Research on tissue rapid breeding technology of endangered plant Cibotiumbarometz (L.) J. Sm.

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Research Article

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Research on tissue rapid breeding technology of endangered plant *Cibotium barometz* (L.) J. Sm.

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Abstract This research aimed to establish the *Cibotium barometz* tissue rapid breeding technology and provide a theoretical basis for its artificial breeding and industrial planting. The spores' germination conditions were selected, along with the optimum sterilization time and spore germination medium. The germination material was incubated in different basic media to select a basal medium suitable for proliferation and differentiation. Based on the results of basic medium screening, the proliferation orthogonal and differentiation experiments were performed to screen the best media, and then the *C. barometz* rooting experiment was performed in two single-factor experiments by using the differentiated sporophytes as materials. The optimal sterilization time of *C. barometz* spores was 11 minutes and the best medium for germination was 1/8MS medium supplemented with 0.02 mg/L IAA. MS medium was suitable for the proliferation and differentiation of tissue culture seedlings, while the WPM medium was suitable for sporophyte differentiation. The best medium for growth was the MS medium, supplemented with 1.0 mg/L IAA and 0.5 mg/L 6-BA. Concentrations of sucrose had a significant effect on the differentiation of tissue culture seedlings, while no sucrose culture had barely any growth or differentiation effect on sporophytes. The effect of proliferation and differentiation was the best when sucrose concentration was set at 20 g/L. Low concentration cannot limit the growth or differentiation, while high concentration may inhibit those processes. The PGRs including IAA, NAA, 6-BA, and GA_3 had better differentiation effects, but the KT was slightly weaker. The rooting rate of sporophytes initially increased and then decreased with the concentrations of ABT, IBA, and IAA. The rooting rate reached the maximum when the concentrations of ABT and IBA were at 1.5 mg•L^{-1} and IAA was at 1.0 mg•L^{-1} (which were 86.82%, 91.52%, and 90.89%, respectively). Compared with ABT and IAA, IBA had a significant effect on the average and the longest root length of *C. barometz*, and the three PGRs had no obvious effect on the average or maximum diameter. The optimal cultivation conditions for the organization of rapid breeding technology from the germination, growth, differentiation, and rooting of *C. barometz* were obtained, and the sporophytes were successfully transplanted to the external environment, and all these results provided a theoretical reference for the industrial planting of *C. barometz*.

Keywords *Cibotium barometz* (L.) J. Sm., Rapid breeding technology, differentiation, tissue culture

Key message

In this study, a more systematic and complete tissue culture rapid propagation system was formed from spore germination, proliferation, differentiation, and rooting, along with the sporophytes for transplanting and culture.

Introduction

The *Cibotium barometz* (L.) J. Sm. is a fern belonging to the family *Dicksoniaceae*, is native to tropical and subtropical Asia and grows primarily in the wet tropical biome (Yang et al. 2015). The rhizome of *C. barometz* is an important traditional Chinese medicine for treating rheumatism, limb pain, and sciatica. The hairs on the rhizome are another medicinal part, and they have been used for hemostasis (Qiu and Deng. 2020). The main active components of rhizomes are volatile oils, phenols, phenolic acids, flavonoids, polysaccharides, terpenes, and trace elements (Shi et al. 2016). Among them, polysaccharide is the most enriched compound (Xu et al. 2011), while phenolic acids are the primary

active components of *C. barometz* (Jia et al. 2000). However, *C. barometz*'s presence in nature is endangered due to the destruction of the ecological environment and high demand as a raw material for medicine. *C. barometz* has been listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (Appendix II) (CITES 2023) and the Category Ⅱ of national essential protected wild plants in China (Forestry Administration. 2021).

Currently, *C. barometz* in China has a total distribution area of around 7 000 hm^2, an annual output of about 850 000 Kg, an annual consumption of about 800 000 Kg, and a growth and renewal cycle of about five to seven years (Yang et al. 2015). The ground component of the *C. barometz* is considerably larger than that of regular ferns since it has underdeveloped roots, and it requires much more water and nutrients than regular ferns. However, the underground portion of plants, such as common ferns with their shallow adventitious roots that are soft and slender, has a very limited capacity for absorption, which is an innate limitation on the viability of *C. barometz* plants (Zhang and Zhang. 2010). Additionally, the wild *C. barometz*'s reserves are shrinking annually and it has been added to the List of Wild Plants under State Key Protection (the first batch, 2021) due to its relatively slow growth rate, low efficiency of natural renewal, poor ability to adapt to the change of external environments like soil and light conditions, and the ongoing degradation of the world's ecological environment by over-exploitation of nature and poor production practices. Besides, *C. barometz* is an indispensable gradient in many proprietary Chinese medicines, such as Jinmao Goji Pills, Liuwei Dihuang Pills, Anti-Bone Hyperplasia Capsules, and Strong Waist Health Kidney Pills (Yu et al. 2023). The vulnerability of *C. barometz* and its high medicinal values bring significance to the research of *C. barometz* tissue culture and in vitro rapid propagation technology, which can promote the protection of *C. barometz* resources in nature.

Most artificial cultivation techniques currently in use are propagation methods, with sluggish and poor propagation coefficients and yields that fall far short of satisfying the demands of the pharmaceutical industry (Botanical Society of China. 2013; Ye et al. 2020). Although previous studies have been reported on *C. barometz* artificial cultivation technology recently (Wu and Cen. 2016; Weng. 2012), its rooting and other issues have not made significant strides. In vitro culture is one of the techniques for ex-situ reproduction and protection of rare and endangered ferns (Isnaini and Praptosuwiryo. 2020). The rapid breeding technology of plant tissue has the advantages of controllable culture conditions, high breeding efficiency, fast growth, and small occupation space, which can make up for the deficiency of traditional breeding technology. Therefore, this study focuses on the tissue-based rapid breeding techniques of *C. barometz* to provide a theoretical basis for the artificial breeding of *C. barometz* to meet its demands from the market while minimizing the consumption of natural *C. barometz* resources.

Methods

Plant materials

The spores of *C. barometz* were collected from Guangxi Botanical Garden of Medicinal Plants in July 2022, and the material of the *C. barometz* was identified as the *Cibotium barometz* (L.) J. Sm. by researcher Wei Kunhua and material from the lamellae period obtained by using *C. barometz* spores as ex-plants in 1/8MS medium. The mature *C. barometz* spores were sterilized with 75% (v/v) ethanol for 15 s, followed by 0.1% (w/v) mercuric chloride solution for 5, 8, 11, and 14 min, respectively. Finally, the spores were rinsed with sterile water 4-5 times and inoculated in 1/8MS medium. There were 4 sterilization schemes in total, with each scheme consisting of 30 ex-plants. The spores germinated at 25 ℃ under a 12/12 h photoperiod, with cool white fluorescent light (1 000 lx). Germination rates were

recorded over the 60-day culture.

Effect of different basic media on tissue seedling growth in *C. barometz*

The MS medium was selected as the basal medium in this study. An orthogonal test was used to select the best combination and concentration of PGRs for promoting spore germination. For the test, three PGRs, namely, 6-BA, NAA, and IAA, were used at four concentrations (0, 0.02, 0.04, and 0.06 mg/L) each. The orthogonal design was repeated three times, and each treatment consisted of 10 ex-plants. The spores germinated at 25 ℃ under a 12/12 h photoperiod, with cool white fluorescent light (1 000 lx). Following 60 d of culture, germination rates were recorded.

Effect of PGSs on the growth of tissue seedlings in *C. barometz*

To choose the optimal basal medium for gametophyte proliferation, gametophyte was cultured on MS, 1/2MS, WPM, White, N6, and B5 medium. To each medium was added 0.5 mg/L NAA, 1.5 mg/L 6-BA, and 0.25 g/L activated carbon. Thereafter, PGR containing medium at various PGR concentrations (6-BA: 0, 0.5, 1.0, 1.5; NAA and IAA: 0, 0.2, 0.5, and 1.0 mg/L 6-BA, NAA, and IAA) was made by the addition of PGR, and orthogonal design was employed to select the optimum combination and concentration of PGRs. The orthogonal design was repeated three times, and each treatment consisted of 36 ex-plants. The gametophyte was cultured at 25 ℃ under a 12/12 h photoperiod, with cool white fluorescent light (1 000 lx). After 60 days of culture, the multiplication rates and growth state of gametophyte were recorded.

Effect of different concentrations of sucrose and PGRs on tissue seedling differentiation in *C. barometz*

To determine the effect of sucrose and PGRs on sporophyte induction, the gametophyte was transferred to an MS medium with different concentrations of sucrose (0, 10, 20, 30, 40, and 50 g/L) and different concentrations (0.1, 0.5, 1.0, 1.5, and 2.0 mg/L) of different PGRs (IAA, 6-BA, NAA, GA_3, and KT). All the mediums were supplemented with 3% sucrose, 1.5 mg/L 6-BA, 0.5 mg/L NAA, and 0.25 g/L activated carbon except those containing specific sucrose or PGRs concentrations. For each treatment, 36 ex-plants were transferred, and each treatment was repeated three times. The plants were cultivated at 25 ℃, with a relative humidity of 40%-55%, and a 12/12 h photoperiod, with cool white fluorescent light (1 000 lx), and after 45 days the number of sporophytes was examined.

Effect of PGRs on tissue seedling rooting in *C. barometz*

Sporophytes were transferred into MS medium supplemented with 0.25 g/L activated carbon and different compositions and concentrations of PGRs: ABT (0.1, 0.5, 1.0, 1.5, and 2.0 mg/L), IBA (0.1, 0.5, 1.0, 1.5, and 2.0 mg/L), and IAA (0.1, 0.5, 1.0, 1.5, and 2.0 mg/L). 36 ex-plants were used for each treatment, which was set in triplicate. The plants were cultivated at 25 ℃, with a relative humidity of 40%-55%, and a 12/12 h photoperiod, with cool white fluorescent light (1 000 lx). The percentage of root induction, root number, root length, the longest root length, root diameter, and the maximum diameter were recorded after 45 days.

Statistical analysis

Germination rate (%) = the number of germination per vial / the number of total inoculum per vialc ×%

Statistical data was analyzed using Excel2010 and SPSS26 statistical software and Duncan multiple comparisons.

Results

Aseptic system establishment and germination of *C. barometz*

As shown in Table 1, the sterilization effects of four treatments for *C. barometz* spores were compared.

Among the four treatments, the optimal sterilization time for spore germination was 11 minutes, which had the highest survival rate (80%) and lower contamination rate (5%). The survival rate of spores initially increased and then decreased with the extension of mercuric chloride solution disinfection time. Although the contamination rate was significantly suppressed by 14-minute exposure of mercuric acid, the reduced survival rate indicated that the optimal *C. barometz* treatment was 11 minutes.

PGRs play a key role in spore germination, and the different concentrations of PGRs significantly affect the germination of *C. barometz* spores. The range analysis indicated that 6-BA had the greatest influence on germination, followed by NAA and IAA (Table 2). Further optimization showed that the spore germination rate ranged from 16.11% ~ 50.09%. The spore germination rate was the highest in the presence of 6-BA (50.09%) (Table 3), while the addition of 0.02 mg/L 6-BA or IAA reduced the germination rate to 44.72% and 43.89%, respectively. The maximum germination range of the same factor at different concentrations was 33.98%, and germination also occurred at 6-BA concentrations from 0 to 0.06 mg/L. The germination rate and the 6-BA content are negatively correlated, indicating that 6-BA may inhibit the germination of *C. barometz* spores. Based on the above results, 1/8MS medium supplemented with 0.02 mg/L IAA would be a better medium for *C. barometz* spore germination.

Table 1 Effects of different disinfection times on germination of *C. barometz*

Sterilization time/min	Number of vaccinations/bottle	Pollution count/bottle	Number of deaths/bottle	Survival number/bottle	contamination rate/%	mortality/%	Success rate/%
5	20	9	0	11	45	0	55
8	20	5	1	13	25	5	70
11	20	3	1	16	15	5	80
14	20	2	6	12	10	30	60

Table 2 Variance analysis of *C. barometz* germination rates on different compositions and concentrations of PGRs

Source of variance	Sum of variance	df	Variance	F-value	P-value
6-BA	2721.770	3	907.257	12.108	0.01<P<0.05
NAA	236.864	3	78.955	1.054	P>0.1
IAA	392.576	3	130.859	1.746	P>0.1
Error	224.794	3	74.931		
Sum	3576.005	12			

Table 3 Range analysis of *C. barometz* germination rates on different compositions and concentrations of PGRs

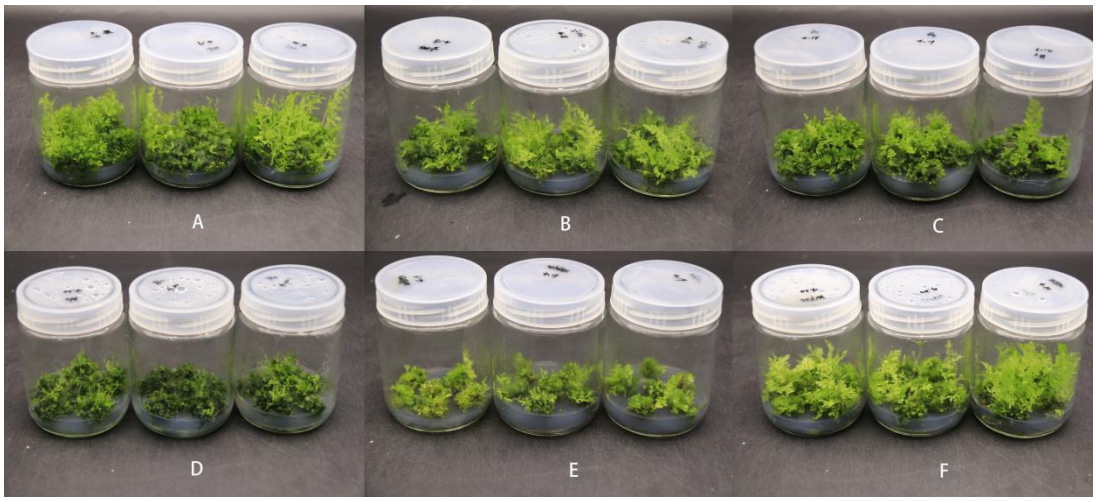
Concentration of phytohormone (mg/L)			Factors		
6-BA	NAA	IAA	A(6-BA)	B(NAA)	C(IAA)
0	0	0	$K_A1/4=50.09$	$K_B1/4=32.31$	$K_C1/4=34.53$
0.02	0.02	0.02	$K_A2/4=44.72$	$K_B2/4=38.89$	$K_C2/4=43.89$
0.04	0.04	0.04	$K_A3/4=16.11$	$K_B3/4=31.94$	$K_C3/4=35.00$

0.06	0.06	0.06	K_A4/4=32.78	K_B4/4=40.56	K_C4/4=30.28
	R(range)		33.98	8.62	13.61

Effects of basal medium on gametophyte proliferation

We noticed variations in the multiplication rate and growth state of gametophytes grown in different basal mediums (Fig. 1; Table 4). Furthermore, the strongest gametophyte proliferation and a great number of sporophytes were observed on the MS medium. However, the proliferation of gametophytes cultured in the WPM medium was slightly lower than that in the MS medium, but the formation of sporophytes was faster in greater quantity. Therefore, the MS medium was suitable for the proliferation and differentiation of *C. barometz* gametophytes while the WPM medium promoted the formation of sporophytes. In addition, we found that N6 and White medium were not conducive to the formation of sporophytes. Considering the growth rate, we chose the MS medium as the optimal basal medium for proliferation.

Fig. 1



Growth status of *C. barometz* with different basal media. A: MS，B：1/2MS，C：B5，D：N6，E：White，F：WPM.

Table 4 Effects of the different basal media on *C. barometz* gametophyte proliferation

Treatment	Growth rate/bottle	Number of sporozoites/bottle	Growth conditions
MS	11.82±0.55a	33.89±1.23a	Large proliferation, plenty of sporophytes, and the sporophyte is larger and green
1/2MS	8.33±0.41b	29.56±2.51b	Small proliferation times, plenty of sporophytes, the sporophyte is green
B5	7.87±0.63b	27.00±2.32b	Small proliferation, plenty of sporophytes, but sporophytes are small
N6	8.13±0.18b	8.28±0.54d	Small proliferation factor, fewer sporophyte count, and smaller sporophytes
White	3.28±0.51c	15.11±1.94c	Small proliferation times, fewer sporophytes, and smaller sporophytes
WPM	8.41±0.02b	35.06±3.20a	Small proliferation times, plenty of sporophytes, large sporophytes, and greener

Different lowercase letters in the same column indicated a significant difference at P <0.05. n=6 indicates six replicates.

Effects of PGRs on gametophyte proliferation

The MS medium was selected as the basal medium to perform orthogonal experiments with three factors and four levels. The concentrations of cytokinins (6-BA) and auxins (NAA and IAA) were screened by orthogonal assays to search for the optimal plant PGRs. In this study, variance analysis revealed that 6-BA, NAA, and IAA had no significant effect on the growth of *C. barometz* gametophyte (Table 5). Further optimization showed that the growth of seedlings ranged from 12.30 to 15.27. When the concentration of IAA was 1.0 mg/L, the growth rate of tissue culture seedlings reached the highest level(14.98), and which was also at high level(14.25) when the concentration of 6-BA was 0.5 mg/L. The maximum growth range of the same factor at different concentrations was 3.75, and it was also observed at IAA concentrations of 0.00 mg/L to 1.0 mg/L, indicating that the concentration of IAA may require further optimization. Based on the above results, we found that the maximum gametophyte proliferation was achieved on MS medium supplementing 1.0 mg/L IAA and 0.5 mg/L 6-BA, respectively. And we may draw a conclusion that the best combination and concentration of PGRs for gametophyte proliferation was the MS medium supplemented with 0.5 mg/L 6-BA and 1.0 mg/L IAA.

Table 5 Variance analysis of *C. barometz* growth rate on different compositions and concentrations of PGRs

Source of variance	Sum of variance	df	Variance	F-value	P-value
6-BA	7.914	3	2.638	0.279	P>0.1
NAA	16.886	3	5.629	1.086	P>0.1
IAA	15.552	3	5.184	0.549	P>0.1
Error	28.320	3	9.440		
Sum	68.672	12			

Table 6 Range analysis of *C. barometz* growth rate on different compositions and concentrations of PGRs

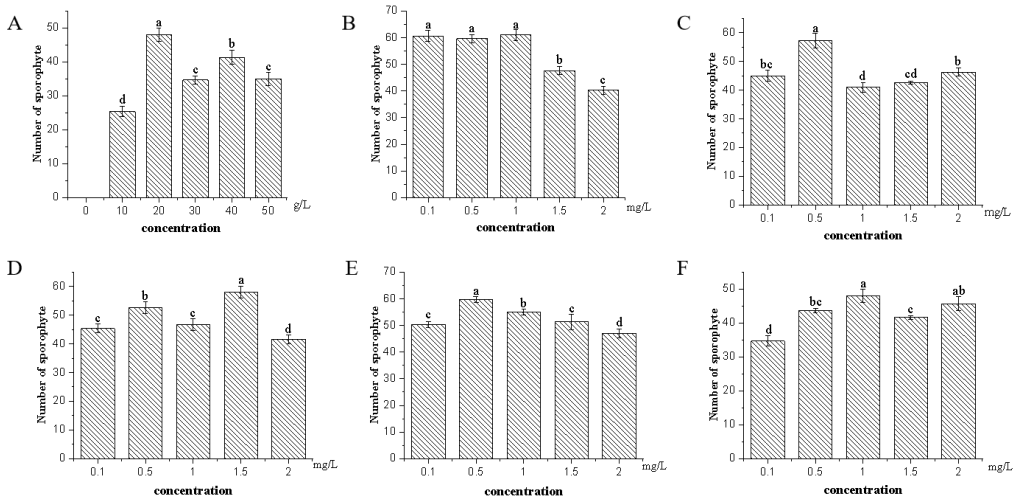
Concentration of phytohormone (mg/L)			Factors		
6-BA	NAA	IAA	A(6-BA)	B(NAA)	C(IAA)
0	0	0	$K_A1/4$=12.41	$K_B1/4$=14.17	$K_C1/4$=12.30
0.5	0.2	0.2	$K_A2/4$=14.25	$K_B2/4$=13.24	$K_C2/4$=13.89
1.0	0.5	0.5	$K_A3/4$=13.72	$K_B3/4$=12.49	$K_C3/4$=13.16
1.5	1.0	1.0	$K_A4/4$=13.95	$K_B4/4$=13.34	$K_C4/4$=14.98
	R(range)		1.85	1.68	3.75

Effects of concentrations of sucrose and PGRs on induction of sporophytes

Sporophytes formation was monitored at various sucrose and PGRs (6-BA, IAA, NAA, GA_3, and KT) concentrations to find the optimal conditions to induce sporophytes formation. Among the various concentration tests (Fig. 2), the highest number of sporophytes was obtained on MS medium with 20 g/L sucrose. However, sporophyte formation was inhibited when the concentration of sucrose was higher than 40 g/L. Furthermore, the concentrations of difference had a significant effect on the differentiation of *C. barometz* gametophytes. Overall, 6-BA, IAA, NAA, and GA_3 promoted the differentiation of gametophyte significantly to a greater extent than the effect of KT. After 45 days of

culture, the maximum sporophyte number (59.33) was obtained on the medium that was supplemented with 0.5 mg/L GA_3. Best differentiation was observed at IAA concentration of 0.5 mg/L with a sporophyte number of up to 57.33 plants per bottle, NAA concentration of 1.5 mg/L with sporophyte number of up to 58 plants per bottle. The differentiation at 0.5 mg/L NAA was close to those treated at 1.5 mg/L, where the number of sporophytes was 52.67 plants per bottle. 6-BA concentrations in the 0.1 mg/L - 1.0 mg/L range resulted in similar sporophytes differentiation and declined at concentration higher than 1.5 mg/L. The sporophyte differentiation level peaked at 0.5 mg/L GA_3 and decreased as the GA_3 concentration increased. The differentiation effect was the strongest when the KT concentration is 1.0 mg/L, where the number of sporophytes was up to 48 plants per bottle

Fig. 2



Effect of different concentrations of sucrose and different PGRs on *C. barometz* gametophyte proliferation. A: Sucrose concentration，B: 6-BA concentration，C: IAA concentration，D: NAA concentration，E: GA_3 concentration，F: KT concentration. Each metric is expressed as mean ± standard deviation, n=6, and different lowercase letters in each treatment indicate the difference between different treatments ($P < 0.05$).

Effects of PGRs on tissue seedling rooting in *C. barometz*

The PGRs ABT, IBA, and IAA had different effects on rooting percentage, mean root length, longest root length, average diameter, and maximum diameter. As shown in Table 7, within a certain concentration range, the rooting rate of *C. barometz* initially increased and then decreased with the amount of ABT, IBA, and IAA concentrations. The maximum percentage of rooting (91.52%) was observed in the MS medium with 1.5 mg/L IBA. Compared with ABT and IAA, IBA had a significant effect on the average root length and longest root length in *C. barometz*, and there was no significant difference in the effects of the three PGRs on average diameter and maximum diameter. The rooting rate of *C. barometz* sporophyte was high, which was conducive to improving the survival rate of transplanting *C. barometz* sporophyte in the later stage and laying a certain foundation for the industrial planting of *C. barometz*.

Table 7 Effects of different concentrations of PGRs on the rooting of *C. barometz* sporophyte seedlings

Hormone	Concentration /mg•L^{-1}	Rooting rate/repetition (%)	Mean root length/ repetition(cm)	Maximum root length/plant(cm)	Mean diameter/ repetition(mm)	Maximum diameter/plant(mm)
ABT	0.1	73.08±6.01b	7.65±0.87b	14.57±2.60bc	0.68±0.08ab	0.87±0.03a
	0.5	83.83±4.57a	6.10±0.65b	11.92±1.17c	0.62±0.07b	0.97±0.21a
	1.0	84.80±6.53a	7.91±0.99ab	18.13±3.23ab	0.78±0.10a	1.04±0.18a
	1.5	86.82±2.45a	7.62±0.84b	15.64±3.46abc	0.77±0.11a	1.15±0.23a
	2.0	83.87±7.55a	9.69±1.45a	20.43±3.23a	0.73±0.01ab	1.28±0.29a
IBA	0.1	77.75±4.61b	7.33±1.36d	16.12±4.31b	0.62±0.12a	0.86±0.12b
	0.5	78.31±0.46b	8.66±1.03cd	16.43±1.44b	0.62±0.02a	0.92±0.21b
	1.0	86.69±0.71a	10.89±0.91bc	20.62±4.42ab	0.65±0.03a	1.09±0.06ab
	1.5	91.52±3.68a	13.30±2.64ab	22.27±2.82ab	0.72±0.02a	1.19±0.04a
	2.0	89.54±1.34a	13.85±0.86a	23.03±1.84a	0.71±0.06a	1.16±0.12a
IAA	0.1	83.43±1.14b	7.05±0.82c	16.39±1.18b	0.54±0.02b	0.76±0.10b
	0.5	73.16±5.06c	7.13±1.06c	16.31±1.23b	0.70±0.08a	1.05±0.17a
	1.0	90.89±1.99a	9.91±0.56b	24.72±4.09a	0.66±0.07a	0.90±0.21ab
	1.5	87.12±0.66ab	9.76±2.42bc	21.49±1.97ab	0.69±0.02a	1.02±0.12ab
	2.0	82.38±5.70b	12.63±1.62a	24.01±4.68a	0.72±0.05a	1.05±0.10a

Note: Each metric is expressed as mean ± standard deviation, n=6, and different lowercase letters in each treatment indicate the difference between different treatments ($P < 0.05$).

Discussion

Effect of disinfection time and PGRs on germination of *C. barometz*

The vast majority of ferns have sporangia on the lower surface of their leaves and reproduce mainly as spores (Yu et al. 2017). As a fern, the mature spore of *C. barometz* is usually used as an ex-plant. In ex-plant sterilization, the selection and treatment of ex-plants are the first step toward the establishment of plant tissue culture (Gong et al. 2010). It is crucial to control the disinfection time of ex-plants, the ex-plants are easily contaminated if the disinfection time is too short, while long-term sterilization is toxic to ex-plants and can even kill them. In this study, we found that the material contamination rate was the highest with 5-minute disinfection, while the mortality rate was the highest with 14-minute disinfection. Sterilization for 8 minutes and 11 minutes led to a lower contamination and higher germination rate . Therefore, the optimal disinfection time of *C. barometz* spores was 11 minutes.

PGRs are an important factor for spore germination. Pu Lili et al. (Pu et al. 2015) showed that the germination rate of *C. barometz* spores was within a certain concentration range and showed to increase with the inorganic salt concentration. Therefore, an orthogonal experimental design was employed to investigate the effect of the compositions and concentrations of PGRs in *C. barometz* spore germination. The results showed that the highest germination rate was observed with the absence of 6-BA addition, and the sporophyte germination rate decreased after 6-BA addition, indicating that 6-BA would inhibit the germination of *C. barometz* spores. Based on the above results, we found that

1/8MS medium supplemented with 0.02 mg/L IAA was the most suitable combination for *C. barometz* spore germination, which is consistent with Pu's research (Pu et al. 2015).

Effects of the basal medium on gametophyte proliferation

The basal medium provides nutritional components for plants in plant tissue culture and plays an important role in plants' micro-propagation. (Hong et al. 2023). MS medium is the most widely used for plant tissues (Cai et al. 2022). The 1/2 MS medium is obtained by halving a large number of elements in MS medium, usually suitable for plant rooting culture, and the WPM medium is specially designed for woody plants. The white medium was the first dedicated medium for tissue culture, contained low concentrations of inorganic salts, and was mainly used to induce adventitious roots. The composition of the N6 medium is relatively simple, containing a higher concentration of KNO_3 and $(NH_4)_2SO_4$, which promotes the formation, growth, and differentiation of plant callus. The B5 medium contains a low concentration of ammonium, making it suitable for most plants. In this study, MS, 1/2MS, WPM, White, N6, and B5 were tested on the proliferation of *C. barometz* gametophytes, we found that these mediums had significant differences in the growth of *C. barometz* gametophytes. The MS, 1/2MS, and WPM media also supported the growth of gametophytes. Within them, the MS medium had a better effect on proliferation, and the WPM medium had a positive effect on the differentiation of sporophytes. However, White, N6, and B5 medium were not conducive to the growth of *C. barometz* gametophyte.

Effects of PGRs on gametophyte proliferation

Proliferative culture is a key step in plant tissue culture, and the composition and concentrations of PGRs affect cell division, morphogenesis, organ differentiation, and development in plants. (Zhang et al. 2021). In our study, the MS medium was selected as the basal medium for exploring the appropriate composition and concentration of PGRs on the growth of *C. barometz* gametophyte. We found that the growth rate of tissue culture seedlings was higher (14.25) when the concentration of 6-BA was 0.5 mg/L, and the medium with the best growth percentage was MS medium, supplemented with 1.0 mg/L IAA and 0.5 mg/L 6-BA. *Cibotium barometz* is a rare and precious plant and it is relatively difficult to reproduce under natural conditions. Therefore, plant tissue culture technology can achieve large-scale production and meet the great demand for *C. barometz* in the medicinal market.

Effects of concentrations of sucrose and PGRs on induction of sporophytes

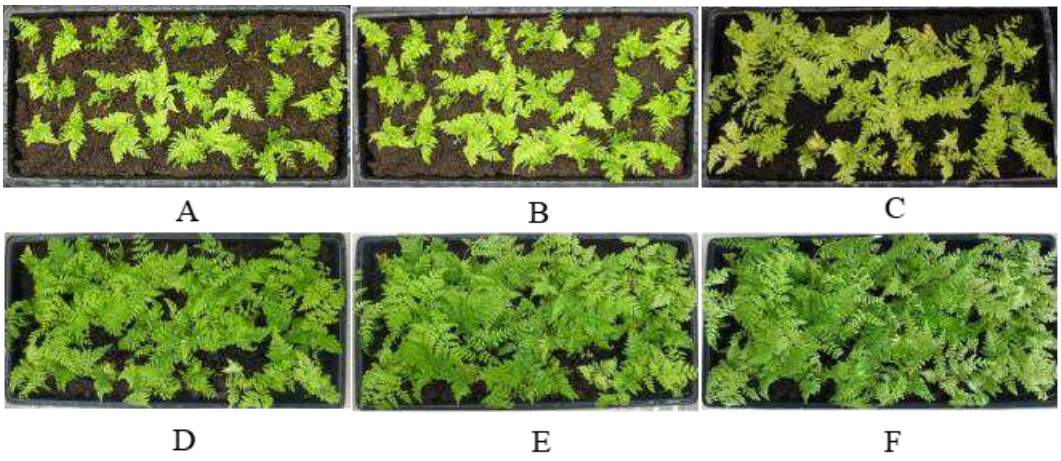
To explore the effects of sucrose concentrations and those of PGRs' species and concentrations on the differentiation of *C. barometz*, we added different concentrations of sucrose and PGRs to the WPM medium. Sucrose provides a carbon source in plant culture that can be used as energy material for plant growth, provide sufficient energy and promote plant growth, and as an osmotic regulator to regulate and maintain plant osmotic pressure, and to prevent plant cells from dehydration or poor growth (Long 2008; Shi et al. 2021). In this study, we found that sucrose-free *C. barometz* gametophytes grew slowly and did not differentiate to sporophytes. This phenomenon may be due to the lack of carbon sources that provide energy for plant growth. The sporophyte's performance was improved on WPM medium supplemented with 20 g/L sucrose. However, high concentration (40 g/L) of sucrose inhibited the growth of gametophytes or the formation of sporophytes, and low concentrations of sucrose (10 g/L) had no significant effect on the growth of gametophytes, with the number of sporophytes decreased. Therefore, appropriate sucrose content is conducive to the growth and differentiation of plants. Lower or higher concentrations of sucrose cause higher-than-threshold level of plant cell osmotic pressure, thus inhibiting the growth and differentiation of plants. In addition, PGRs play an important role in the cell division, morphogenesis, organ differentiation, and development of plants. They also have a

significant effect on the differentiation of *C. barometz* sporophytes. In general, IAA, NAA, 6-BA, and GA_3 were beneficial to *C. barometz* sporophyte formation, but KT slightly inhibited the *C. barometz* sporophyte differentiation.

Effects of phytohormones on tissue seedling rooting in *C. barometz*

The underdeveloped root system of *C. barometz* is an important factor in the continuous decline of their wild resources (Zhang and Zhang. 2010). Only when the *C. barometz* sporophytes seedling has a complete root system can they better adapt to the external environment and large-scale cultivation. After transferring the differentiated sporophytes into the medium-supplemented PGRs, the rooting rate was from 73.08% to 91.52%. In our study, the optimal concentrations for inducing rooting of ABT, IBA, and IAA were 1.5, 1.5, and 1.0 mg/L, respectively, with the rooting rate of 86.82%, 91.52%, and 90.89%. Compared with ABT and IAA, the treatment of IBA promotes root elongation, but has no significant effects on the diameter of the root. After rooting induction, acclimatization is required for *C. barometz* sporophytes grown in in vitro conditions to adapt to the external environment. In vitro-produced sporophytes of *C. barometz* were successfully acclimated and survived in a glass greenhouse after about six weeks of transplanting (Fig. 3), laying a certain foundation for the industrial cultivation of *C. barometz*. Incomplete tissue culture can be also used to summarize induced differentiation in different matrices to form sporophytes. Hu et al (Hu et al. 2023) found that the optimal transformation matrix of sporophytes was peaty soil-vermiculite, the formation time of sporophytes was 40 days, and the conversion rate was 84.42%. The seedlings that were transplanted into the substrate of peaty soil-vermiculite-perlite reached a survival rate of more than 90%.

Fig. 3



The plantlets of *C. barometz* sporophytes after acclimatization for different time. A:2 weeks，B:3 weeks，C:4 weeks，D:5 weeks，E:6 weeks，F:7 weeks.

Conclusion

In this study, we developed the systematic and complete rapid propagation system on *C. barometz.* The scope of this research ranged from the growth and development process of spore germination to proliferation, differentiation and rooting. We also discussed how the sporophyte should be transplanted and cultured to achieve good growth status and high survival rate. All these provided a theoretical basis for the industrial production of *C. barometz*.

Data availability All data generated or analysed in this study are included in this article.

Abbreviations

C.barometz	*Cibotium barometz* (L.) J. Sm.
IAA	Indoleacetic acid
6-BA	6-benzyl adenine
PGRs	Plant growth regulators
NAA	1-Naphthaleneacetic
GA_3	Gibberellic acid
ABT	ABT rooting powder
IBA	Indole-3-Butyric acid
KT	Kinetin

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Declarations

Conflict of interest The authors declare that they have not conflict of interest.