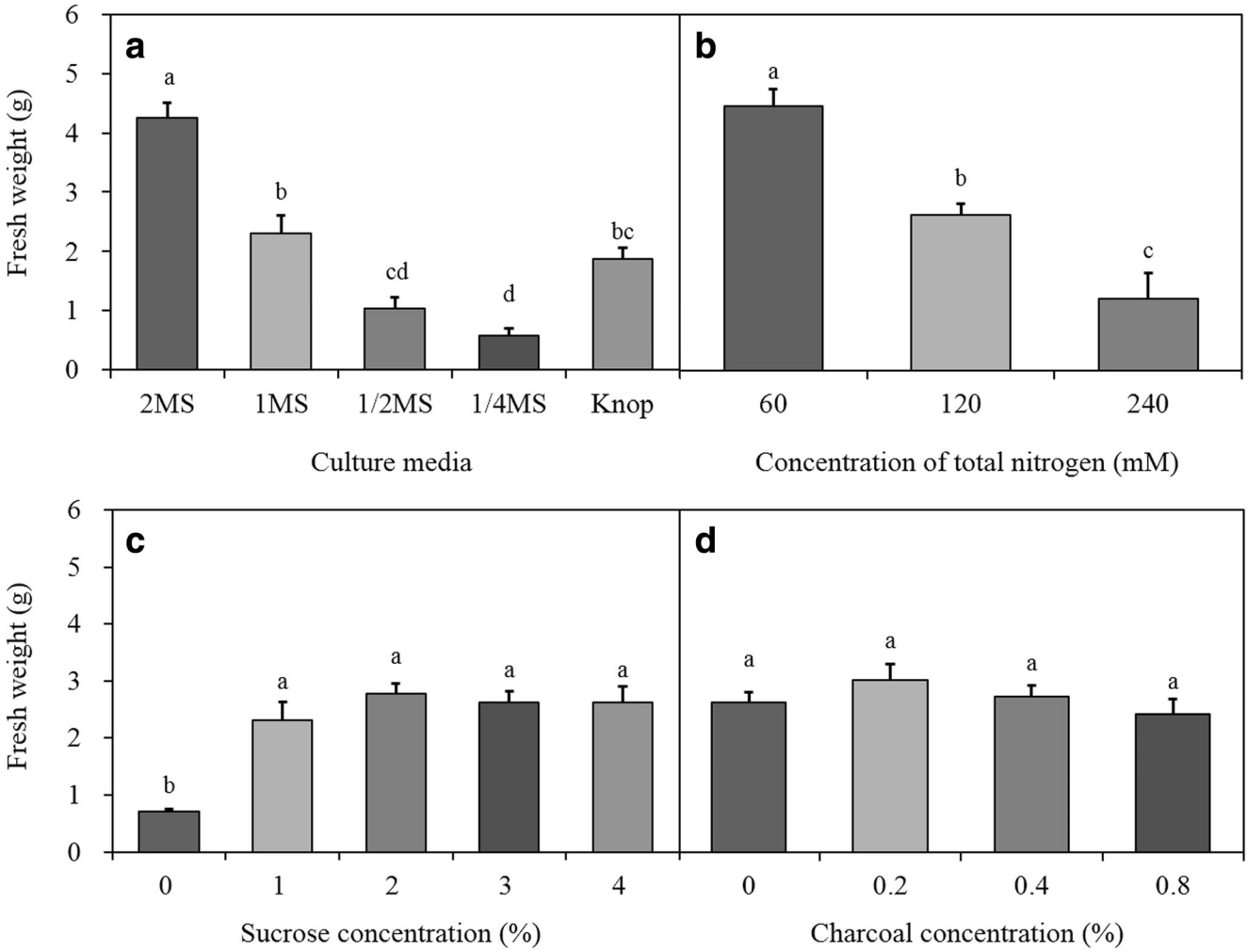

Fig. 2. Effect of medium components on *Lemmaphyllum microphyllum* C. Presl prothallus growth cultured for 8 wk: (*A*) type of culture media (quarter-strength Murashige and Skoog, 1/4 MS; half-strength, 1/2MS; full-strength, 1MS; and, double-strength, 2MS); (*B*) total nitrogen concentration; (*C*) sucrose concentration; (*D*) charcoal concentration. The *columns* represent means and *vertical bars* indicate standard error ($n = 4$).

components over time. Therefore, to increase the fresh weight of the fragmented gametophyte, optimal medium conditions should be developed.

Mechanical fragmentation of gametophytes using a hand blender is considered to be the most effective method for large-scale production of sporophytes (Fernández *et al.* 1999; Jang *et al.* 2019a, b). Young sporophytes were obtained from only 1 g of fragmented gametophyte that was sown on the surface of the soil substrate, which demonstrated that a large number of sporophytes can be obtained from small amounts of gametophytes. However, the number of sporophytes formed was affected by the type of soil substrate; sporophytes failed to form in soil that contained peat moss. Peat moss has high cation exchange capacity and water holding capacity, ample pore space, and high air permeability, which provides favorable conditions for plant growth (Nelson 1991; Robertson 1993; Stamps and Evans 1999). However, in the culture environment of this study, the soil substrate with a large amount of peat moss was very moist because of the continuous supply of water through sub-irrigation. Such a humid environment could act as an obstacle to the growth of fragmented gametophytes. In another study, very humid environments inhibited the formation of *Dryopteris erythrosora* (D.C. Eaton) Kuntze sporophytes in soil substrate with added peat moss (Jang *et al.* 2019a). These results indicate that mechanical fragmentation of gametophytes can yield a large number of sporophytes; however, soil substrates and moist soils influence the formation of sporophytes. Therefore, soil substrates with good drainage and aeration should be used.

Conclusions

A secure proliferation method is proposed for gametophyte proliferation and sporophyte formation that is suitable for production of large quantities of *L. microphyllum*. Mechanical

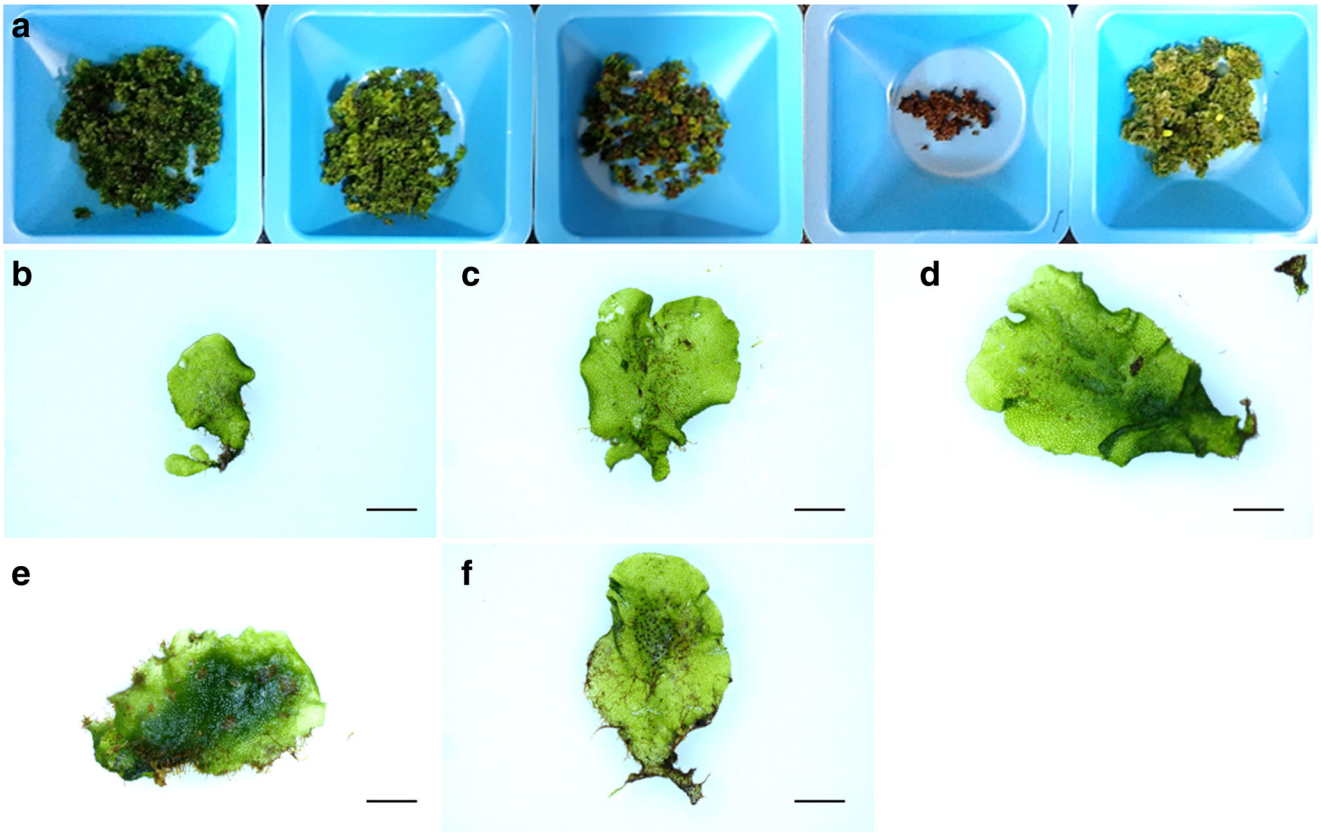

Fig. 3. Growth response and organ formation of *Lemmaphyllum microphyllum* C. Presl prothallus cultured in different media: (*A*) prothallus after 8 wk of culture (left to right: double-, full-, half-, and quarter-strength Murashige and Skoog [MS] media, and Knop medium); (*B*)–(*F*), double-, full-, half-, quarter-strength MS media and Knop medium, respectively. *Scale bars* are 1 mm.

Table 1 Effects of different soil substrates on sporophyte formation and development of *Lemmaphyllum microphyllum* C. Presl.

Substrate[z]	No. of sporophytes/pot[y]	No. of leaves/plant	Leaf length (mm)	Leaf width (mm)
Hs1	424.5 ± 15.19 a	2.5 ± 0.22 a	10.0 ± 0.55 a	4.8 ± 0.17 b
Hs2-Pr1	382.5 ± 59.01 a	2.5 ± 0.22 a	9.4 ± 0.34 a	5.7 ± 0.26 a
Hs2-D1	430.0 ± 12.02 a	3.0 ± 0.33 a	10.6 ± 0.28 a	5.2 ± 0.27 ab
Hs1-Pt1-Pr1[x]	0.0 ± 0.00 b	0.0 ± 0.00 b	-	-
Hs1-Pt1-D1[x]	0.0 ± 0.00 b	0.0 ± 0.00 b	-	-

Substrate	No. of roots/plant	Root length (mm)	Fresh weight (mg/sporophyte)	
			Aerial part	Underground part
Hs1	2.4 ± 0.16 a	10.7 ± 0.89 b	13.8 ± 1.39 b	0.3 ± 0.05 c
Hs2-Pr1	2.2 ± 0.29 a	13.0 ± 0.75 b	23.2 ± 2.93 a	2.7 ± 0.46 a
Hs2-D1	2.5 ± 0.22 a	15.3 ± 0.63 a	23.6 ± 2.70 a	1.4 ± 0.31 b
Hs1-Pt1-Pr1[x]	0.0 ± 0.00 b	-	-	-
Hs1-Pt1-D1[x]	0.0 ± 0.00 b	-	-	-

Lowercase letters indicate significant difference based on Tukey's honestly significant difference test at 5% probability

[z] *Hs*, horticultural substrate; *Pt*, peat moss; *Pr*, perlite; *D*, decomposed granite; numbers 1 or 2 represents volume ratio of each soil

[y] Pot (75 × 75 × 75 mm) containing 0.3 L culture medium was used for this experiment

[x] For empty cells, no measurements were possible because sporophytes did not form

fragmentation using a scalpel and blender was the most effective way to produce a large number of gametophytes and sporophytes from small amounts of gametophyte. These results can be proposed commercially as a convenient cultivation method *ex vitro*. It can also be evaluated for propagation of other ferns that form gametophytes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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