

In vitro propagation and long-term observation of acclimated plants in endangered tree fern *Alsophila costularis*

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Abstract

Alsophila costularis Barker (*Cyathea costularis*), an endangered tree fern with tree-like erect stem, attracts gardening enthusiasts as a special ornamental plant. In vitro propagation can be advantageous for germplasm conservation and commercial application of *A. costularis*. Here, we described in vitro propagation of *A. costularis* via spore culture and green globular bodies (GGBs) system, as well as the long-term observation of acclimated plants regenerated from GGBs. In spore culture, the low concentration of mineral salt (1/8 MS) was beneficial for sporophyte formation on gametophytes, but sporophytes per conical flask was only 8 plantlets. In GGB system, cytokinin thidiazuron (TDZ) was essential for GGB induction and multiplication. The maximum of GGB induction frequency (93.33%) was obtained on 1/2MS medium with 2.0 mg/l TDZ by using juvenile sporophytes as explants, and the same medium was optimal for GGB multiplication. 1/4 MS supplemented with 0.1% (w/v) activated carbon (AC) was appropriate for plantlet regeneration from GGB, GGB differentiation frequency was 100%, and 42.40 plantlets could be regenerated from one piece of GGBs. The maximum of plantlet height (4.64 cm) was obtained on 1/2 MS with 0.1% (w/v) AC. After 6 years of acclimatization cultivation for plantlets regenerated from GGBs, plants in the plastic pots with diameter of 60 cm showed the excellent vegetative and reproductive growth, and the mature spores of these plants could produce sporophytes. Morphological and histological observation demonstrated that *A. costularis* GGBs was a green structure consisting of multiple single GGBs with hair-like structures. One single GGB could develop into one plantlet.

Introduction

Cyatheaceae, a well-known family, is the largest group of tree fern (Korall et al., 2007). The tree-like erect stems make Cyatheaceae distinguished from other fern species, and attracts the attention of gardening enthusiasts as a special ornamental plant (Jones 1987). For commercial purpose, the erect stems or 'trunk' of tree ferns are dug, transported and replanted. As long as the crown of erect stems remains intact, these tree ferns can still continue to grow (FPA Biodiversity Program 2012). Due to overexploitation and natural habitat destruction of Cyatheaceae, all species in Cyatheaceae are listed in Appendix I of the Convention for International Trade on Endangered Species of Wild Fauna and Flora (CITES, 2022), and also be considered as endangered fern species in China (National Forestry and Grassland Administration 2021). *Alsophila costularis* Barker (*Cyathea costularis*) is a tree fern mainly distributed in Yunnan, Tibet and Guangxi of China (Zhang 2004), and lacks effective in vitro propagation method. Establishment of in vitro propagation protocol is urgently needed for germplasm conservation and commercial application of *A. costularis*.

Spore culture is a conventional method for in vitro propagation of ferns (Barnicoat et al. 2011). Spore germination is affected by many factors, such as light, temperature, concentration of mineral salts and sugar (Chang et al. 2007; Wu et al. 2010). Moreover, there is a significant difference among species in the time from spore inoculation to sporophyte formation (Fernández & Revilla 2003). Some species exhibit a long period to form sporophytes, such as *Asplenium nidus* (about 6 ~ 8 months) and *Anemia tomentosa*

var. *anthriscifolia* (about 7 months), which severely affects the commercial application of ferns (Fernández & Revilla 2003; Castilho et al. 2018) .

In vitro propagation via green globular bodies (GGBs), a unique tissue culture system in fern plants, was firstly reported by Higuchi et al. (1987), and was successfully applied for some fern plants, e.g., *Nephrolepis cordifolia* (Higuchi et al. 1987), *Platyserium bifurcarum* (Liao & Wu 2011), *Pteris aspericaulis* var. *tricolor* (Yu et al. 2021a) and so on. Compared with the conventional propagation via spore culture, in vitro propagation via GGB system is more efficient, for example 160,000 plantlets of *N. cordifolia* could be obtained from one runner tips via GGB system in 6 months (Higuchi et al. 1987). GGBs, a special structure of ferns that is different from callus, is used not only for in vitro propagation, but also for cryopreservation (Pence 2015) and somatic embryogenesis (Li et al. 2015).

Cytokinins play an important role in GGB system. In most fern species with GGB system, such as *N. cordifolia* (Higuchi et al. 1987), *A. nidus* (Higuchi & Amaki 1989), and *Cibotium barometz* (Yu et al. 2017), GGB induction is highly dependent on cytokinin 6-benzylaminopurine (BAP) or thidiazuron (TDZ), and GGB organogenesis is also significantly controlled by cytokinins, i.e., the presence of cytokinins inhibits GGBs developing into plantlets, and removal of cytokinins promotes GGB differing into plantlets. This is beneficial for the effective control of organogenesis in GGB system by regulating the cytokinin content (Amaki & Higuchi 1991).

Since propagation of tree ferns is a great challenge, few species are propagated by plant nurseries for commercial purpose. Some species of Cyatheaceae can be propagated by spores, but they cannot be propagated by in vitro vegetative propagation methods because it is difficult to obtain the excellent explants from 'trunk' (Large & Braggins 2004; Yu et al. 2017). In recent years, in vitro propagation by spore culture, observation of gametophyte and sporophyte development have been reported in some species of Cyatheaceae (Marcon et al. 2017; Parajuli and Joshi 2014; Rechenmacher et al. 2010; Reis Moura et al. 2010; Vargas and Droste 2014). Moreover, the somatic embryogenesis in *Cyathea delgadii* has been deeply explored from various factors, i.e., explant type, light conditions mineral salt concentration, endogenous hormones and sugar (Grzyb et al. 2017, 2018; Grzyb and Mikuła 2019; Mikuła et al. 2015a, b). However, in vitro propagation via GGB system and long-term observation of acclimated plants in Cyatheaceae have never been reported. Here, the present work is related to *Alsophila costularis*, a specie of Cyatheaceae. We establish an in vitro propagation protocol of *A. costularis* via spore culture and GGB system, and describe the morphological and histological features of GGBs, as well as long-term observation of acclimated plants obtained via GGB system. Our new approach will be beneficial to germplasm conservation and commercial application of *A. costularis*.

Materials And Methods

Plant materials

The mature spores of *Alsophila costularis* were collected from the individual plant in Pinbian, a place of Yunnan province in China.

Spore culture

After soaked with distilled water for 3 hours, spores were surface-sterilized with 5% sodium hypochlorite for 12 min, then rinsed 5 times with sterilized distilled water. Afterward, the surface-sterilized spores were made into a suspension, then dripped into 100 ml conical flasks containing media with different concentrations of mineral salt, i.e. 1/8-, 1/4-, 1/2- or full MS (Murashige and Skoog 1962). Firstly, surface-sterilized spores were cultured in the dark for 24 h, then transferred to light culture (light intensity $40 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, photoperiod 16 h light/8 h dark). The relative humidity and temperature of culture room was $40 \pm 2 \%$ and $25 \pm 2 \text{ }^{\circ}\text{C}$, respectively. All culture conditions described below are the same as the light culture. Gametophyte and sporophyte formation were observed every two days, and the time for abundant gametophyte formation and sporophyte formation were recorded. After 16 weeks of culture, the number of sporophytes in each conical flask was investigated.

GGB induction and multiplication

In order to investigate the influence of TDZ on GGB induction, in vitro juvenile sporophytes (2 fronds) produced via spore culture on 1/8 MS medium were used as explants, and cultured on 1/2 MS media supplemented with different TDZ concentrations (0, 0.1, 0.5, 1.0, 1.5, and 2.0 mg/l). The time for GGB formation from juvenile sporophyte was recorded. After 8 weeks of induction culture, GGB induction was evaluated by following formula: GGB induction frequency = (the number of explants producing GGBs / the total number of explants) $\times 100 \%$.

For GGB multiplication, GGBs induced on 1/2 MS medium containing 2.0 mg/l TDZ were uniformly divided into small pieces, then cultured on 1/2 MS media supplemented with different TDZ concentrations (0, 0.1, 0.5, 1.0, 1.5, and 2.0 mg/l). The initial diameter and fresh weight of each piece of GGBs before multiplication culture was 3.0 mm and 5.0 mg, respectively. After 8 weeks of culture, the final fresh weight of GGBs was investigated.

Plantlet regeneration from GGBs and plantlet culture

To determine the effect of mineral salt concentration and activated carbon (AC) on plantlet regeneration from GGBs, GGBs multiplied from 1/2 MS medium with 2.0 mg/l TDZ were divided into small pieces as above, then inoculated on media with different concentrations of mineral salt (1/4-, 1/2- or full MS) and 0.1% (w/v) AC or not. After 8 weeks of culture, GGB differentiation was evaluated by following formula: GGB differentiation frequency = (the number of GGBs developing into more than 5 plantlets / total number of GGBs) $\times 100 \%$, and the plant regeneration efficiency was measured by the number of plantlets regenerated from one piece of GGBs. Afterward, the plantlets were transferred to bottle containing the same media as above for plantlet culture. After 8 weeks of culture, the plantlet height was measured.

Plant acclimatization

240 rooted plantlets (height ≥ 4.0 cm) cultured on 1/2 MS medium with 0.1% (w/v) AC were chosen for acclimatization. Firstly, these plantlets were washed with water to remove residual medium, then planted in the plastic trays containing peat and covered with film. After 4 weeks, the film covering the plastic trays was removed. The plantlets were continuously cultured in the opened plastic trays for another 4 weeks, and the survival rate was calculated as the number of survival plantlets divided by the total number of plantlets. The survival plantlets (height ≥ 10.0 cm) were transplanted in the plastic pots (diameter 10.0 cm) containing a mixture (peat: perlite = 8: 1, v/v). After 6 months of cultivation, these plantlets were transferred again to the plastic pots with various diameter (60, 30, 20, 12 cm) to explore the influence of pot size on the plant growth. The plants were cultivated in the greenhouse under natural light condition, as well as irrigated every 4-5 days and fertilized with compound fertilizer (N 15%,w/w; P₂O₅ 15%, w/w; K₂O 15%, w/w) every month.

After 6 years of cultivation, the size of erect stems and fronds, as well as sporangium formation in all treatments were recorded. The mature spores collected from acclimated plants were cultured as the method described above to investigate the spore activity of acclimated plants.

Morphological and histological observation of GGBs

To explore the structure of GGBs, GGBs obtained from multiplication and differentiation stages were used for morphological and histological observation. Morphological observation was conducted under Leica MZ16. For histological observation, the paraffin sections of GGBs were prepared according to the method described by Zhou et al. (2013). The paraffin sections were observed under Leica DM 6000B microscope, and photographed by using Leica DFC 450C camera.

Statistical analysis

The experiments were arranged in completely randomized design. Each treatment consisted of 5 replicates. In GGB system, 6 samples were contained in each replicate. Data were statistically analyzed for least significant difference (LSD) using SPSS V16.0 statistical software (SPSS Inc., Chicago, IL, USA).

Results

In vitro propagation via spore culture

We tested the influence of mineral salt concentration on sporophyte formation of *A. costularis*. In all treatments, abundant gametophyte formation on MS medium (25.60 days) was significantly later than that of other treatments, and no sporophyte was obtained on MS medium. Abundant gametophyte formation on 1/8-, 1/4- and 1/2- MS media were observed at a similar time, i.e. 21.60 ~ 22.80 days after spores sowed on the media (Table 1, Fig.1a). Moreover, a few of sporophytes were obtained on 1/8, 1/4 and 1/2 MS media after 70 ~ 88 days of spores sowed (Fig.1b). The maximum of sporophytes per conical flask (8 plantlets) and the shortest time for sporophyte formation (70 days) were both obtained

on 1/8 MS medium (Table 1), which indicated that 1/8 MS medium was suitable for in vitro propagation of *A. costularis* via spore culture.

GGB induction and multiplication

Using a plant growth regulator-free medium as a control, we studied the effect of TDZ on GGB induction and multiplication. The shoot tips of juvenile sporophytes in the control and 0.1 mg/l TDZ treatment maintained the normal morphology, and no GGBs were formed (Table 2). A small number of GGBs were induced on the medium supplemented with 0.5 mg/l TDZ, but GGB induction frequency was just 6.67 % (Table 2). Increasing TDZ concentration, GGB induction frequency was improved significantly, and the time for GGB induction from the shoot tips of juvenile sporophytes was shortened. Both the maximum of GGB induction frequency (93.33%) and the shortest time of GGB induction (41.60 days) appeared in the treatment of 2.0 mg/l TDZ (Fig. 2a, Table 2).

During GGB multiplication, not only GGB fresh weight increased, but also plantlets regenerated from GGB appeared (Table 3), which was harmful to maintain the normal morphology of GGBs. As illustrated in Table 3, degree of plantlet regeneration decreased when TDZ concentration increased to 1.0 mg/l. Plantlet regeneration at the highest TDZ concentration of 2.0 mg/l was inhibited effectively (Fig. 2b), although GGB final fresh weight (51.10 mg) was lower than other treatments. The results indicated that 2.0 mg/l TDZ was optimal for both GGB induction and multiplication.

Plantlet regeneration from GGBs and plantlet culture

To select the ideal medium for plantlet regeneration from GGBs, the media with different concentrations of mineral salt and AC were designed for GGB cultivation. The formation of juvenile fronds on GGBs was observed in all treatments from the third week of culture (Fig. 2c). After 8 weeks of culture, GGB differentiation frequency and regeneration efficiency in all treatments were respectively above 83.33% and more than 24 plantlets regenerated from one piece of GGBs. The maximum of GGB differentiation frequency (100%) and regeneration efficiency (42.40 plantlets) were both obtained on 1/4 MS medium with 0.1% (w/v) AC, which could be regarded as the appropriate medium for plantlet regeneration from GGB. GGB differentiation frequency and regeneration efficiency in the treatments of 1/4-, 1/2- and full- MS media without AC did not show significant difference from each other (Table 4). For the treatments containing same concentration of mineral salt, adding the AC increased GGB differentiation, but the difference was not significant (Table 4). However, adding AC into 1/4-, 1/2 MS media improved the plantlet regeneration efficiency significantly (Table 4).

During plantlet cultivation, the plantlet height on 1/2 MS media with AC or not was higher than that on other treatments of 1/4-, full MS, which indicated that 1/2 MS was appropriate mineral salt concentration for plantlet cultivation (Table 4). Moreover, 1/2 MS medium supplemented with 0.1% AC obtained the maximum of plantlet height (4.64 cm) after 12 weeks of cultivation (Fig. 2d), and this medium was optimal for plantlet growth.

Vegetative and reproductive growth of acclimated plants

For acclimatization, 240 rooted plantlets (height ≥ 4.0 cm) cultured on 1/2 MS medium with 0.1% (w/v) AC were transferred to plastic trays containing peat. 85 % of plantlets survived after 8 weeks of cultivation. As the height was above 10.0 cm after 12 weeks of culture (Fig. 2e), the survival plantlets were transplanted in the plastic pots (diameter 10.0 cm) containing a mixture (peat : perlite = 8: 1, v/v). After 6 months of cultivation, part of these plants were planted into the plastic pots with different sizes (diameter 60, 30, 20, 12 cm) for acclimatization cultivation in the greenhouse. Two years after planting, the plants in the plastic pots with diameter of 60 cm formed the prominent erect stem (Fig. 1f), and the same phenomenon was not observed in other pot sizes.

After 6 years of cultivation, there was significant difference in vegetative and reproductive growth among the plants in the plastic pots with different sizes (Fig. 3, 4). The plants in the plastic pots with diameter of 60 cm exhibited the characteristics of tree ferns very well (Fig. 3a, 4). The height and diameter of their erect stems was 71.33 cm and 8.04 cm, respectively, which was excellent in all treatments (Fig. 4a). Moreover, the frond size of tree ferns in plastic pots with diameter of 60 cm was significantly larger than that in other size pots (Fig. 4b). With the decrease of pot sizes, the size of erect stems and frond decreased significantly (Fig. 4), which indicated that we can choose different pot sizes for the different purpose of cultivation.

From May to August, the sporangium formation was observed on the back of mature fronds in the plastic pots with diameter of 60 and 30 cm, but only plants in the plastic pots with diameter of 60 cm produced the mature spores (Fig. 3b). These mature spores were collected and cultured in 1/8 MS medium, and sporophyte formation was observed after about 70 days of spore culture (Fig. 3c).

Morphological and histological observation of GGBs

GGBs obtained from multiplication culture was a green structure that consisting of multiple single GGBs (Fig. 5a). The single GGB contained two discrete bipolar structures, i.e. the green shoot meristem on the top and the brown root meristem on the bottom (Fig. 5b, c). The single GGB could proliferate into the new single GGB (Fig. 5b, c). During the differentiation stage, frond formation was observed from the top view of GGBs (Fig. 5d). The shoot meristem and root meristem of single GGB initiated the development of juvenile frond and root, respectively (Fig. 5e). One single GGB produced one plantlet (Fig. 5f).

As histological analysis shown in Fig. 6a, the shoot meristem of single GGB from multiplication culture possessed numerous meristem cells with the big and dense nucleus. Although a number of hair-like structures surrounding the shoot meristem, the top region of shoot meristem was no hair-like structures observed (Fig. 6a). The top region of shoot meristem on the single GGB obtained from multiplication culture was smooth (Fig. 6a). Differently, the same region of single GGB obtained from the differentiation stage bulged outward significantly (Fig. 6b). Moreover, the brown tissues were observed on the bottom of single GGB obtained from the multiplication and differentiation stages (Fig. 6c), which should be the root meristem in morphological observation above (Fig. 5b, c).

Discussion

For in vitro propagation of fern plants via spore culture, the mineral salt concentration is one of the key factors influencing spore germination, gametophyte development, and sporophyte formation (Fernández et al 1997; Fernández & Revilla 2003). The tree fern *Cyathea australis* preferred forming sporophytes on 1/2 MS medium supplemented with 150 mg/L NaH_2PO_4 and 20 g/L sucrose (Reis Moura et al. 2010). Conversely, the gametophytes of *Cyathea lepifera* could produce sporophytes on 1/10 ~ 1/80 MS media, but not on 1/2 MS (Kuriyama et al. 2004). In the present study, high mineral salt concentration significantly inhibited the sporophyte formation of *A. costularis*. No sporophyte was obtained on MS medium, and the lower mineral salt concentration 1/8 MS was optimal for sporophyte formation (Table 1).

Only 0.4 ~ 8.0 sporophytes per conical flask were obtained in treatments of 1/2 ~ 1/8 MS studied here (Table 1). The low efficiency of sporophyte formation, i.e., 0.14 ~ 1.43 sporophytes per tested tube with 1/10 ~ 1/80 MS, was also observed in *Cyathea lepifera* (Kuriyama et al., 2004). As the observation of 11 *Cyathea* species reported by Goller and Rybczynski (2007), the time for sporophyte formation arranged from 4 to 12 months. This time for *A. costularis* in the present study was 70.0 ~ 88.0 days (Table 1). The low efficiency and long period of sporophyte formation might be an obstacle for commercial application of tree ferns in *Cyatheaceae*.

Considering the low efficiency of in vitro propagation via spore culture (8 plantlets per conical flask), we established the in vitro propagation method of *A. costularis* via GGB system, which the regeneration efficiency reached 42.40 plantlets per GGBs (Table 4). Similar with *Nephrolepis cordifolia* (Higuchi et al., 1987), *Asplenium nidus* (Higuchi & Amaki, 1989) and *Cibotium barometz* (Yu et al., 2017), GGB induction of *A. costularis* was significantly dependent on the presence of cytokinins, and GGB induction frequency was increased with enlarging the cytokinin concentration. In the present study, the regeneration from GGBs of *A. costularis* was effectively inhibited by adding high concentration of cytokinin (2.0 mg/l TDZ), and the inhibition was released by culturing GGB on the medium without cytokinin (Table 3), which confirmed cytokinins controlling GGB organogenesis firstly proposed by Higuchi et al. (1987). Different from the role of cytokinins in GGB system of fern plants, cytokinin TDZ effectively induced protocorm-like body (PLB) formation from root tips of *Doritaenopsis* (Park et al., 2003), cytokinin BA was beneficial to the shoot multiplication of *Malus orientalis* (Amirchakhmaghi et al. 2019). Although the resurrection plant *Selaginella pulvinata* belongs to fern-allies, not GGBs but adventitious buds were induced by culturing the sterilized frond tips on the media containing various concentrations of BAP or TDZ (Yu et al. 2021b). Moreover, unlike cytokinin-dependent GGB induction of *A. costularis* studied here, somatic embryo of tree fern *Cyathea delgadii* could be induced on the medium without cytokinins and other plant growth regulators by using explants (length 2.5 mm) excised from the youngest leaf of an etiolated sporophyte (Mikuła et al., 2015a, b).

Mineral salt concentration and AC have different effects on regeneration of different plants. GGBs of *A. costularis* in our study were prone to develop into plantlets in all treatments. The mineral salt

concentration and addition of AC exerted a negligible influence on GGB differentiation frequency, but adding AC into 1/4-, 1/2 MS media improved the plantlet regeneration efficiency significantly (Table 4). Moreover, the mineral salt concentration significantly affected the plantlet growth of *A. costularis*, and the maximum of plantlet height (4.64 cm) was obtained in 1/2 MS medium with AC (Table 4). Differently, plantlet regeneration from GGBs and plantlet growth in *Pteris aspericaulis* var. *tricolor* preferred 1/4 MS, and the addition of AC inhibited plantlet growth and easily led to leaf chlorosis (Yu et al., 2021a). The proper mineral salt concentration for plantlet regeneration of *Rumohra adiantiformis* 'Florida' and *Pteris ensiformis* 'Victoriae' was MS and 1/2 MS, respectively (Amaki & Higuchi, 1991). In *Matteuccia struthiopteris*, 1/4 MS supplemented with 1.0% AC favored plantlet regeneration from meristematic nodules (MN), as well as the development of roots and fronds (Thakur et al. 1998). Mikuła et al. (2015a) reported the necessity of using 1/2 MS to stimulate normal plantlet regeneration from somatic embryo of tree fern *Cyathea delgadii*, and 1/8 MS resulted in the excessive elongation of fronds.

GGBs of *A. costularis* was a green structure that consisting of multiple single GGBs (Fig. 5a). Its morphology was distinguished from callus and PLB (Ekmekçigil et al. 2019; Srinivasan et al. 2021), and the color was different from the yellow-green GGBs of *Cibotium barometz* (Yu et al., 2017). A single GGB of *A. costularis* possessing two discrete bipolar structures, i.e., shoot meristem and root meristem (Fig. 5b, c), could develop into a single plantlet (Fig. 5e). Similarly, a single PLB in orchid or a single somatic embryo in *Cyathea delgadii* or other seed plants, also could form a single plantlet (Gantait and Sinniah 2012; Mikuła et al. 2015a, b; Ng and Saleh 2011). This indicated that the single GGB was a structure with similar properties to a single PLB or somatic embryo.

The pot size affects plant biomass significantly. The small pot size led to a biomass reduction in *Pinus contorta* (Endean & Carlson 1975). The main reason is that a small pot implied a small quantity of soil or other substrate and thereby, a reduction in the availability of water and nutrients to the plant as well as impediment of root growth (Poorter et al. 2012). With the decrease of pot size, the decrease of erect stem height and frond size was observed in *A. costularis* studied here (Fig. 4). That would be beneficial to produce *A. costularis* plants with different size to meet the different needs of horticulture.

Conclusion

In this study, we described the in vitro propagation methods of *A. costularis* via spore culture and GGB system, respectively. Although sporophyte plantlets were obtained by these two methods, the efficiency of GGB system (42.40 plantlets regenerated from one GGB segment) was significantly higher than that of spore culture (8 plantlets per conical flask). 1/2MS with 2.0 mg/l TDZ was appropriate for both GGB induction and multiplication. The optimal medium for plantlet regeneration from GGBs and plantlet growth was 1/4 MS with 0.1% (w/v) AC and 1/2 MS with 0.1% (w/v) AC, respectively. After 6 years of acclimatization cultivation for plantlets regenerated from GGBs, plants in the plastic pots with diameter of 60 cm showed the excellent vegetative and reproductive growth, and the mature spores of these plants could produce sporophytes.

Declarations

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Author contributions:

CY and RY designed the experiments and contributed to manuscript revisions. YP and QS performed the experiments, analyzed data and wrote the manuscript. GW and LW assisted in long-term observation of acclimated plants. All authors approved the final draft of the manuscript.

Data availability

The data sets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Tables

Table 1

Effects of mineral salt concentration on sporophyte formation of *Alsophila costularis* during in vitro propagation via spores

Mineral salt concentrations	Time for abundant gametophyte formation (day)	Number of sporophytes per conical flask	Time for sporophyte formation (day)
1/8 MS	21.60 ± 0.75 b	8.00 ± 0.55 a	70.00 ± 0.63 c
1/4 MS	22.80 ± 0.49 b	3.80 ± 0.37 b	74.80 ± 0.49 b
1/2 MS	22.80 ± 0.80 b	0.40 ± 0.24 c	88.00 ± 2.00 a
MS	25.60 ± 0.75 a	0.00 ± 0.00 c	–
Data represent mean ± standard error. Means within a single column followed by different letters are significantly different according to LSD test at $P = 0.05$.			

Table 2
Effects of TDZ concentration on GGB induction of *Alsophila costularis*

TDZ concentration (mg L ⁻¹)	GGB induction frequency (%)	Time for GGB formation (day)
0	0.00 ± 0.00 d	–
0.1	0.00 ± 0.00 d	–
0.5	6.67 ± 4.08 d	59.00 ± 1.00 a
1.0	23.33 ± 4.08 c	58.60 ± 0.75 a
1.5	36.66 ± 3.33 b	50.00 ± 0.63 b
2.0	93.33 ± 4.08 a	41.60 ± 0.75 c
Data represent mean ± standard error. Means within a single column followed by different letters are significantly different according to LSD test at <i>P</i> = 0.05.		

Table 3
Effects of TDZ concentration on GGB multiplication of *Alsophila costularis*

TDZ concentration (mg L ⁻¹)	Final fresh weight (mg)*	Plantlet regeneration during GGB multiplication**
0	60.60 ± 1.76 a	+++
0.1	59.99 ± 1.71 a	+++
0.5	58.66 ± 1.51 a	+++
1.0	57.35 ± 1.41 a	++
1.5	52.43 ± 1.42 b	++
2.0	51.10 ± 2.00 b	+
Data represent mean ± standard error. Means within a single column followed by different letters are significantly different according to LSD test at <i>P</i> = 0.05.		
* The initial diameter and fresh weight of GGBs was 3.0 mm and 5.0 mg, respectively.		
** +++ high frequency of plantlet regeneration from GGBs, ++ medium frequency of plantlet regeneration from GGBs, + low frequency of plantlet regeneration from GGBs		

Table 4

Effects of mineral salt concentration and activated carbon (AC) on the plantlet regeneration from GGB and plantlet growth of *Alsophila costularis*

Mineral salt concentration	AC (% w/v)	GGB differentiation frequency (%)	Plantlet regeneration efficiency*	Plantlet height (cm)
1/4 MS	0	90.00 ± 4.08 ab	26.20 ± 1.36 c	2.66 ± 0.19 d
1/4 MS	0.1	100.00 ± 0.00 a	42.40 ± 1.63 a	2.70 ± 0.13 d
1/2 MS	0	86.67 ± 3.33 b	24.80 ± 1.39 c	4.08 ± 0.19 b
1/2 MS	0.1	93.33 ± 4.08 ab	33.20 ± 1.36 b	4.64 ± 0.24 a
MS	0	83.33 ± 7.45 b	24.00 ± 2.10 c	3.02 ± 0.13 cd
MS	0.1	90.00 ± 4.08 ab	27.80 ± 1.91 c	3.42 ± 0.17 c
Data represent mean ± standard error. Means within a single column followed by different letters are significantly different according to LSD test at $P = 0.05$.				
* Plantlet regeneration efficiency indicates the number of plantlets regenerated from one piece of GGBs, and the initial diameter and fresh weight of GGBs was 3.0 mm and 5.0 mg, respectively.				

Figures

Figure 1

In vitro propagation of *A. costularis* via spore culture. (a) Abundant gametophyte formation on 1/8 MS medium after 22 days of spores sowed. (b) Sporophyte formation on 1/8 MS medium after 70 days of spores sowed, arrow indicates the sporophyte plantlet.

Figure 2

In vitro propagation of *A. costularis* via green globular bodies (GGBs) system. (a) Arrow indicates GGB formation from juvenile sporophyte after 8 weeks of induction culture on 1/2 MS supplemented with 2.0 mg/l TDZ. (b) GGB multiplication after 4 weeks of culture on 1/2 MS with 2.0 mg/l TDZ. (c) GGB differentiation in the third week of culture on 1/4 MS supplemented with 0.1% AC. (d) Plantlets after 8 weeks of culture on 1/2 MS supplemented with 0.1% AC. (e) Plantlets planted plastic trays containing

peat after 12 weeks of cultivation. (f) Plant transplanted into the plastic pots with diameter of 60 cm after two years of cultivation.

Figure 3

Vegetative and reproductive growth of acclimated plants of *A. costularis*. (a) Plants in the plastic pots with different sizes after 6 years cultivation, the diameter of plastic pots arranged form left to right is 60, 30, 20 and 12 cm, respectively. (b) Mature sporangium on the back of frond from plant in the plastic pot (diameter 60 cm) after 6 years of cultivation. (c) Juvenile sporophyte obtained via spore culture of acclimated plants in the plastic pot (diameter 60 cm) after about 70 days of culture on 1/8 MS.

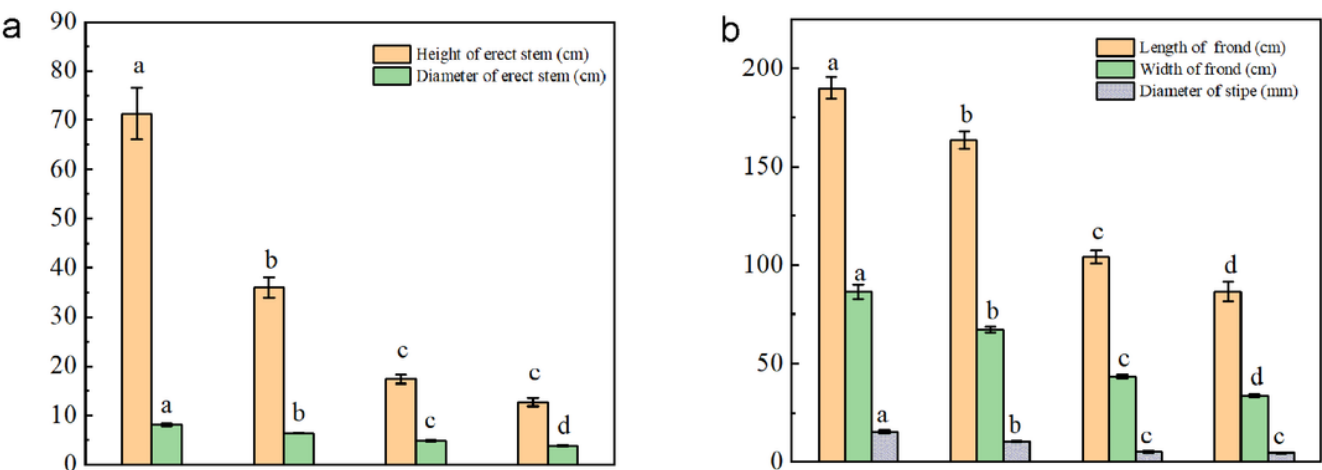


Figure 4

Vegetative growth of acclimated plants of *A. costularis* in the plastic pots with different sizes. (a) Height and diameter of erect stems. (b) Characteristic of fronds.

Figure 5

Morphology of green globular bodies (GGBs) in *A. costularis*. (a) Top view of GGBs in multiplication stage, arrows indicate the single GGB. (b)-(c) Single GGB in multiplication stage, arrow 1 indicates the mother single GGB, arrow 2 indicates the new single GGB. (d) Top view of GGBs in differentiation stage. (e) Single GGB in differentiation stage, arrow indicates the frond formation. (f) Plantlet formation from the single GGB.

Figure 6

Histology of green globular bodies (GGBs) in *A. costularis*. (a) Single GGB in multiplication stage, arrow 1 indicates the smooth top region of single GGB, arrow 2 indicates hair-like structure. (b) Single GGB in differentiation stage, arrow indicates that top region of single GGB bulges outward. (c) Arrow indicates the brown tissues.

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