

Article

Optimization of Spore Germination, Gametophyte Liquid Culture, and Sporophyte Induction in the Endangered Medicinal Fern *Cibotium barometz*

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Highlights

What are the main findings?

- A continuous in vitro propagation workflow was established, encompassing spore germination, gametophyte liquid proliferation, 5 L air-lift bioreactor culture, and sporophyte induction.
- Treatment S-5 (MS + 0.4 mg L⁻¹ NAA + 0.4 mg L⁻¹ TDZ + 0.8 mg L⁻¹ GA₃) produced the highest spore germination rate and gametophyte longest-axis length at 75 d.
- In liquid culture, M-6 was most suitable for rapid fresh-biomass proliferation, whereas M-5 provided a more balanced performance in dry weight, total flavonoid yield, and total phenolic yield.
- In the 5 L air-lift culture system, integrated evaluation of biomass, metabolite yield, electrical conductivity, principal component analysis, and clustering identified 45 d as an appropriate culture period.
- Proliferated gametophytes retained the capacity to form sporophytes; the best induction system achieved a sporophyte conversion rate of 63.02% and an acclimatization survival rate of 91.77%.

What are the implications of the main findings?

- The system provides a technical platform for ex situ conservation, nursery-scale propagation, and reduced pressure on wild *Cibotium barometz* resources.
- Liquid culture links gametophyte biomass production with subsequent sporophyte regeneration, improving the consistency and traceability of propagation material.
- Electrical conductivity combined with multivariate statistical evaluation can serve as an auxiliary tool for culture-period determination and process monitoring.
- The continuous linkage of the spore–gametophyte–sporophyte stages provides a basis for completing the artificial life-cycle propagation of *Cibotium barometz*.
- This workflow may also provide a methodological reference for other rare fern species that are difficult to propagate.



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Abstract

This study established a continuous in vitro propagation system for *Cibotium barometz* encompassing spore germination, liquid proliferation of gametophytes, air-lift bioreactor culture, and sporophyte induction. An L9(3⁴) orthogonal design was used to screen combinations of NAA, TDZ, and GA₃, and an L16(4⁵) design was used to estimate the main effects of MS salt strength, sucrose, 6-BA, and IAA on gametophyte proliferation. Biomass, total flavonoids, total phenolics, soluble sugars, and electrical conductivity (EC) were monitored in a 5 L air-lift bioreactor. Treatment S-5 produced the highest 75 d germination rate (78.91%) and gametophyte longest-axis length (68.00 µm). M-6 yielded the greatest fresh biomass, whereas M-5 provided a more balanced dry-matter and metabolite yield. At 45 d, fresh weight, dry weight, and total flavonoid yield reached 35.02 g L⁻¹, 2.79 g L⁻¹, and 74.73 mg L⁻¹, respectively. EC was negatively correlated with biomass and phenolic yields, and the first two principal components explained 97.48% of the variation. The best sporophyte-induction treatment achieved a conversion rate of 63.02% and an acclimatization survival rate of 91.77%. This system provides a methodological basis for ex situ conservation and scalable propagation of *C. barometz*.

Keywords: *Cibotium barometz*; spore germination; gametophyte liquid culture; sporophyte induction; principal component analysis; cluster analysis

1. Introduction

Cibotium barometz (L.) J. Sm. is a large medicinal fern of the genus *Cibotium* in the family Cibotiaceae, as recognized by the Pteridophyte Phylogeny Group I classification [1]. Its rhizome is an important source of the traditional Chinese medicine *Cibotii Rhizoma* and contains phenolic acids, flavonoids, polysaccharides, terpenoids, and other constituents with medicinal, ornamental, and ecological value [2–5]. Destructive rhizome harvesting, habitat degradation, slow natural regeneration, and increasing market demand have placed sustained pressure on wild populations. *Cibotium barometz* is listed as a Grade II nationally protected wild plant in China and is included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [6,7]. Establishing a sustainable and traceable propagation system that does not depend on collecting wild rhizomes is therefore important for both conservation and production [8–12].

Cibotium barometz exhibits an alternation of generations between the sporophyte and gametophyte. Sporangia on the abaxial surface of diploid sporophyte fronds produce haploid spores through meiosis. After germination, the spores sequentially form a protonemal cell, a filamentous stage, and a laminar gametophyte. Mature gametophytes produce antheridia and archegonia; motile sperm fertilize the egg to form a zygote, which subsequently develops into a new sporophyte. Gametophytes bear rhizoids but do not form rhizomes, whereas rhizomes are sporophytic organs [13–18]. These life-cycle characteristics allow both spores and gametophytes to be used as starting materials for in vitro propagation.

Previous studies have shown that basal-salt strength, sucrose concentration, and plant growth regulators markedly affect spore germination, gametophyte proliferation, and sporophyte differentiation in *C. barometz*. Half-strength MS or other low-salt media often favor early germination, whereas full-strength MS, WPM, and different plant growth regulator combinations exert stage-dependent effects on subsequent proliferation and differentiation [13,14,19]. Recent multi-omics evidence further indicates that sugar metabolism and endogenous hormone signaling participate in regulating differ-

ent developmental stages of *C. barometz* tissue-cultured plantlets [20]. However, most previous studies have focused on a single culture stage or small-volume vessels. A continuously integrated system combining plant growth regulator screening, gametophyte liquid proliferation, bioreactor time-course evaluation, and sporophyte regeneration, together with a comprehensive assessment of biomass, metabolites, and process indicators, remains lacking.

Accordingly, this study aimed to: (1) Use an orthogonal design to screen plant growth regulator combinations that promote spore germination and early gametophyte development in *C. barometz*; (2) evaluate the main effects of MS basal-salt strength, sucrose, 6-BA, and IAA on gametophyte liquid proliferation and metabolite accumulation; (3) characterize temporal changes in gametophyte biomass, metabolites, and culture-medium electrical conductivity in a 5 L air-lift bioreactor and determine an appropriate culture period; and (4) compare the regeneration and acclimatization performance of different sporophyte-induction systems. The overall objective was to establish a continuous in vitro propagation workflow from spore germination through gametophyte liquid proliferation to sporophyte formation, thereby providing a technical basis for germplasm conservation and scalable seedling production. For clarity, the principal favorable treatments are identified throughout the manuscript by both code and composition: S-5, full-strength MS medium without sucrose supplemented with 0.4 mg L^{-1} NAA, 0.4 mg L^{-1} TDZ, and 0.8 mg L^{-1} GA₃; M-6, 1/2-strength MS basal salts with the standard MS vitamin mixture maintained at full strength, 1% sucrose, 0.1 mg L^{-1} 6-BA, and 1.2 mg L^{-1} IAA; M-5, 1/2-strength MS basal salts with full-strength vitamins, 4% sucrose, 0.4 mg L^{-1} 6-BA, and 0.8 mg L^{-1} IAA; and SI-1, 1/2-strength MS solid medium containing 2% sucrose and 7.0 g L^{-1} agar without plant growth regulators.

2. Materials and Methods

2.1. Plant Material and Spore Pretreatment

Mature fertile pinnae were collected from Nanshan Forest Park, Yunfu, Guangdong Province, China, in June 2025. The material was identified as *Cibotium barometz* (L.) J. Sm. (Cibotiaceae) by Professor Gang Xu of Guangdong Pharmaceutical University. Fertile pinnae were placed in clean paper bags and maintained under dry, well-ventilated conditions for approximately 2 d to allow natural spore release. After removing fragments of leaf tissue and sporangia, the dried spores were stored at 4 °C. All aseptic manipulations were performed in an SW-CJ-2FD-II vertical laminar-flow clean bench (Suzhou Antai Airtech Co., Ltd., Suzhou, China). Precisely 1.5 mg of mature spores was weighed using an ATY124 analytical balance (Shimadzu Philippines Manufacturing Inc., Rosario, Cavite, Philippines) and transferred to a 5 mL centrifuge tube, mixed thoroughly with 5 mL of sterile water, and soaked at room temperature for 12 h to allow complete imbibition. After settling for approximately 5 min, the supernatant was removed, and the spores were surface-sterilized with approximately 5 mL of 5% sodium hypochlorite solution for 6–8 min with gentle inversion. The disinfectant was discarded, and the spores were washed 3–5 times with sterile water. During each wash, the suspension was mixed thoroughly, allowed to settle, and the supernatant was removed to minimize residual disinfectant. Finally, the spores were resuspended in 5 mL of sterile water to obtain a homogeneous spore suspension. Germination was defined as rupture of the spore wall followed by emergence of a chlorophyllous protonemal cell and/or a colorless, transparent rhizoid through the germination aperture.

2.2. Morphological Observation of Spore Germination and Plant Growth Regulator Orthogonal Experiment

Full-strength liquid MS medium [21], prepared from commercial MS basal salts and the standard MS vitamin mixture (Qingdao Hope Bio-Technology Co., Ltd., Qingdao, China), without sucrose was used as the basal medium. An L9(3⁴) orthogonal array was used to evaluate three factors, NAA, TDZ, and GA₃, each at 0.1, 0.4, and 0.8 mg L⁻¹. The fourth column of the array was left unassigned, and no interaction terms were allocated. The medium pH was adjusted to 5.8 ± 0.1 before autoclaving at 121 °C for 20 min in a GMJ-80L-I vertical pressure steam sterilizer (Shanghai Shenan Medical Instrument Factory, Shanghai, China). Each culture unit consisted of a 240 mL glass culture vessel containing 40 mL of treatment medium and 20 µL of homogeneous spore suspension. Cultures were maintained at 26 ± 1 °C under a 12 h light/12 h dark photoperiod and a light intensity of approximately 2000 lx (approximately 30 µmol m⁻² s⁻¹). The experiment was conducted in three temporally independent runs, each comprising three independently prepared, inoculated, and cultured vessels per treatment, for a total of nine independent culture units. The individual culture vessel was the experimental and statistical unit. At 45 and 75 d, at least 100 spores per vessel were examined using an MF23-M fluorescence biological microscope (Guangzhou Micro-shot Technology Co., Ltd., Guangzhou, China) to determine germination, and the longest-axis length of 30 randomly selected gametophytes per vessel was measured using calibrated microscopy software. The mean of the within-vessel observations was used for statistical analysis.

2.3. Optimization of the Gametophyte Liquid-Culture Medium

Green gametophytes derived from the same batch of aseptic spore cultures, cultured for 35–50 d, and exhibiting complete and uniform morphology were selected and cut into approximately 3–5 mm explants of similar size, each retaining an intact growth point. An L16(4⁵) orthogonal array was used to evaluate MS basal-salt strength, sucrose, 6-BA, and IAA. The fifth column served as an unassigned error column, and no interactions were allocated. MS, 1/2 MS, 1/4 MS, and 1/8 MS denote MS basal salts supplied at 1, 1/2, 1/4, and 1/8 strength, respectively, while the vitamin mixture was maintained at full strength. Sucrose was supplied at 1%, 2%, 3%, or 4%, and both 6-BA and IAA were supplied at 0.1, 0.4, 0.8, or 1.2 mg L⁻¹. Sucrose was obtained from Tianjin Damao Chemical Reagent Factory (Tianjin, China). The medium pH was adjusted to 5.8 ± 0.1 before autoclaving at 121 °C for 20 min. Each 250 mL glass culture vessel contained 100 mL of liquid medium and was inoculated with 2.00 ± 0.02 g fresh weight of gametophyte material. Cultures were maintained on an HY-6A double-layer orbital shaker (Yineng Experimental Instrument Factory, Changzhou, China) at 120 rpm for 30 d under the temperature and photoperiod conditions described in Section 2.2. Each treatment was evaluated in three temporally independent experimental runs, with three independently prepared and inoculated vessels per run, yielding nine independent culture units. Fresh weight, dry weight, total flavonoid yield, total phenolic yield, soluble sugar content, and electrical conductivity were determined at the end of culture.

2.4. Culture in a 5 L Air-Lift Bioreactor

Based on the main-effect means from the L16 orthogonal experiment, a candidate formulation predicted to favor fresh- and dry-biomass accumulation (1/2-strength MS basal salts + 4% sucrose + 0.1 mg L⁻¹ 6-BA + 1.2 mg L⁻¹ IAA) was selected for scale-up. This formulation was not one of the individual treatment rows directly tested in the L16 array and was not compared directly with M-5 and M-6 within the same shake-flask experiment. Accordingly, the bioreactor experiment was interpreted as a scale-up validation

of a main-effect-predicted combination rather than confirmation of a unique optimum. A laboratory-built 5 L glass internal-loop air-lift bioreactor (Guangdong Pharmaceutical University, Guangzhou, China) was used with a working volume of 2 L and an inoculum of 20.00 ± 0.02 g gametophyte fresh weight. Oil-free air was sterilized through a $0.22 \mu\text{m}$ hydrophobic filter and continuously supplied through a bottom sparger at 1.0 L min^{-1} (0.5 vvm). Cultures were maintained at $25 \pm 2^\circ\text{C}$ under a 12 h light/12 h dark photoperiod. Three independent complete bioreactor runs were performed. Samples were collected at 9, 18, 27, 36, 45, 54, and 61 d during each run. Three technical measurements were performed at each sampling time, and their mean was used as the value for that independent run; therefore, the number of independent replicates in the bioreactor time-course experiment was $n = 3$. Fresh weight, dry weight, total flavonoids, total phenolics, soluble sugars, and electrical conductivity were measured to characterize culture-period and nutrient-consumption dynamics.

2.5. Determination of Total Flavonoids, Total Phenolics, Soluble Sugars, and Electrical Conductivity

Harvested gametophytes were washed twice with water to remove residual culture medium, blotted to remove surface moisture, and weighed to determine fresh weight. Samples were dried at 50°C to constant weight using a DHG-9203A forced-air drying oven (Shanghai Donglu Instrument Equipment Co., Ltd., Shanghai, China) for dry-weight determination. Total flavonoids and total phenolics were extracted from the same ethanolic extract. Briefly, 0.50 g of dried powder was mixed with 10 mL of 70% ethanol prepared from analytical-grade ethanol (Guangzhou Chemical Reagent Factory, Guangzhou, China) (solid-to-liquid ratio 1:20, w/v), ultrasonically extracted at 40°C for 40 min using a KQ5200E ultrasonic cleaner (Dongguan Keqiao Ultrasonic Equipment Co., Ltd., Dongguan, China), and centrifuged at $8000 \times g$ for 10 min using a TGL-16B high-speed benchtop centrifuge (Shanghai Anting Scientific Instrument Factory, Shanghai, China). The supernatant was collected and adjusted to a final volume of 10 mL. Total flavonoids were determined using the $\text{NaNO}_2\text{-Al}(\text{NO}_3)_3\text{-NaOH}$ colorimetric method [22]. Sodium nitrite and aluminum nitrate were obtained from Guangzhou Chemical Reagent Factory (Guangzhou, China), sodium hydroxide was obtained from Xilong Scientific Co., Ltd. (Shantou, China), and the rutin reference standard was obtained from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). Absorbance was measured at 510 nm using a UV-1500 UV-visible spectrophotometer (Shanghai Meixi Instrument Co., Ltd., Shanghai, China). Total phenolics were determined using the Folin–Ciocalteu method [23]. Folin–Ciocalteu reagent was obtained from Tianjin Huasheng Chemical Reagent Co., Ltd. (Tianjin, China), and the protocatechuic acid reference standard was obtained from Shanghai Shifeng Biotechnology Co., Ltd. (Shanghai, China). Absorbance was measured at 760 nm using the UV-1500 spectrophotometer. For soluble sugar determination, 0.10 g of dried tissue was extracted twice with 10 mL of distilled water in an HWS-2 thermostatic water bath (Shanghai Yiheng Scientific Instrument Co., Ltd., Shanghai, China) at boiling temperature for 30 min. The combined extracts were analyzed using the anthrone-sulfuric acid method [24], with a glucose reference standard obtained from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China) and absorbance measured at 620 nm using the UV-1500 spectrophotometer. Each biological sample was analyzed in triplicate, and the technical measurements were averaged. Total flavonoid and total phenolic values were converted to yields per unit culture volume (mg L^{-1}), whereas soluble sugars were expressed on a tissue dry-weight basis (mg g^{-1}). A 20 mL aliquot of culture medium was filtered through a $0.20 \mu\text{m}$ membrane, and EC was measured at room temperature using an SW-302 temperature-compensated TDS/EC tester (China) calibrated with a standard conductivity solution. EC was expressed as mS cm^{-1} .

2.6. Sporophyte Induction and Acclimatization

Gametophytes obtained by liquid proliferation were transferred to three sporophyte-induction systems: SI-1, 1/2-strength MS solid medium containing 2% sucrose; SI-2, full-strength MS solid medium without plant growth regulators; and SI-3, a sterilized peat:perlite substrate (2:1, *v/v*). The SI-1 and SI-2 media were solidified with 7.0 g L⁻¹ agar, adjusted to pH 5.8 ± 0.1 before autoclaving at 121 °C for 20 min, and dispensed at 40 mL per 240 mL culture vessel. A gametophyte explant approximately 3–5 mm in diameter with an intact growth point was defined as one inoculation unit. Equal numbers of inoculation units were transferred to each vessel and individually numbered and counted. Culture temperature, photoperiod, and light intensity were the same as those described in Section 2.2. The number of inoculation units, the number of units producing at least one visible sporophyte, the time to first sporophyte emergence, and the time to stable sporophyte establishment were recorded. Sporophytes were acclimatized after developing a visible root system and at least one expanding juvenile frond. The culture medium was removed from the roots, and plantlets were transplanted into sterilized peat:perlite (2:1, *v/v*). Acclimatization lasted 30 d at 25 ± 2 °C under a 12 h light/12 h dark photoperiod and approximately 2000 lx. During days 1–7, transparent covers were used to maintain 90–95% relative humidity. During days 8–14, cultures were ventilated for 15 min daily, and during days 15–21, ventilation was extended to 1 h daily. From day 22 onward, covers were removed completely, relative humidity was gradually reduced to 70–75%, and sterile water was sprayed according to substrate surface moisture. Plants entering this procedure were defined as acclimatized plants; plants that remained alive and resumed new-frond growth after 30 d were defined as surviving plants.

Sporophyte conversion rate (%) = number of gametophyte units forming sporophytes/number of inoculated gametophyte units × 100.

Acclimatization survival rate (%) = number of surviving plants/number of plants entering acclimatization × 100.

2.7. Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 26.0 (IBM Corp., Armonk, NY, USA), and results are presented as mean ± standard error (SE). In the spore-germination, shake-flask liquid-culture, and sporophyte-induction experiments, each culture vessel was independently prepared, inoculated, and cultured. Three temporally independent runs with three vessels per run yielded *n* = 9 independent culture units; the culture vessel, rather than the microscopic field or an individual observation within a vessel, was the independent statistical unit. The bioreactor experiment comprised three independent runs, and the mean of three technical measurements within each run was used; thus, the independent replicate number for the bioreactor time course was *n* = 3. The L9(3⁴) and L16(4⁵) orthogonal experiments were used to estimate main effects only. Because no interaction columns were assigned, interaction effects were neither estimated nor subjected to significance testing. To compare the nine or sixteen directly observed culture combinations, the combinations were treated as levels of a one-way analysis of variance, followed by Duncan's multiple range test [25] at *p* < 0.05. Two-sided Pearson correlations were calculated using the means of the seven bioreactor sampling times. These analyses were considered exploratory associations within a time series and were not interpreted as causal evidence. PCA was performed on a Z-score-standardized multivariate matrix, and composite scores were calculated by weighting retained principal-component scores by their variance contributions. Hierarchical clustering was performed using Ward's method and squared Euclidean distance. Graphs were prepared using OriginPro 2024 (OriginLab

Corporation, Northampton, MA, USA), and scale bars were added to micrographs using ImageJ1.54f (National Institutes of Health, Bethesda, MD, USA).

3. Results

3.1. Spore Germination and Gametophyte Development

Spores of *C. barometz* were yellow, nearly tetrahedral, and trilete; each laesura was approximately 7 μm long and did not extend to the equatorial plane (Figure 1A,B). Within 48 h after inoculation, the spores imbibed water, expanded, and became nearly spherical (Figure 1C). At approximately 9 d, the spore wall ruptured along the laesurae and a green protonemal cell emerged (Figure 1D), followed by formation of a colorless rhizoid oriented approximately perpendicular to the protonemal cell (Figure 1E). Successive transverse divisions produced a 3–4-celled filament (Figure 1F). A wedge-shaped apical meristematic cell then developed (Figure 1G), divided laterally, and established a laminar gametophyte (Figure 1H). Continued indentation of the growth region and expansion of the two wings produced an approximately symmetrical cordate gametophyte bearing numerous rhizoids on the basal ventral surface (Figure 1I). Mature gametophytes formed archegonia and antheridia (Figure 1J,K).

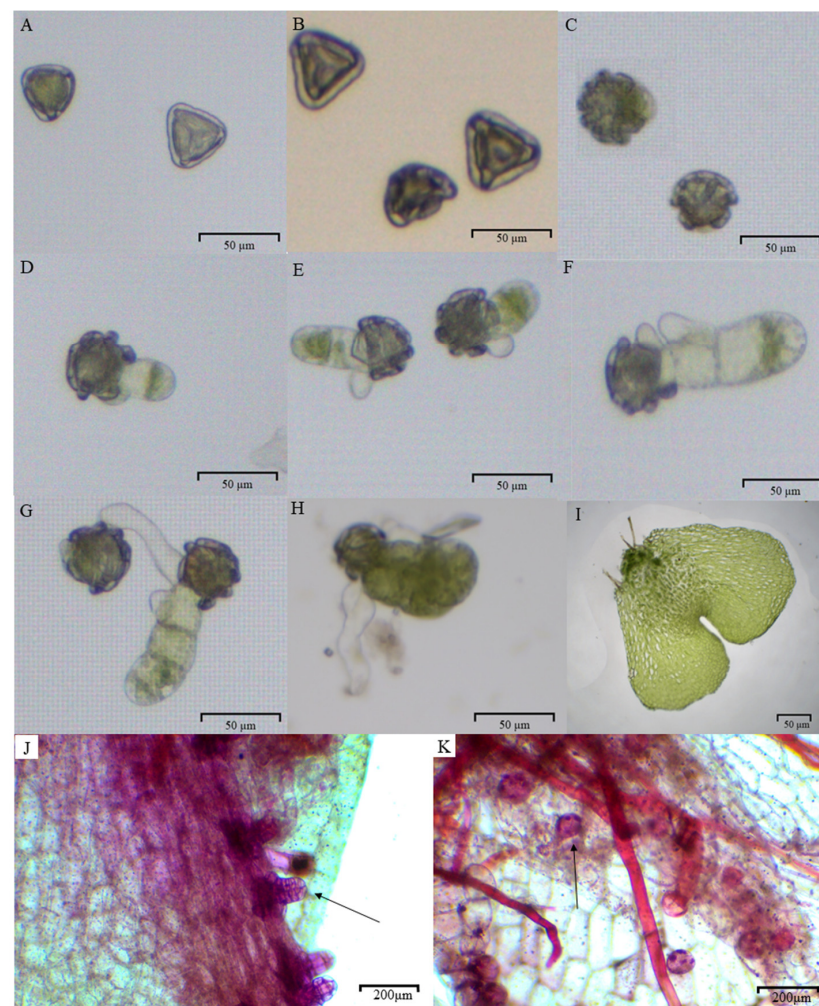


Figure 1. Spore germination and gametophyte development in *Cibotium barometz*. (A,B), mature spores; (C), imbibed spore; (D), emergence of the protonemal cell; (E), formation of the primary rhizoid; (F), 3–4-celled filament; (G), apical wedge-shaped meristematic cell; (H), laminar gametophyte; (I), cordate gametophyte; (J), archegonia; (K), antheridia.

3.2. Effects of Plant Growth Regulator Combinations on Spore Germination and Early Gametophyte Growth

Different combinations of NAA, TDZ, and GA₃ significantly affected spore germination and gametophyte longest-axis length (Table 1). At 75 d, S-5 produced the highest germination rate ($78.91\% \pm 2.14\%$) and longest-axis length ($68.00 \pm 2.00 \mu\text{m}$), both assigned to Duncan group a. From 45 to 75 d, germination increased by only approximately 0.95–3.01 percentage points across treatments, whereas gametophyte longest-axis length continued to increase by 2.00–10.00 μm . This comparison represents a descriptive temporal trend because the time $\times$ treatment interaction was not evaluated using a repeated-measures model. Main-effect range analysis indicated that the order of influence on 75 d germination was NAA ($R = 34.74$) > TDZ ($R = 33.34$) > GA₃ ($R = 23.60$), whereas the order for gametophyte longest-axis length was NAA ($R = 27.93$) > GA₃ ($R = 21.93$) > TDZ ($R = 18.27$). Factor-level means indicated that 0.4 mg L⁻¹ NAA and 0.8 mg L⁻¹ GA₃ were generally favorable. Because interactions were not estimable in this design, S-5 was selected as the preferred treatment under the present conditions on the basis of its directly observed performance.

Table 1. Effects of orthogonal plant growth regulator treatments on spore germination and gametophyte longest-axis length in *Cibotium barometz*.

Treatment	NAA (mg L ⁻¹)	TDZ (mg L ⁻¹)	GA ₃ (mg L ⁻¹)	45 d Germ. (%)	45 d Length (μm)	75 d Germ. (%)	75 d Length (μm)
S-1	0.1	0.1	0.1	8.01 ± 1.54 f	8.00 ± 1.20 g	8.96 ± 1.58 f	10.00 ± 1.19 g
S-2	0.1	0.4	0.4	18.04 ± 1.67 e	14.00 ± 1.17 f	19.84 ± 1.65 e	16.70 ± 1.20 f
S-3	0.1	0.8	0.8	65.95 ± 2.14 b	40.00 ± 1.88 c	68.96 ± 2.09 b	45.80 ± 1.92 c
S-4	0.4	0.1	0.4	61.02 ± 2.13 b	43.00 ± 1.98 b	62.93 ± 2.10 c	49.20 ± 2.01 bc
S-5	0.4	0.4	0.8	75.93 ± 2.09 a	58.00 ± 2.04 a	78.91 ± 2.14 a	68.00 ± 2.00 a
S-6	0.4	0.8	0.1	58.13 ± 2.11 c	34.00 ± 2.08 d	60.14 ± 2.05 c	39.10 ± 2.01 d
S-7	0.8	0.1	0.8	22.04 ± 1.59 e	21.00 ± 1.18 e	23.08 ± 1.64 e	24.50 ± 1.21 e
S-8	0.8	0.4	0.1	29.19 ± 1.56 d	20.00 ± 1.25 e	31.05 ± 2.11 d	23.40 ± 1.20 e
S-9	0.8	0.8	0.4	64.06 ± 2.04 b	47.00 ± 2.03 b	65.90 ± 2.15 bc	53.60 ± 2.09 b

Note: Values are means $\pm$ SE from nine independent culture units ($n = 9$). Germ., germination; length, gametophyte longest-axis length. Within each column, different lowercase letters indicate significant differences among treatments according to Duncan's multiple range test ($p < 0.05$).

3.3. Optimization and Comprehensive Evaluation of the Gametophyte Liquid-Culture Medium

Combinations of MS basal-salt strength, sucrose, 6-BA, and IAA significantly affected gametophyte biomass and metabolite yields (Table 2). M-6 produced the greatest fresh weight ($65.53 \pm 1.54 \text{ g L}^{-1}$) and was therefore most suitable when rapid fresh-biomass proliferation was the primary objective. M-5 produced a dry weight of $3.67 \pm 0.36 \text{ g L}^{-1}$, a total flavonoid yield of $94.52 \pm 12.69 \text{ mg L}^{-1}$, and a total phenolic yield of $72.14 \pm 13.48 \text{ mg L}^{-1}$, representing a more balanced overall performance. Main-effect range analysis showed that the order of effects on fresh weight was 6-BA ($R = 24.07$) > IAA ($R = 15.79$) > sucrose ($R = 12.36$) > MS basal-salt strength ($R = 6.59$). For dry weight, the order was 6-BA > sucrose > IAA > MS basal-salt strength, and 6-BA also had the largest range for both total flavonoid and total phenolic yields. Overall, relatively low 6-BA, relatively high IAA, 4% sucrose, and 1/2-strength MS salts favored one or more target traits, although the optimal levels differed among traits. PC1 and PC2 explained 85.63% and 10.17% of the variation, respectively, for a cumulative total of 95.80%. PC1 was positively associated with fresh weight, dry weight, total flavonoid yield, and total phenolic yield, whereas PC2 more strongly represented differences between soluble sugar content and biomass. The five highest composite scores were obtained for M-6, M-5, M-1, M-15, and M-9 (Figure 2). The candidate formulation used for bioreactor validation was derived from main-effect prediction and was not one of the directly observed treatments in Table 2; it therefore does not replace the empirical ranking of M-5 or M-6.

Table 2. Biomass, metabolite yields, and PCA-based composite ranking of liquid-medium optimization treatments.

Treatment	MS Salts	Sucrose	6-BA (mg L ⁻¹)	IAA (mg L ⁻¹)	Fresh wt. (g L ⁻¹)	Dry wt. (g L ⁻¹)	Flavonoid Yield (mg L ⁻¹)	Phenolic Yield (mg L ⁻¹)	PCA Rank
M-1	MS	4%	0.1	0.1	47.36 ± 1.00 b	3.56 ± 0.11 a	78.57 ± 4.02 b	54.49 ± 4.80 b	3
M-2	MS	1%	0.4	0.4	36.16 ± 1.01 d	1.82 ± 0.05 d	32.17 ± 2.11 d	30.93 ± 2.78 c	9
M-3	MS	2%	0.8	0.8	22.84 ± 0.80 f	1.49 ± 0.06 e	36.40 ± 3.57 d	17.07 ± 1.78 d	10
M-4	MS	3%	1.2	1.2	20.70 ± 1.21 f	1.40 ± 0.09 e	36.74 ± 3.25 d	14.55 ± 1.76 d	11
M-5	1/2 MS	4%	0.4	0.8	44.61 ± 3.70 c	3.67 ± 0.36 a	94.52 ± 12.69 a	72.14 ± 13.48 a	2
M-6	1/2 MS	1%	0.1	1.2	65.53 ± 1.54 a	3.06 ± 0.08 b	73.90 ± 6.30 b	45.56 ± 5.55 b	1
M-7	1/2 MS	2%	1.2	0.1	21.22 ± 1.06 f	1.21 ± 0.06 e	20.89 ± 1.61 d	18.62 ± 2.23 d	12
M-8	1/2 MS	3%	0.8	0.4	20.31 ± 0.97 f	1.41 ± 0.08 e	21.58 ± 2.15 d	15.24 ± 1.37 d	14
M-9	1/4 MS	4%	0.8	1.2	40.06 ± 1.51 d	3.37 ± 0.16 a	52.74 ± 7.49 c	62.43 ± 7.48 a	5
M-10	1/4 MS	1%	1.2	0.8	22.00 ± 0.67 f	1.05 ± 0.03 f	18.21 ± 1.15 e	13.27 ± 0.92 d	15
M-11	1/4 MS	2%	0.1	0.4	35.72 ± 1.81 d	1.78 ± 0.08 d	45.62 ± 8.36 c	26.36 ± 2.35 c	8
M-12	1/4 MS	3%	0.4	0.1	30.02 ± 1.82 e	2.00 ± 0.12 d	59.70 ± 6.54 c	34.59 ± 4.40 c	6
M-13	1/8 MS	4%	1.2	0.4	21.46 ± 0.37 f	1.54 ± 0.03 e	13.95 ± 1.31 e	18.49 ± 1.60 d	13
M-14	1/8 MS	1%	0.8	0.1	20.32 ± 1.20 f	0.90 ± 0.07 f	14.07 ± 1.54 e	12.92 ± 1.73 d	16
M-15	1/8 MS	2%	0.4	1.2	50.50 ± 1.27 b	3.20 ± 0.08 b	46.90 ± 4.25 c	49.67 ± 5.20 b	4
M-16	1/8 MS	3%	0.1	0.8	33.04 ± 2.68 e	2.36 ± 0.14 c	33.27 ± 4.27 d	35.13 ± 5.05 c	7

Note: Values are means ± SE from nine independent culture units (n = 9). wt., weight. Within each column, different lowercase letters indicate significant differences among treatments according to Duncan's multiple range test ($p < 0.05$).

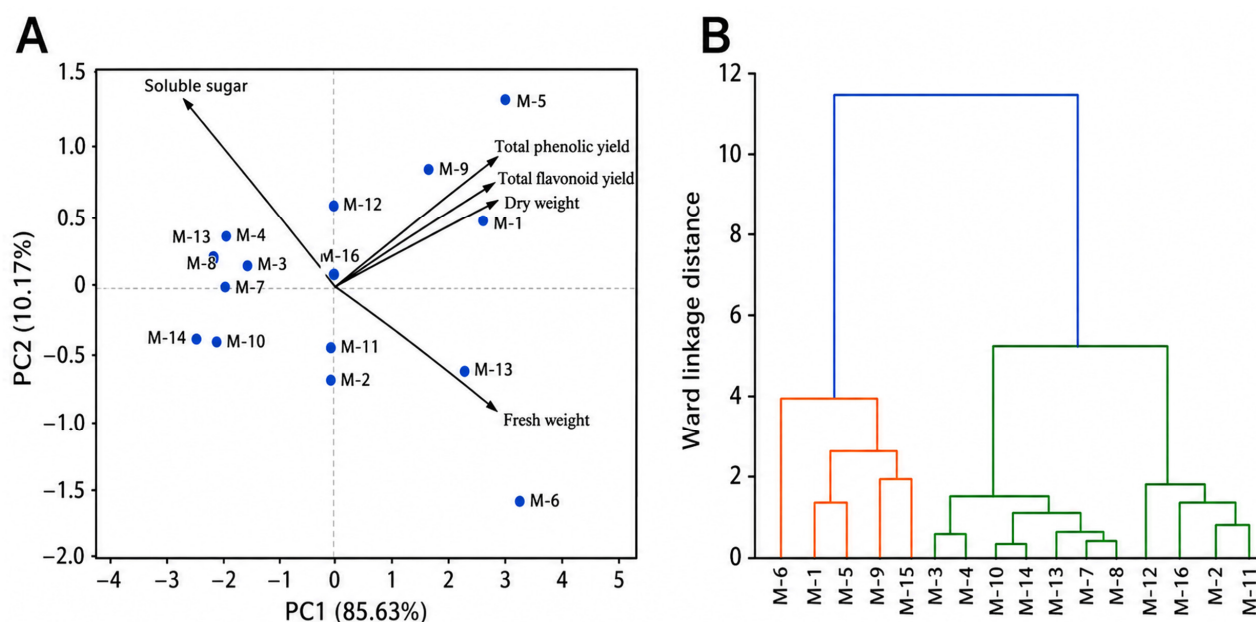


Figure 2. PCA-based comprehensive evaluation and Ward hierarchical clustering of liquid-medium optimization treatments. (A) PCA biplot; PC1 and PC2 explain 85.63% and 10.17% of the variation, respectively. (B) Ward hierarchical clustering.

3.4. Selection of the Air-Lift Bioreactor Culture Period

Fresh weight, dry weight, and total flavonoid yield generally increased and then decreased as culture duration increased (Table 3). Fresh weight reached $35.02 \pm 1.22 \text{ g L}^{-1}$ at 45 d and was significantly greater than at all other sampling times. Dry weight at both 45 and 54 d was assigned to Duncan group a; however, total flavonoid yield was greatest at 45 d ($74.73 \pm 5.25 \text{ mg L}^{-1}$) and was significantly higher than at 54 d. After 54 d, fresh weight and total flavonoid yield declined, while EC increased from $0.82 \pm 0.02 \text{ mS cm}^{-1}$ at 45 d to $1.11 \pm 0.03 \text{ mS cm}^{-1}$ at 61 d. Considering biomass, metabolite yields, and EC dynamics together, 45 d was identified as an appropriate culture period under the present experimental conditions.

Table 3. Dynamics of biomass, metabolite yields, and electrical conductivity during culture in a 5 L air-lift bioreactor.

Culture Time (d)	Fresh Weight (g L^{-1})	Dry Weight (g L^{-1})	Total Flavonoid Yield (mg L^{-1})	Total Phenolic Yield (mg L^{-1})	Soluble Sugars (mg g^{-1})	EC (mS cm^{-1})
9	$12.41 \pm 1.03 \text{ f}$	$0.98 \pm 0.07 \text{ e}$	$22.45 \pm 2.22 \text{ d}$	$15.69 \pm 2.63 \text{ c}$	$2.41 \pm 0.12 \text{ c}$	$1.62 \pm 0.04 \text{ a}$
18	$16.04 \pm 0.95 \text{ e}$	$1.21 \pm 0.16 \text{ d}$	$22.41 \pm 4.95 \text{ d}$	$14.71 \pm 2.43 \text{ c}$	$2.96 \pm 0.07 \text{ b}$	$1.48 \pm 0.04 \text{ b}$
27	$20.53 \pm 0.86 \text{ d}$	$1.48 \pm 0.07 \text{ d}$	$32.66 \pm 2.60 \text{ c}$	$19.83 \pm 1.66 \text{ b}$	$3.29 \pm 0.11 \text{ a}$	$1.32 \pm 0.03 \text{ c}$
36	$26.63 \pm 1.00 \text{ c}$	$2.42 \pm 0.10 \text{ b}$	$54.34 \pm 4.04 \text{ b}$	$36.78 \pm 2.73 \text{ a}$	$2.96 \pm 0.11 \text{ b}$	$1.08 \pm 0.03 \text{ d}$
45	$35.02 \pm 1.22 \text{ a}$	$2.79 \pm 0.11 \text{ a}$	$74.73 \pm 5.25 \text{ a}$	$38.07 \pm 2.83 \text{ a}$	$2.82 \pm 0.09 \text{ b}$	$0.82 \pm 0.02 \text{ f}$
54	$31.84 \pm 1.06 \text{ b}$	$2.51 \pm 0.10 \text{ a}$	$61.21 \pm 4.49 \text{ b}$	$32.05 \pm 2.48 \text{ a}$	$2.64 \pm 0.08 \text{ c}$	$0.94 \pm 0.03 \text{ e}$
61	$26.30 \pm 0.99 \text{ c}$	$2.05 \pm 0.09 \text{ c}$	$43.29 \pm 3.43 \text{ c}$	$23.62 \pm 1.93 \text{ b}$	$2.54 \pm 0.08 \text{ c}$	$1.11 \pm 0.03 \text{ d}$

Note: Values are means $\pm$ SE from three independent bioreactor runs ($n = 3$); the three technical measurements within each run were averaged before analysis. Within each column, different lowercase letters indicate significant differences among culture times according to Duncan's multiple range test ($p < 0.05$).

3.5. Electrical Conductivity Correlations, PCA, and Cluster Analysis

Exploratory Pearson correlation analysis was conducted using the means of the seven bioreactor sampling times. EC was significantly negatively correlated with fresh weight, dry weight, total flavonoid yield, and total phenolic yield, with correlation coefficients of -0.999 ($p < 0.0001$), -0.986 ($p < 0.0001$), -0.970 ($p = 0.0003$), and -0.909 ($p = 0.0046$), respectively (Table 4; Figure 3). The temporal correspondence between EC and fresh weight

was strongest, indicating that changes in the ionic status of the culture medium may serve as an auxiliary process indicator of gametophyte biomass accumulation. Because the analysis included only seven time points and all variables changed with time, these correlations were not interpreted as independent causal relationships.

Table 4. Summary of electrical conductivity correlations, PCA, and cluster analysis.

Analysis Target	Statistic	<i>p</i> Value/ Cumulative Variance	Interpretation
EC-fresh weight	$r = -0.999$	$p < 0.0001$	Very strong negative correlation; auxiliary biomass process indicator
EC-dry weight	$r = -0.986$	$p < 0.0001$	Very strong negative correlation
EC-total flavonoid yield	$r = -0.970$	$p = 0.0003$	Total flavonoid yield increased as EC decreased
EC-total phenolic yield	$r = -0.909$	$p = 0.0046$	Mid-to-late total phenolic yield changed synchronously with decreasing EC
Bioreactor PCA (PC1)	80.76%	97.48%	Explained most multivariate variation
Medium-optimization PCA (PC1)	85.63%	95.80%	Supported comprehensive medium screening

Note: Pearson correlations were based on the means of seven bioreactor sampling times, and *p* values were obtained from two-sided tests. PCA was performed on a Z-score-standardized multivariate matrix.

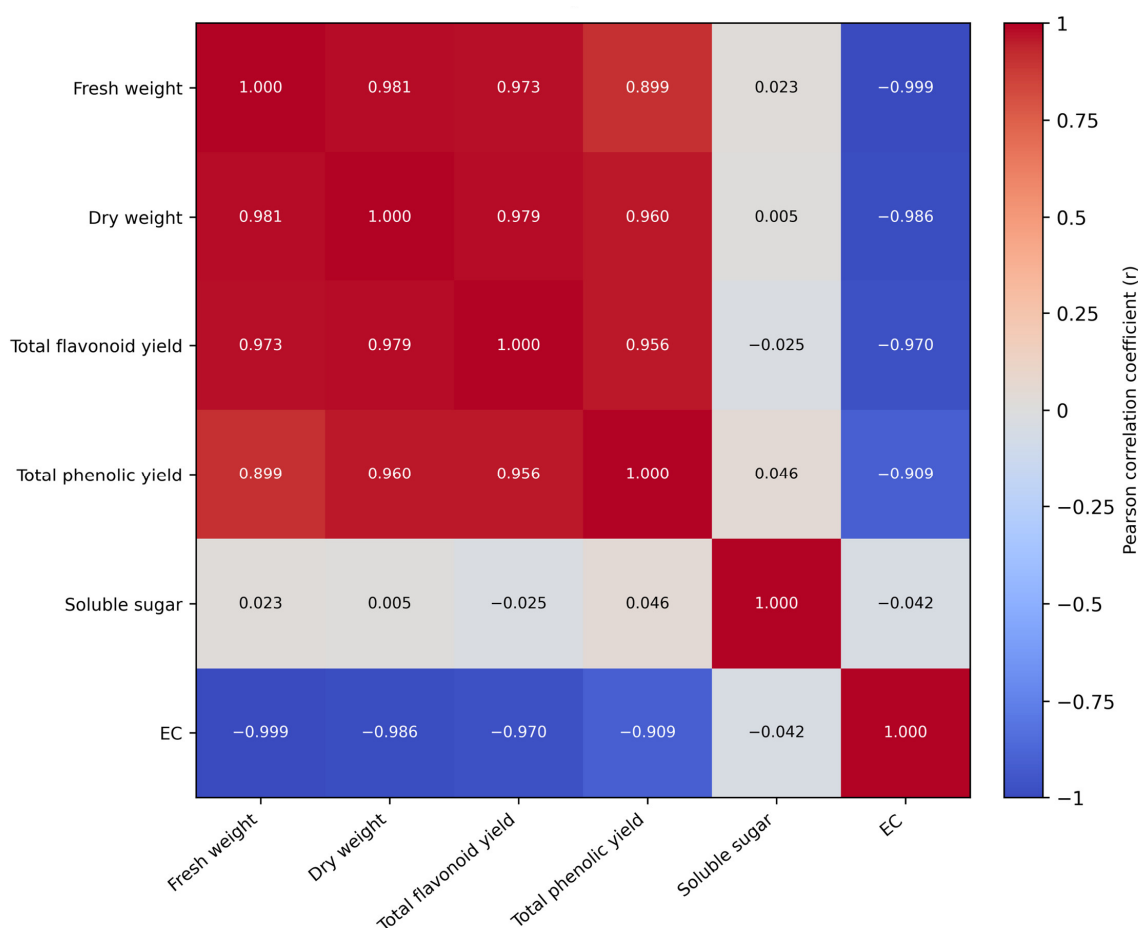


Figure 3. Pearson correlation heatmap of multiple indices during air-lift bioreactor culture.

In the multivariate PCA of the bioreactor time course, PC1 and PC2 explained 80.76% and 16.72% of the variation, respectively, for a cumulative total of 97.48% (Figure 4). PC1 represented a composite gradient of increasing fresh weight, dry weight, and metabolite yields accompanied by decreasing EC, whereas PC2 mainly represented soluble sugar content and culture-stage differences. Ward clustering classified 9 d as the early stage, 18–27 d as the rapid-growth stage, and 36–61 d as a high-biomass-to-decline transition

stage. The 45 d time point occupied the core of the high-yield stage, consistent with the Duncan groupings and individual-variable trends.

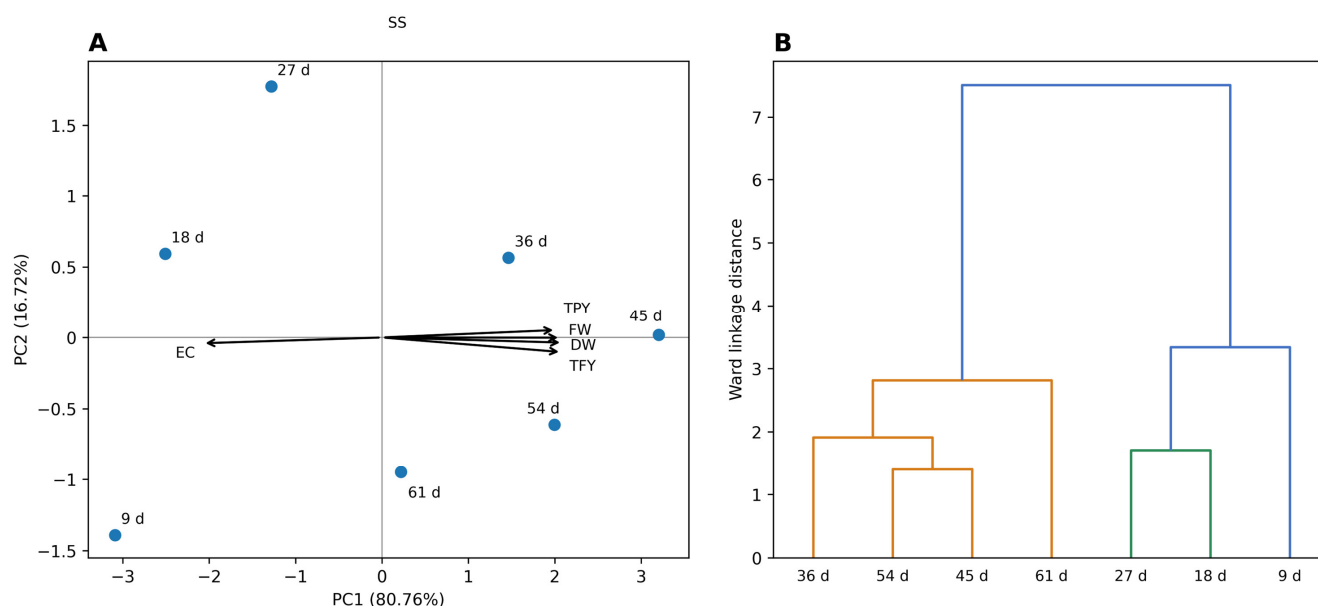


Figure 4. PCA biplot (A) and Ward hierarchical clustering (B) of bioreactor culture times. PCA variables included fresh weight, dry weight, total flavonoid yield, total phenolic yield, soluble sugar content, and EC.

3.6. Sporophyte Induction from Proliferated Gametophytes

Gametophytes obtained after liquid proliferation retained the ability to form sporophytes, and significant differences were observed among the three induction systems (Table 5). SI-1 produced the highest sporophyte conversion rate ($63.02\% \pm 1.95\%$), which was significantly higher than those of SI-2 and SI-3. SI-1 also produced the shortest times to first sporophyte emergence and stable sporophyte establishment, at 28.89 ± 0.31 d and 44.67 ± 0.53 d, respectively, and its acclimatization survival rate was $91.77\% \pm 1.81\%$. Relative to the peat:perlite system (SI-3), SI-1 increased the conversion rate by 27.86 percentage points and acclimatization survival by 11.01 percentage points, indicating that an aseptic solid-medium stage improved the consistency and stability of sporophyte induction. Representative photographs of gametophyte liquid culture and sporophyte regeneration are provided in Figure 5.

Table 5. Effects of different induction systems on sporophyte formation and acclimatization survival from proliferated gametophytes.

Treatment	Induction System	Conversion (%)	First Emergence (d)	Stable Establishment (d)	Survival (%)
SI-1	Proliferated gametophytes → 1/2-strength MS solid medium (2% sucrose)	63.02 ± 1.95 a	28.89 ± 0.31 a	44.67 ± 0.53 a	91.77 ± 1.81 a
SI-2	Proliferated gametophytes → full-strength MS solid medium without plant growth regulators	46.02 ± 1.99 b	32.00 ± 0.50 b	49.89 ± 0.59 b	85.29 ± 2.01 b
SI-3	Proliferated gametophytes → sterilized peat:perlite substrate (2:1)	35.16 ± 2.19 c	35.44 ± 0.41 c	54.22 ± 0.46 c	80.76 ± 2.63 b

Note: Values are means $\pm$ SE (n = 9). Within each column, different lowercase letters indicate significant differences among treatments according to Duncan's multiple range test ($p < 0.05$). Shorter times to first emergence and stable establishment indicate faster induction.

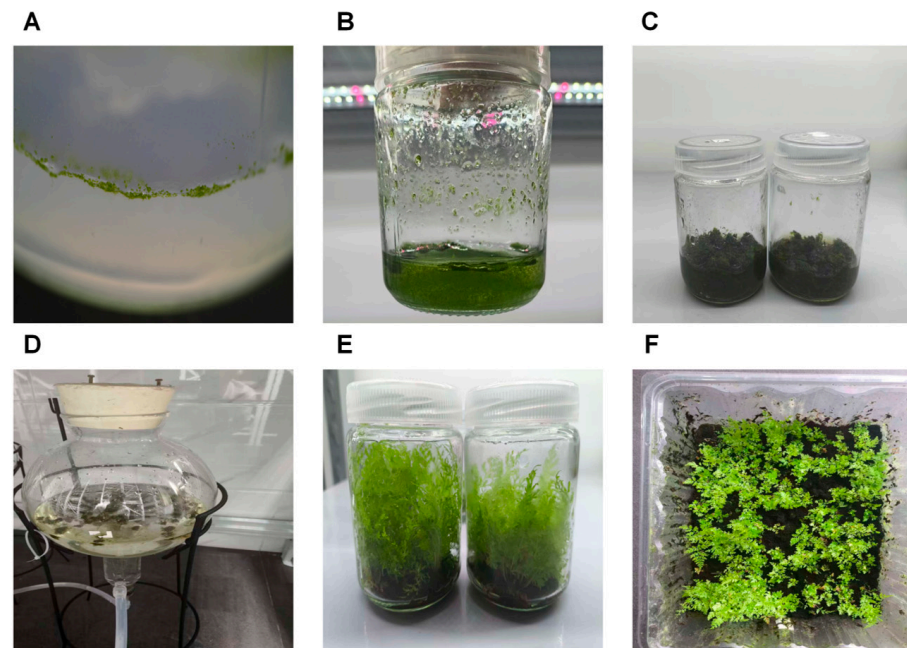


Figure 5. Representative stages of the continuous in vitro propagation of *Cibotium barometz*. (A) Spore germination; (B) gametophytes before liquid culture; (C) gametophytes after shake-flask liquid culture; (D) gametophyte culture in a 5 L air-lift bioreactor; (E) sporophyte induction; (F) acclimatized sporophytes.

4. Discussion

Plant growth regulators exert stage-dependent effects on fern spore germination and gametophyte establishment [13–19]. In the present study, S-5 (full-strength MS medium without sucrose supplemented with 0.4 mg L^{-1} NAA, 0.4 mg L^{-1} TDZ, and 0.8 mg L^{-1} GA₃) increased both germination and gametophyte longest-axis length, indicating that this defined combination of NAA, TDZ, and GA₃ favors early cell division and laminar expansion. Isnaini and Praptosuwiryo [13] reported that 1/2-strength MS medium promoted early germination of *C. barometz*, whereas Yu et al. [19] obtained high germination in a low-salt medium, demonstrating that mineral nutrition and plant growth regulator effects should be interpreted in relation to developmental stage. Main-effect analysis indicated that NAA had the largest range for both 75 d germination and gametophyte longest-axis length. However, the best directly observed combination, S-5, was not equivalent to a simple assembly of the individually best factor levels, supporting the need to evaluate complete combinations. Because the L9 design did not allocate columns for interactions, this difference was not interpreted as statistical evidence of interaction but rather as a basis for subsequent full-factorial validation.

The liquid-culture results further demonstrated that maximum fresh weight and optimum overall dry-matter and metabolite yield did not coincide. M-6 (1/2-strength MS basal salts with full-strength vitamins, 1% sucrose, 0.1 mg L^{-1} 6-BA, and 1.2 mg L^{-1} IAA) maximized fresh biomass, whereas M-5 (1/2-strength MS basal salts with full-strength vitamins, 4% sucrose, 0.4 mg L^{-1} 6-BA, and 0.8 mg L^{-1} IAA) provided a better balance of dry matter, total flavonoid yield, and total phenolic yield. 6-BA produced relatively large ranges for the four major yield traits, and higher 6-BA levels were generally unfavorable for biomass accumulation. In contrast, 1.2 mg L^{-1} IAA and 4% sucrose favored fresh biomass, dry biomass, and phenolic yields in different ways. Sucrose acts both as a carbon source and an osmotic regulator; insufficient sucrose may restrict rapid proliferation, whereas higher concentrations can favor dry-matter and secondary-metabolite accumulation [19,26–29]. Recent multi-omics analysis of *C. barometz* tissue-cultured plantlets also showed that sugar

metabolism and signaling by IAA, cytokinins, and GAs are important during transitions among filamentous, gametophytic, and sporophytic stages [20]. Culture-medium selection should therefore be guided by an explicitly defined objective, such as proliferation-oriented or metabolite-oriented production, rather than by fresh weight alone. Importantly, the medium used in the 5 L bioreactor was 1/2-strength MS basal salts with full-strength vitamins, 4% sucrose, 0.1 mg L^{-1} 6-BA, and 1.2 mg L^{-1} IAA, a candidate combination predicted from the L16 main-effect means, rather than M-5 or M-6 from Table 2. Because these three formulations were not directly compared in the same shake-flask experiment, the bioreactor medium is interpreted only as a scale-up validation formulation and is not claimed to outperform all directly tested combinations.

In the 5 L air-lift bioreactor, 45 d combined the greatest fresh weight, high dry weight, and the greatest total flavonoid yield, whereas biomass and flavonoid yield declined after 54 d as EC began to increase. Air-lift bioreactors improve mass transfer through circulation without mechanical agitation and can reduce shear injury, making them useful for scaling up horticultural and medicinal plant cultures [26,27]. The strong temporal correspondence between declining EC and biomass accumulation in the present study suggests that rapid or online EC monitoring may help identify nutrient-ion consumption and culture stage. However, the correlation analysis included only seven time points, and shared temporal trends may have contributed to the high coefficients. EC should therefore be considered an auxiliary signal requiring calibration against biomass and metabolite measurements, rather than an independent causal indicator or sole harvest criterion.

The sporophyte-induction results illustrate the complementary roles of aseptic culture systems and low-cost substrates. SI-1 (1/2-strength MS solid medium containing 2% sucrose and 7.0 g L^{-1} agar, without plant growth regulators) achieved higher sporophyte conversion and acclimatization survival than either full-strength hormone-free MS solid medium or the sterilized peat:perlite substrate (2:1, *v/v*), suggesting that a compositionally controlled solid medium with a low contamination risk provides more consistent water availability, mineral nutrition, and explant performance during early sporophyte formation. Peat and other simple substrates, in contrast, are inexpensive and operationally convenient for post-regeneration acclimatization and nursery production, but their moisture, microbial, and nutrient conditions are more difficult to standardize [14]. The advantage of the present system is therefore not the complete replacement of inexpensive substrates. Rather, it supports a two-stage strategy in which consistent spore germination, gametophyte proliferation, and sporophyte formation are first achieved under controlled *in vitro* conditions, followed by transfer to a low-cost peat-perlite substrate for acclimatization. This approach balances propagation efficiency, quality control, and production cost.

This study combined orthogonal screening, Duncan grouping, PCA, clustering, and EC process monitoring to evaluate the continuous culture sequence from spore to sporophyte. Main-effect analysis addressed the limitations of comparing only the 16 observed combinations, whereas PCA and clustering revealed coordinated changes among multiple indicators. Nevertheless, several limitations remain. The L9 and L16 designs did not estimate interactions; the bioreactor correlation analysis included only seven sampling times and a limited number of independent bioreactor replicates; the main-effect-predicted bioreactor formulation has not yet been compared directly with M-5 and M-6 in the same experimental run; and genetic stability, seedling production cost, and performance at larger scales remain to be evaluated. Future studies should use full-factorial or response-surface designs to test key interactions, directly validate candidate media, and assess ploidy, genetic fidelity, long-term transplant performance, and production economics.

Overall, gametophytes produced by liquid proliferation can serve both as biomass-production material and as propagation material for sporophyte regeneration, thereby

reducing dependence on wild rhizomes and donor plants. For *C. barometz*, a Grade II nationally protected species subject to international trade controls [6,7], a traceable spore–gametophyte–sporophyte–acclimatized-plantlet workflow may facilitate ex situ conservation, standardized seedling production, and sustainable resource use.

5. Conclusions

A continuous in vitro propagation system was established for *C. barometz*, extending from spore germination and gametophyte liquid proliferation to 5 L air-lift bioreactor culture and sporophyte induction. S-5 (full-strength MS medium without sucrose supplemented with 0.4 mg L^{−1} NAA, 0.4 mg L^{−1} TDZ, and 0.8 mg L^{−1} GA₃) performed best for spore germination and early gametophyte growth; M-6 (1/2-strength MS basal salts with full-strength vitamins, 1% sucrose, 0.1 mg L^{−1} 6-BA, and 1.2 mg L^{−1} IAA) was most suitable for rapid fresh-biomass proliferation, whereas M-5 (1/2-strength MS basal salts with full-strength vitamins, 4% sucrose, 0.4 mg L^{−1} 6-BA, and 0.8 mg L^{−1} IAA) better balanced dry-matter and phenolic yields. Based on the integrated multivariate evaluation, 45 d was the recommended culture period under the tested bioreactor conditions, and EC can serve as an auxiliary process indicator when interpreted together with biomass and metabolite measurements. SI-1 (1/2-strength MS solid medium containing 2% sucrose and 7.0 g L^{−1} agar, without plant growth regulators) achieved a sporophyte conversion rate of 63.02% and an acclimatization survival rate of 91.77%. The system provides a methodological basis for ex situ conservation and scalable seedling production of *C. barometz*. However, the candidate bioreactor formulation still requires direct comparison with M-5 and M-6, and key equipment parameters, factor interactions, and larger-scale production performance require further validation.

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Abbreviations

The following abbreviations are used in this manuscript:

6-BA	6-benzylaminopurine
EC	Electrical conductivity
GA ₃	Gibberellic acid
IAA	Indole-3-acetic acid
MS	Murashige and Skoog medium
NAA	1-Naphthaleneacetic acid

PCA	Principal component analysis
SE	Standard error
TDZ	Thidiazuron

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