CrossMark

ORIGINAL ARTICLE

In vitro propagation of the endangered tree fern *Cibotium barometz* through formation of green globular bodies

Rongpei Yu[1,2] · **Guangfei Zhang[3]** · **Han Li[1,2]** · **Hua Cao[1,2]** · **Xijun Mo[1,2]** · **Min Gui[1,2]** ·
Xuhong Zhou[1,2] · **Yalian Jiang[1,2]** · **Shenchong Li[1,2]** · **Jihua Wang[1,2]**

Abstract *Cibotium barometz* is an endangered tree fern, used both as ornamental plant and traditional Chinese medicinal plant. In this study, an effective in vitro propagation protocol was obtained through formation of green globular bodies (GGBs) from in vitro juvenile sporophytes. The effect of plant growth regulators (PGRs) on GGB induction and multiplication, as well as mineral salt concentration and active charcoal (AC) on plantlet regeneration from GGBs was evaluated. Thidiazuron (TDZ; 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea) had a significant effect on GGB induction and multiplication ($P < 0.001$), while a-naphthaleneacetic acid (NAA) did not ($P > 0.05$). GGB induction rate was above 80 % on 1/2 Murashige and Skoog (MS) media supplemented with TDZ (1.0 mg L^{-1}) and NAA (0.1, 0.3 or 0.5 mg L^{-1}). The same media were also optimal for GGB multiplication. GGBs cultured on 1/4 MS media supplemented with 0.1 or 0.2 % (w/v) AC showed a high rate of GGB development into plantlets above 90 %. 1/2 MS media supplemented with 0.1 or 0.2 % AC were the most effective for plantlet growth. Regenerated plantlets were successfully acclimatized (80 %) in greenhouse conditions. Morphological and histological analysis revealed that *C. barometz* GGBs was a yellow-green globular structure composed of the single GGB with meristems and hair-like structures, and new single GGBs were initiated from the epidermal cells of meristem zone.

Keywords *Cibotium barometz* · In vitro propagation · Green globular bodies · Thidiazuron

Abbreviations
2iP	N^6-(2-Isopentenyl) adenine
AC	Active charcoal
BA	N^6-benzyladenine
CITES	Convention for international trade on endangered species
GGBs	Green globular bodies
LSD	Least significant difference
MS	Murashige and Skoog (1962)
NAA	a-Naphthaleneacetic acid
PGR	Plant growth regulator
PLBs	Protocorm-like bodies
TDZ	Thidiazuron (1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea)

Introduction

Cibotium barometz (L.) J. Sm. is a tree fern listed in Appendix II of the Convention for International Trade on Endangered Species of Wild Fauna and Flora (CITES 2015). *C. barometz* is the only Dicksoniaceae family member found in mainland China, it is considered as an endangered species (Zhang et al. 2002). However, the species has varied utilities in China. It is not only an ornamental plant because of its attractive fibrous trunk covered with special golden hairs, but it is also a medicinal plant in traditional Chinese medicine as the rhizome shows anti-inflammation, pain

✉ Shenchong Li
lsc0618@163.com

✉ Jihua Wang
wjh0505@gmail.com

1 Flower Research Institute, Yunnan Academy of Agricultural Sciences, Kunming 650205, China

2 National Engineering Research Center for Ornamental Horticulture, Kunming 650205, China

3 Institute of Ecology and Geobotany, Yunnan University, Kunming 650091, China

relief and anti-osteoporosis activities (Cuong et al. 2009; Wu and Yang 2009; Zhao et al. 2011). Unfortunately, due to its overexploitation and the destruction of its natural habitats in recent years, the species is under severe threat and requires urgent protection (Zhang et al. 2002). Therefore, there is an urgent need to produce high quality plantlets for horticultural, medicinal, and conservation uses without resourcing to harvest from the remaining wild population.

Conventional propagation of ferns from spores is generally a time-consuming procedure (Liao and Wu 2011). At the same time, the low multiplication rate makes the vegetative propagation also inefficient (Thakur et al. 1998). However, a variety of in vitro vegetative propagation methods have been successfully practiced in many fern species through shoot organogenesis from rhizomes (Fernández et al. 1997; Winarto and Teixeira da Silva 2012), juvenile leaves (Camloha et al. 1994), homogenized sporophytic tissues (Teng and Teng 1997), bud scales (Ambrožič-Dolinšek and Camloh 1997), callus (Hegde et al. 2006), somatic embryos (Mikuła et al. 2015a, b) or green globular bodies (GGBs) (Amaki and Higuchi 1991). Among these methods, in vitro propagation through GGBs is particularly efficient (Higuchi et al. 1987; Higuchi and Amaki 1989).

Higuchi et al. (1987) first named rapidly proliferating tissues as GGBs, which were inducted from the runner tips of *Nephrolepis cordifolia* cultured on medium containing N^6-benzylaminopurine (BA), and converted to plantlets easily when cultured on plant growth regulator (PGR)-free medium. GGBs were successfully inducted in *Asplenium nidus* (Higuchi and Amaki 1989), *Pteris ensiformis, Rumohra adiantiformis, Adiantum raddianum*, (Amaki and Higuchi 1991), *Blechnum spicant* (Fernández et al. 1996) and *Polypodium cambricum* (Bertrand et al. 1999) using different sporophytic explants. Cytokinin is generally regarded as an important PGR for in vitro propagation through GGBs, since it promotes GGB formation while also inhibiting plantlet regeneration from GGBs. This allows for tight organogenesis control during in vitro propagation via the regulation of cytokinin content (Amaki and Higuchi 1991). GGBs are different from callus, but similar to protocorm-like bodies (PLBs) in orchids (Higuchi et al. 1987). Interestingly, GGBs from different species show distinctive external morphological features such as scale- or hair-like structures (Amaki and Higuchi 1991; Higuchi et al. 1987). However, all GGBs possess multiple meristems inside or on their periphery. Some meristems come to the surface and develop into new GGBs, which results in a high multiplication rate (Liao and Wu 2011). Thus, GGBs is considered as an important material for in vitro vegetative propagation of ferns. In addition, GGBs can also serve as plant materials for cryopreservation (Li et al. 2013; Pence 2015) or the research of somatic embryogenesis (Li et al. 2015).

There are only few studies on in vitro propagation of *C. barometz* and limited descriptions of fern GGBs. Goller and Rybczyński (2007) reported in vitro culture of *C. glaucum* and *C. schiedei* using spores as explants, but sporophytes were only obtained in *C. schiedei*. Until now, GGB induction or other in vitro vegetative propagation methods for genus *Cibotium* have never been reported, because of the difficulty in obtaining proper explants from tree ferns. Here, we describe an in vitro propagation protocol for *C. barometz* through formation of GGBs using in vitro juvenile sporophytes as explants. Furthermore, we describe the morphology and histology of *C. barometz* GGBs. Our new protocol is set to largely improve the propagation of *C. barometz*.

Materials and methods

Plant material

C. barometz spores were obtained from mature fronds collected in Pingbian (Yunnan province, China). The fronds were wrapped in paper bags and dried at room temperature for 1 week to release spores, and spores were stored at 4 °C in dark until use. Spores (10 mg) were soaked with distilled water for 1 h, then surface-sterilized for 5 min with 0.5 % sodium hypochlorite. Afterward, they were rinsed five times with sterile distilled water by centrifugation (3000 rpm; 2 min) and resuspension. Finally, the spores were suspended in 10 mL of sterile distilled water and 2 mL of spore suspension was inoculated in a conical flask (100 mL volume), which contained 40 mL 1/2 MS (Murashige and Skoog 1962) medium supplemented with 3 % (w/v) sucrose and 0.7 % (w/v) plant agar, and adjusted to pH 5.8 before being autoclaved. Cultures were maintained under the light intensity of 40 μmol m^{-2} s^{-1} provided by cool-white fluorescent lights (photoperiod 16 h light/8 h dark) in a controlled environment room at 25 ± 2 °C. Gametophytes that developed from spores were periodically watered to induce the sporophyte formation. In vitro juvenile sporophytes with 2–3 fronds (about 5.0 mm in length of the first frond) were used for the study.

GGB induction from juvenile sporophytes

For GGB induction, in vitro juvenile sporophytes used as explants were cultured on 1/2 MS supplemented with PGRs. Using PGR-free 1/2 MS medium as a control, the effect of two different PGRs was evaluated together at different concentrations: thidiazuron (TDZ) (0.2, 0.5 or 1.0 mg L^{-1}) and a-naphthaleneacetic acid (NAA) (0.1, 0.3 or 0.5 mg L^{-1}). After 4 weeks of preculture, shoot tips of juvenile sporophytes became swollen and formed

primordia in PGR treatments, but not in the control. Then primordia (diameter ≥2.0 mm) were excised from juvenile sporophytes and subcultured on the same medium. Every 2 days, GGB formation was observed to calculate the time for GGB formation from primordia. After 8 weeks of subculture, GGB induction rate was recorded. Cultures were incubated under the conditions described above.

GGB multiplication

To determine the effect of PGRs on GGB multiplication, GGBs (diameter ≥ 5.0 mm) induced on 1/2 MS supplemented with 1.0 mg L^{-1} TDZ and 0.3 mg L^{-1} NAA, were cut into segments with the diameter of 2.0 mm, which the initial fresh weight was 4.0 mg. Each segment was cultured on 1/2 MS supplemented with TDZ (0.5, 1.0 or 1.5 mg L^{-1}) and NAA (0.1, 0.3 or 0.5 mg L^{-1}) in combination. In all sets of experiments, PGR-free 1/2 MS medium was used as a control. Cultures were incubated under the conditions described above. After 4 weeks of culture, GGB final fresh weight and plantlet regeneration from GGBs were assessed.

Plantlet regeneration from GGBs and acclimatization

In order to regenerate plantlets from GGBs, GGBs (diameter ≥ 5.0 mm) from 1/2 MS supplemented with 1.0 mg L^{-1} TDZ and 0.1 mg L^{-1} NAA after 4 weeks of multiplication culture, were cut into segments with the diameter of 2.0 mm and cultured on PGR-free media consisting of different mineral salt concentrations (1/4 MS, 1/2 MS or MS), along with various concentrations of activated charcoal (AC) (0, 0.1 % or 0.2 %, w/v). Cultures were incubated under the conditions described above. After 8 weeks of culture, the rate of GGB development into plantlets was assessed. Subsequently, plantlets regenerated from GGBs were transferred to the same fresh medium, and plantlet height was recorded after 8 weeks of plantlet cultivation.

For plantlet acclimatization, regenerated plantlets higher than 2.5 cm and with well-developed roots were gently washed in 0.100–0.125 % (w/v) chlorothalonil solution and transferred to plastic boxes with covers, which contained a 5:1 (v/v) mixture of sterilized peat and perlite. After 5 weeks, covers of plastic boxes were gradually opened for several days, then plantlets were moved to plastic pots containing a 5:1 (v/v) mixture of peat and perlite and irrigated with tapwater every 4–5 days. These plantlets were maintained in the greenhouse with the shade (70 %) at 25 ± 5 °C under natural photoperiod conditions.

Morphological and histological observation of GGBs

For morphological observation of GGBs, samples were collected in GGB multiplication and differentiation stages and dissected under a stereoscope (Leica MZ16, Germany) and photographed with a camera (Leica DFC 450C, Germany).

To observe the histology of GGBs, the same samples were collected again, and immediately fixed in FAA (70 % ethanol: glacial acetic acid: formalin, 18:1:1) for 24 h, then dehydrated through a graded series of ethanol solutions, and embedded in paraffin wax. The samples were sectioned to a thickness of 8–10 μm and stained with hematoxylin (Zhou et al. 2013), then observed under the light microscope (Leica DM 6000B, Germany) and photographed with a camera (Leica DFC 450C, Germany).

Scanning electron microscopy

To observe the surface structure of GGB, samples collected in GGB multiplication and differentiation stage were fixed in FAA. After dehydration through a series of ethanol rinses, samples were critical-point dried and coated with molybdenum-palladium alloy using an ion-sputter apparatus (Hitachi E-1010, Japan), and imaged under a scanning electron microscope (Hitachi S-3000N, Japan) with an accelerating voltage of 15.0 kV.

Data analysis

Experiments were performed in a completely randomized design. Experiments of GGB induction, GGB multiplication and plantlet regeneration were repeated three times, using five replicates each containing six explants. The data were analyzed by means of ANOVA analysis of variance and least significant difference (LSD) procedure using the software SPSS V16.0 for Windows (SPSS Inc., Chicago, IL, USA). Significance was set at the 0.05 level.

Results

GGB induction from juvenile sporophytes

We tested the influence of PGRs on GGB induction using PGR-free medium as a control. GGB induction in *C. barometz* is highly dependent on the presence of PGRs. With the exception of the control, all tested treatments had primordium formation from juvenile sporophytes after 4 weeks of preculture (Fig. 1a), and then got GGB formation from primordia. Table 1 summarizes the effect of PGRs on GGB induction. Explants cultured on the medium supplemented with 1.0 mg L^{-1} TDZ and 0.3 mg L^{-1} NAA resulted in the highest GGB induction rate (86.67 %) and the shortest time for GGB formation from primordia (32.39 days). TDZ had a significant effect on GGB induction rate and the time for GGB formation from primordia ($P < 0.001$). With the increase of TDZ concentration, GGB
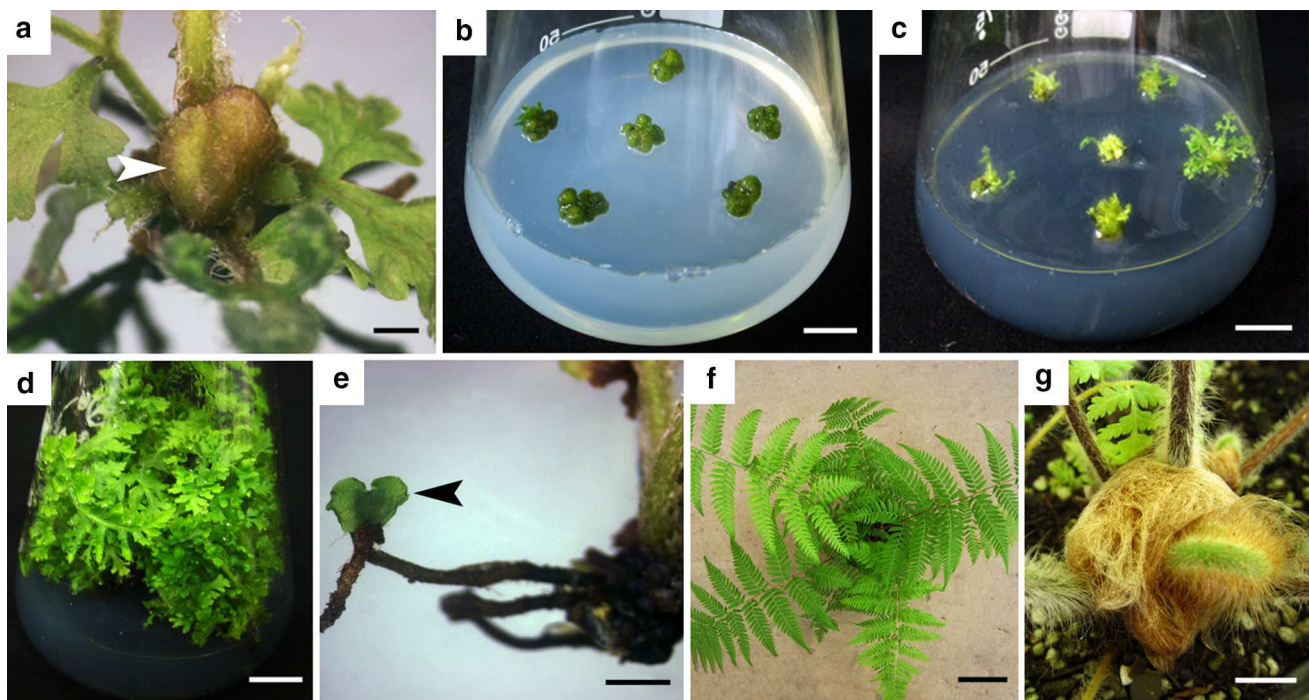
Fig. 1 In vitro propagation of *Cibotium barometz* through formation of GGBs. **a** *Arrow* indicates primordium formation from an in vitro grown juvenile sporophyte after 4 weeks of preculture on 1/2 MS medium containing 1.0 mg L^{-1} TDZ and 0.3 mg L^{-1} NAA, *bar* = 1 mm. **b** GGB multiplication on 1/2 MS medium containing 1.0 mg L^{-1} TDZ and 0.1 mg L^{-1}NAA after 4 weeks of culture, *bar* = 1 cm. **c** GGB differentiation on 1/4 MS medium containing 0.1 % (w/v) active charcoal (AC), *bar* = 1 cm. **d** Sporophyte plantlets developed from GGBs on 1/2 MS medium containing 0.1 % (w/v) AC, *bar* = 1 cm. **e** Formation of aposporous gametophyte on the root of a plantlet cultured on 1/2 MS medium containing 3 % (w/v) sucrose without AC, *arrow* indicates aposporous gametophyte, *bar* = 1 mm. **f** Acclimatized plant in the greenhouse after 5 months, *bar* 10 cm. **g** A crown covered with *golden hairs* of acclimatized plant, *bar* = 5 mm

induction rate increased, and the time for GGB formation from primordia decreased. At the highest TDZ concentration (1.0 mg L^{-1}), GGB induction rate was above 80 %, and the time for GGB formation from primordia was between 32 and 35 days. On the contrary, NAA had no significant effect on GGB induction ($P > 0.05$). Moreover, there was no statistically significant interaction between the concentration of TDZ and NAA ($P > 0.05$).

GGB multiplication

With the multiplication, GGBs increased in size and fresh weight, and became loose and easy to be separated from each other (Fig. 1b). Table 2 reports the effect of TDZ and NAA concentrations on GGB multiplication. GGBs on PGR-free medium produced the highest GGB final fresh weight (72.14 mg), but also result in the highest rate of plantlet regeneration from GGBs (67.78 %). As a result, GGB morphology changed, which was not ideal for GGB multiplication. TDZ had a significant effect on GGB final fresh weight and plantlet regeneration from GGBs ($P < 0.001$). With the increase of TDZ concentration, GGB final fresh weight decreased. Furthermore, the rate

of plantlet regeneration from GGBs also decreased, which was helpful to maintain GGB morphology. However, even at the highest TDZ concentration (1.5 mg L^{-1}), plantlet regeneration from GGBs could not be inhibited completely. In consideration of GGB final fresh weight as well as inhibition of plantlet regeneration from GGBs, 1.0 mg L^{-1} TDZ were the optimal concentration for GGB multiplication, with GGB final fresh weight above 42 mg and a low rate of plantlet regeneration from GGBs. We observed no significant effect of NAA concentration on GGB final fresh weight and plantlet regeneration from GGBs ($P > 0.05$), and there was no statistically significant interaction between the concentrations of TDZ and NAA ($P > 0.05$).

Plantlet regeneration from GGBs and acclimatization

In order to obtain plantlets, GGBs were cultivated on PGR-free medium. The effect of mineral salt concentration and AC on GGB development and plantlet growth was investigated (Table 3). Plantlet formation was observed from the fourth week of culture in most of treatments (Fig. 1c). After 8 weeks of culture, the rate of GGB development into plantlets was above 65 % for the all treatments, and

Table 1 Influence of plant growth regulators on GGB induction in *Cibotium barometz*

Plant growth regulators (mg L^{-1})		GGB induction rate (%)[a]	Time for GGB formation from primordia (day)[b]
TDZ	NAA		
0	0	0	–
0.2	0.1	48.89 ± 2.08 d	43.96 ± 0.74 a
0.2	0.3	46.67 ± 3.33 d	46.43 ± 1.05 a
0.2	0.5	52.22 ± 1.36 d	44.64 ± 0.79 a
0.5	0.1	65.56 ± 2.08 c	36.57 ± 1.07 b
0.5	0.3	65.56 ± 2.72 c	35.34 ± 1.00 bc
0.5	0.5	64.44 ± 3.33 c	36.94 ± 1.05 b
1.0	0.1	82.22 ± 2.08 ab	34.98 ± 1.18 bcd
1.0	0.3	86.67 ± 2.22 a	32.39 ± 0.94 d
1.0	0.5	81.11 ± 2.22 b	32.64 ± 0.63 cd
Sig	TDZ	**	**
	NAA	NS	NS
	TDZ×NAA	NS	NS

Data are presented as the mean ± S.E. (n = 5). Values followed by the same lowercase letter in each column indicate that means do not differ significantly at the 5 % level (LSD test)

NS no significant difference (*P* > 0.05)

**Significant difference (*P* < 0.001)

[a] GGB induction rate = (number of GGBs formed/total number of explants) *100 %

[b] GGB formation was record every 2 days, and time for GGB formation from primordia was calculated after 8 weeks of subculture. Primordia (diameter ≥ 2.0 mm) were excised from juvenile sporophytes after 4 weeks of preculture

Table 2 Influence of plant growth regulators on GGB multiplication in *Cibotium barometz*

Plant growth regulators (mg L^{-1})		GGB final fresh weight (mg)[a]	Rate of plantlet regeneration from GGBs (%)[b]
TDZ	NAA		
0	0	72.14 ± 3.38 a	67.78 ± 3.24 a
0.5	0.1	56.55 ± 2.17 b	46.67 ± 2.83 b
0.5	0.3	57.91 ± 3.27 b	43.33 ± 3.24 b
0.5	0.5	52.26 ± 2.07 bc	42.22 ± 3.77 b
1.0	0.1	46.91 ± 1.94 cd	23.33 ± 2.08 c
1.0	0.3	43.30 ± 1.78 de	21.11 ± 2.72 c
1.0	0.5	42.21 ± 2.40 de	24.44 ± 3.33 c
1.5	0.1	36.85 ± 1.80 ef	16.67 ± 3.04 c
1.5	0.3	33.08 ± 2.19 f	20.00 ± 2.83 c
1.5	0.5	34.09 ± 2.23 f	18.89 ± 2.83 c
Sig	TDZ	**	**
	NAA	NS	NS
	TDZ×NAA	NS	NS

Data are presented as the mean ± S.E. (n = 5). Values followed by the same lowercase letter in each column indicate that means do not differ significantly at the 5 % level (LSD test)

NS no significant difference (*P* > 0.05)

**Significant difference (*P* < 0.001)

[a] GGB initial fresh weight was 4.0 mg, GGB final fresh weight was assessed after 4 weeks of culture

[b] Rate of plantlet regeneration from GGBs = (number of GGBs with more than one regenerated fronds/total number of GGBs) *100 %

it was significantly affected by mineral salt concentration (*P* < 0.001) and AC (*P* < 0.05). Compared with other treatments, 1/4 MS media supplemented with 0.1 or 0.2 % (w/v) AC produced a higher rate of GGB development into plantlets, above 90 %. Moreover, during plantlet cultivation, mineral salt concentration (*P* < 0.001) and AC (*P* < 0.05) also significantly affected plantlet height. Plantlets on 1/2 MS media supplemented with 0.1 or 0.2 % (w/v) AC were higher than those on other treatments, with plantlet height above 3.2 cm after 8 weeks of plantlet cultivation (Fig. 1d). However, there was no statistically significant interaction between mineral salt concentration and AC on the rate of GGB development into plantlets and plantlet height (*P* > 0.05). In addition, aposporous gametophytes were observed on the roots of 4 plantlets cultured on 1/2 MS containing 3 % (w/v) sucrose without AC during plantlet cultivation (Fig. 1e).

Plantlets from all treatments had roots. Regenerated plantlets were successfully acclimatized (80 %) in plastic pots containing a 5:1 (v/v) mixture of peat and perlite under greenhouse conditions. Surviving plants showed a normal phenotype (Fig. 1f). Particularly, a small crown covered with golden hairs appeared at the apex after 5 months of acclimatization (Fig. 1g).

Morphological and histological observation of GGBs

Top view showed that GGBs during multiplication was a yellow-green globular structure, which was composed of the single GGB (Fig. 2a). From the bottom view, single GGBs were connected to each other, and the brown root-like structures were gathered in the center of GGBs (Fig. 2b). To some degree, a single GGB had two discrete bipolar structures: shoot meristem on the top, as well as root meristem on the bottom that was always attached to the brown root-like structures (Fig. 2c). During differentiation, shoot meristem on the top of the single GGB developed into young fronds (Fig. 2d). At the same time, root meristem on the bottom of the single GGB formed more roots or root-like structures (Fig. 2d). As a result, one single GGB developed into one plantlet (Fig. 2d). Protrusions were present on the surface of GGBs during differentiation, due to the formation of young fronds (Fig. 2e). Finally, one cluster of GGBs developed into one cluster of plantlets (Fig. 2f).

Table 3 Influence of mineral salt concentration and AC on GGB differentiation and plantlet growth in *Cibotium barometz*

Mineral salt concentration	AC (%, w/v)	Rate of GGB development into plantlets (%)[a]	Plantlet height (cm)
1/4 MS	0	78.89 ± 3.24 cd	1.75 ± 0.13 g
1/4 MS	0.1	91.11 ± 2.83 a	2.22 ± 0.20 ef
1/4 MS	0.2	90.00 ± 2.08 ab	1.91 ± 0.12 fg
1/2 MS	0	71.11 ± 3.24 d	2.99 ± 0.13 bc
1/2 MS	0.1	83.33 ± 3.93 bc	3.52 ± 0.09 a
1/2 MS	0.2	80.00 ± 3.77 cd	3.25 ± 0.15 ab
MS	0	68.89 ± 2.83 d	2.18 ± 0.21 ef
MS	0.1	74.44 ± 4.51 cd	2.57 ± 0.16 de
MS	0.2	76.67 ± 4.44 cd	2.71 ± 0.09 cd
Sig	Mineral salt	**	**
	AC	*	*
	Mineral salt × AC	NS	NS

Data are presented as the mean ± S.E. (n = 5). Values followed by the same lowercase letter in each column indicate that means do not differ significantly at the 5 % level (LSD test)

NS no significant difference ($P > 0.05$)

*Significant difference ($P < 0.05$)

**Significant difference ($P < 0.001$)

[a]Rate of GGB development into plantlets = (number of GGBs with more than 5 regenerated plantlets/total number of GGBs) *100 %
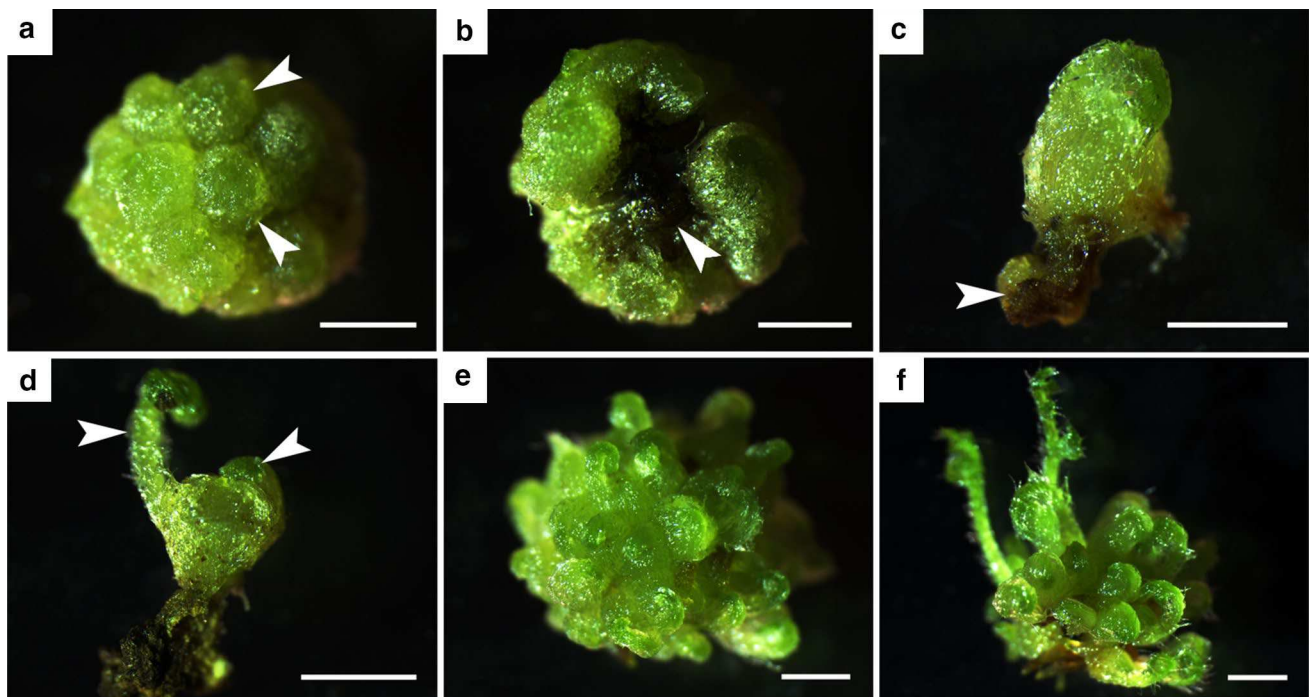

Fig. 2 Morphological observation of *Cibotium barometz* GGBs in the multiplication and differentiation stage. **a–c** GGBs or a single GGB in the multiplication stage. **a** GGB morphology from top view, *arrow* indicates a single GGB. **b** GGB morphology from bottom view, *arrow* indicates *brown* root-like tissues. **c** Single GGB mor-
phology, *arrow* indicates *brown* root-like tissue. **d–f** GGBs or single GGB development into sporophyte plantlet. **d** One single GGB development into one plantlet, *arrow* indicates young fronds. **e** Young frond formation on GGBs. **f** GGBs development into a cluster of plantlets. *bar* 1 mm

Fig. 3 Histological analysis of *Cibotium barometz* GGBs in the multiplication and differentiation stage. **a–c** Single GGBs in the multiplication stage. **a** *Arrow* indicates meristems with dense cytoplasm on the top of the single GGB, *bar* 100 µm. **b** *Arrow* indicates protrusion formation from epidermal cells, that gives rise to the new single GGB, *bar* 100 µm. **c** Single mother GGB (*M*) and single daughter GGB (*D*) produce a new single GGB at the same time, *arrow* indicates the formation of a new single GGB, *bar* 50 µm. **d** Single GGB in the differentiation stage, *arrow* indicates that the top surface of a single GGB extends outward, *bar* 50 µm
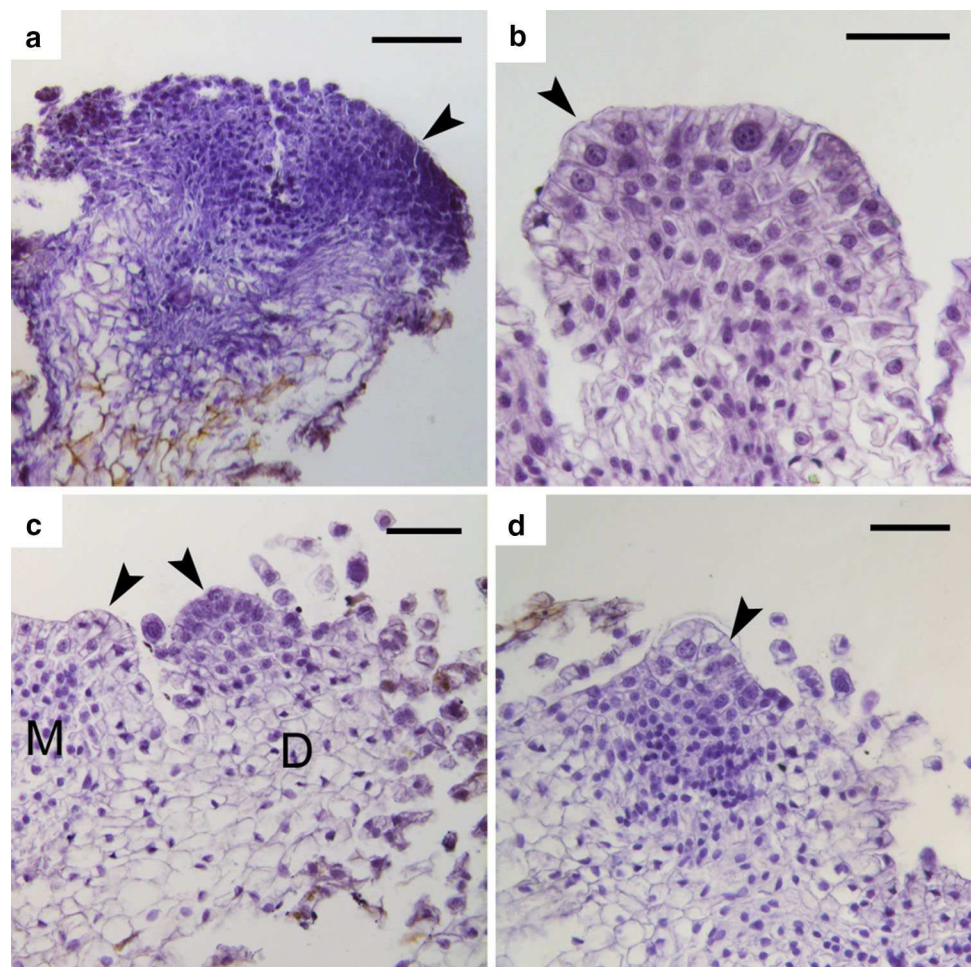
Histological analysis of GGB during multiplication revealed that there were meristems on the top of the single GGB (Fig. 3a). Compared with other cells, meristem cells were cytoplasmically denser (Fig. 3b), and the epidermal cells of meristem zone were bigger in size and nucleus (Fig. 3b). As the epidermal cells divided, dome-shaped protrusions formed on the surface of the single mother GGB (Fig. 3b, c), then developed into the single daughter GGB (Fig. 3c). Interestingly, the single daughter GGB, as well as the single mother GGB produced new single GGBs at the same time (Fig. 3c). As a result, GGBs tended to multiply fast. The single GGB during multiplication had flat or dome-shape top surface (Fig. 3a), while the single GGB during differentiation extended outward (Fig. 3d), which indicated that the single GGB turned into shoot organogenesis.

Scanning electron microscopy

We evaluated GGB surface structure during multiplication and differentiation using scanning electron microscopy imaging. The single GGB was covered with hair-like structures (Fig. 4a), but few hair-like structures were observed on the protrusions (Fig. 4b) and new single GGBs (Fig. 4c). Protrusions were observed on the surface of GGBs during multiplication (Fig. 4b). These protrusions gave rise to new single GGBs (Fig. 4c). During differentiation, sheet-like structures, likely early embryonic leaves, appeared on the top of the single GGB (Fig. 4d, e). In addition, new single GGBs were also found in the differentiation stage, but their top surface was slightly pointier than new single GGBs in the multiplication stage (Fig. 4f), indicating that new single GGBs formed in the differentiation stage probably differentiated directly instead of multiplication.

Discussion

In this study, we used in vitro juvenile sporophytes as explants for GGB induction in *C. barometz*, because of the difficulty in getting proper explants from tree fern individuals. With this method, GGB induction rate was above 45 % except the control (Table 1). Similarly, in vitro juvenile
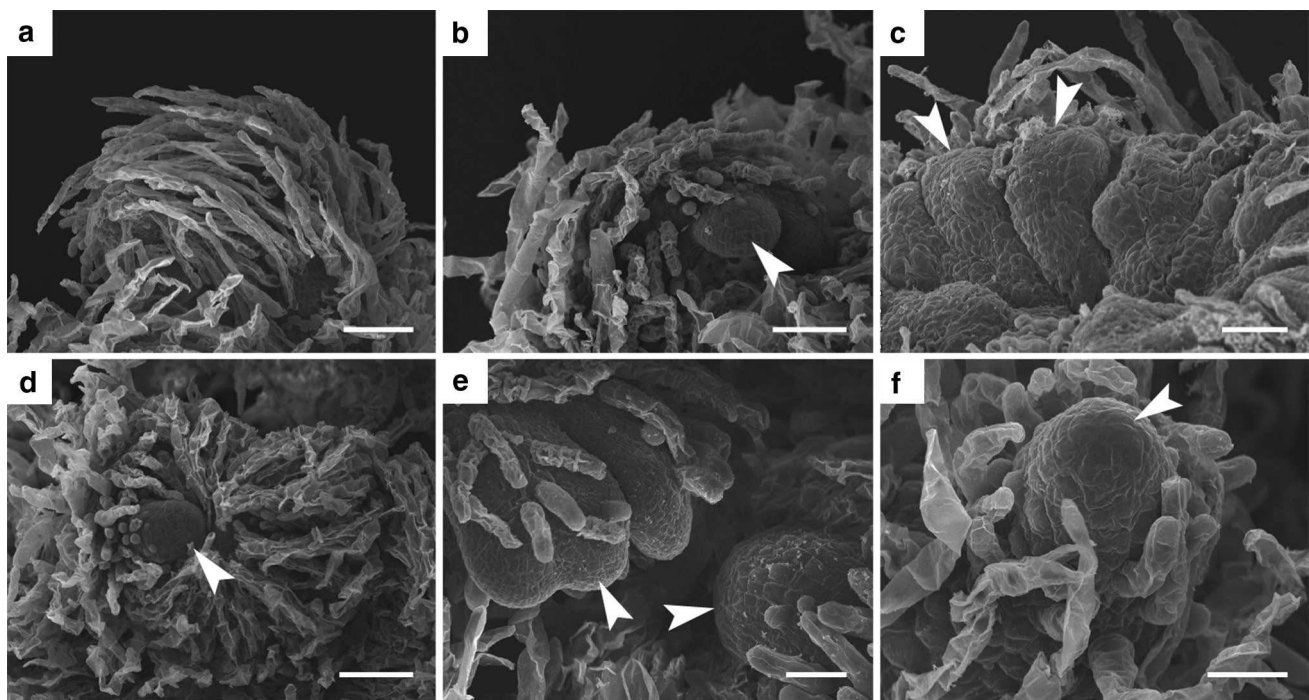
Fig. 4 Scanning electron microscopy of *Cibotium barometz* GGBs in the multiplication and differentiation stage. **a–c** Single GGBs in the multiplication stage. **a** Single GGB covered with hair-like structures, *bar* 100 µm. **b** *Arrow* indicates protrusion on the surface of the single GGB, that gives rise to a new single GGB, *bar* 100 µm. **c** *Arrow* indicates numbers of new single GGBs on the surface, *bar* 100 µm. **d–f** Single GGBs in the differentiation stage. **d** *Arrow* indicates the formation of sheet-like structures, *bar* 100 µm. **e** *Arrow* indicates sheet-like structures on the top of the single GGB, *bar* 50 µm. **f** Morphology of a new single GGB in the differentiation stage, *arrow* indicates that its top surface is slightly pointy, *bar* 50 µm

sporophytes were also used to initiate somatic embryo formation in the tree fern *Cyathea delgadii* (Mikuła et al. 2015a, b). Therefore, in vitro juvenile sporophytes are potential explants for in vitro vegetative propagation of tree ferns, especially those with in vitro propagation protocols from spores.

GGB induction in *C. barometz* was significantly dependent on TDZ, which promoted GGB induction (Table 1). Cytokinins, such as BA and TDZ, are often used to induce adventitious buds in many spermatophytes (Skała et al. 2015; Zhang and Finer 2015), and PLBs in orchids (Malabadi et al. 2004; Ng and Saleh 2011; Park et al. 2003). BA is also optimal for GGB induction in some herbaceous fern species, such as *Nephrolepis cordifolia* (Higuchi et al. 1987), and *Asplenium nidus* (Higuchi and Amaki 1989). However, some species seem to behave differently. For example, increasing the concentration of cytokinin decreased GGB formation in *Platycerium bifurcatum*, while the medium supplemented with 5.37 µM NAA alone displayed the maximum GGB induction (Liao and Wu 2011). In *Blechnum spicant*, rhizomes of juvenile sporophytes were cultivated on the medium with BA for one month preculture. Subculture of these rhizomes on the same medium with BA induced the development of

aposporous gametophytes. Conversely, subculture on the medium with NAA promoted GGB formation (Fernández et al. 1996). In our study, NAA had no significant effect on GGB induction in *C. barometz*, similarly to what reported for *Nephrolepis cordifolia* and *Asplenium nidus* (Higuchi and Amaki 1989; Higuchi et al. 1987).

Cytokinin BA controls the organogenesis of GGBs (Higuchi et al. 1987). BA inhibited the plantlet regeneration from GGBs in *Asplenium nidus* (Higuchi and Amaki 1989), *Adiantum raddianum, Pteris ensiformis*, and *Rumohra adiantiformis* (Amaki and Higuchi 1991). BA also decreased GGB final fresh weight in *Asplenium nidus* (Higuchi and Amaki 1989). Removal of BA from medium promotes GGB differentiation (Higuchi et al. 1987). The medium with PGR alone or combinations that supports rapid growth of GGBs without plantlet regeneration is optimal for GGB multiplication (Liao and Wu 2011). Our data obtained here show that TDZ plays a similar role in GGB multiplication of *C. barometz* as BA in other fern species. However, even at the highest TDZ concentration (1.5 mg L^{-1}), plantlet regeneration from GGBs was not be inhibited completely (Table 2), indicating that it is difficult to absolutely control organogenesis of *C. barometz* GGBs by a single cytokinin at low concentration. Considering the balance between

the inhibition of plantlet regeneration and GGB final fresh weight, we found the optimal TDZ concentration for GGB multiplication in *C. barometz* to be 1.0 mg L^{-1}. Our results also showed that NAA had no significant effect on GGB multiplication in *C. barometz*. In contrast, Liao and Wu (2011) found that the combination of NAA and cytokinin N^6-(2-Isopentenyl)adenine (2iP) was more suitable than using 2iP alone for GGB multiplication in *Platycerium bifurcatum*.

For plantlet regeneration of *C. barometz*, mineral salt concentration and AC had a significant effect on GGB differentiation and plantlet growth (Table 2). Mineral salt concentration is associated with plantlet regeneration in some fern species. For example, mineral salt concentration was associated with a greater frequency of abnormal regenerated sporophytes in *Cyathea delgadii*, 1/2 MS medium was needed to stimulate normal sporophytes from somatic embryos (Mikuła et al. 2015b). Similarly, 1/2 MS medium without sucrose favored leaf expansion of *Pteris ensiformis* (Fernández et al. 1999). In our study, 1/4 MS was an optimal for GGB development into plantlets in *C. barometz*, and 1/2 MS was best for plantlet growth. According to Thomas (2008), AC promotes a morphogenic responses in some ferns in vitro. In *Platycerium bifurcatum* and *Matteuccia struthiopteris*, AC enhanced sporophyte regeneration (Teng 1997; Thakur et al. 1998). Our results also confirmed that addition of AC was beneficial for GGB development and plantlet growth in *C. barometz*.

We observed aposporous gametophytes on the roots of plantlets cultured on 1/2 MS containing 3 % (w/v) sucrose without AC during plantlet cultivation (Fig. 1e). In other fern species, aposporous gametophytes were induced successfully, that influenced by various factors, such as PGRs and sucrose-limiting conditions (Ambrožič-Dolinšek et al. 2002; Fernández et al. 1996; Martin et al. 2006; Teng and Teng 1997). Here, we could not determine the reason for aposporous gametophyte formation in *C. barometz*, but it may be related to residual PGRs from GGB multiplication.

Higuchi et al. (1987) first reported GGB propagation system of *Nephrolepis cordifolia*, but with the limited morphological and histological description of GGBs. *C. barometz* GGBs during multiplication were composed of the single GGB with meristems on its top (Figs. 2a, 3a). New single GGBs were initiated from the epidermal cells of meristem zone (Fig. 3b), confirming previous observations in *Asplenium nidus* (Higuchi and Amaki 1989). Moreover, our results showed that the single daughter GGB and the single mother GGB were able to produce new single GGBs at the same time (Fig. 3c), explaining the efficient multiplication of GGBs. In general, GGBs of different fern species have distinctive features on their surface, such as scale-like structures in *Nephrolepis cordifolia* and *Adiantum raddianum*, hair-like structures in *Pteris ensiformis* and *Rumohra adiantiformis* (Amaki and Higuchi 1991). These same structures were absent in the somatic embryo of *Cyathea delgadii*, but trichomes were observed, which were smaller and shorter than hair-like structures (Mikuła et al. 2015a). In *C. barometz*, there were hair-like structures on GGB surfaces (Fig. 4a), but no scale-like structures. Additionally, sheet-like structures, likely early embryonic leaves, were found during GGB differentiation (Fig. 4d, e). With differentiation, one single GGB of *C. barometz* developed into one plantlet with fronds and roots (Fig. 2d). Similar observations have been obtained in the development of PLBs in orchids (Teixeira da Silva 2013; Ng and Saleh 2011), somatic embryos in *Cyathea delgadii* (Mikuła et al. 2015a, b) and buibils in some fern species, which are the small rounded leafless structures on the fronds (Jones 1987). Moreover, our observations showed that shoot meristem on the top of the single GGB developed into young fronds (Fig. 2d), and root meristem on the bottom of the single GGB formed roots or root-like structures (Fig. 2d), are similar to PLBs in orchids, but differ from zygotic or somatic embryogenesis that includes several development stages. To some degree, *C. barometz* GGBs could be regarded as a structure which is similar to PLBs in orchids.

Conclusion

This is the first report that describes an in vitro propagation protocol for the endangered tree fern *C. barometz* through formation of GGBs using in vitro juvenile sporophytes as explants. The results of this study demonstrate that GGB induction and multiplication in *C. barometz* are highly dependent on PGRs, especially on the cytokinin TDZ. GGB development and plantlet growth are affected by mineral salt concentration and AC. Moreover, our observation shows that *C. barometz* GGBs is a yellow-green globular structure composed of the single GGB with meristems and hair-like structures, and new single GGBs are initiated from the epidermal cells of meristem zone. This protocol described here could be used to facilitate the rapid propagation of the tree fern *C. barometz* for commercial and conservation purposes.

References

Amaki W, Higuchi H (1991) A possible propagation system of *Nephrolepis, Asplenium, Pteris, Adiantum* and *Rumohra* (Arachniodes) through tissue culture. Acta Hortic 300:237–243

Ambrožič-Dolinšek J, Camloh M (1997) Gametophytic and sporophytic regeneration from bud Scales of the fern *Platycerium bifurcatum* (Cav.) C. Chr. in vitro. Ann Bot 80(1):23–28. doi:10.1006/anbo.1996.0383

Ambrožič-Dolinšek J, Camloh M, Bohanec B, Žel J (2002) Apospory in leaf culture of staghorn fern (*Platycerium bifurcatum*). Plant Cell Rep 20(9):791–796. doi:10.1007/s00299-001-0403-2

Bertrand AM, Albuerne MA, Fernández H, González A, Sánchez-Tamés R (1999) In vitro organogenesis of *Polypodium cambricum*. Plant Cell Tissue Organ Cult 57(1):65–69. doi:10.1023/a:1006348628114

Camloha M, Gogala N, Rode J (1994) Plant regeneration from leaf explants of the fern *Platycerium bifurcatum* in vitro. Sci Hortic 56(3):257–266. doi:10.1016/0304-4238(94)9007-8

CITES (2015). Convention on international trade in endangered species of wild fauna and flora Appendices I, II, and III. https://cites.org/eng/app/appendices.php. Accessed 10 March 2016

Cuong NX, Minh CV, Kiem PV, Huong HT, Ban NK, Nhiem NX, Tung NH, Jung JW, Kim HJ, Kim SY, Kim JA, Kim YH (2009) Inhibitors of osteoclast formation from rhizomes of *Cibotium barometz*. J Nat Prod 72(9):1673–1677. doi:10.1021/np9004097

Fernández H, Bertrand AM, Sánchez-Tamés R (1996) Micropropagation and phase change in *Blechnum spicant* and *Pteris ensiformis*. Plant Cell Tissue Organ Cult 44:261–265. doi:10.1007/BF00048534

Fernández H, Bertrand AM, Sánchez-Tamés R (1997) Plantlet regeneration in *Asplenium nidus* L. and *Pteris ensiformis* L. by homogenization of BA treated rhizomes. Sci Hortic 68(1):243–247. doi:10.1016/S0304-4238(96)00986-7

Fernández H, Bertrand AM, Sánchez-Tamés R (1999) Biological and nutritional aspects involved in fern multiplication. Plant Cell Tissue Organ Cult 56(3):211–214. doi:10.1023/A:1006277229136

Goller K, Rybczyński JJ (2007) Gametophyte and sporophyte of tree ferns in vitro culture. Acta Soc Bot Pol 76(3):193–199. doi:10.5586/asbp.2007.022

Hegde S, Menon VK, Noronha R, D'Souza L (2006) Callus culture and an unconventional pattern of sporophyte regeneration in *Drynaria quercifolia*—a medicinal fern. In Vitro Cell Dev Boil Plant 42(6):508–513. doi:10.1079/IVP2006810

Higuchi H, Amaki W (1989) Effects of 6-benzylaminopurine on the organogenesis of *Asplenium nidus* L. through in vitro propagation. Sci Hortic 37(4):351–359. doi:10.1016/0304-4238(89)90146-5

Higuchi H, Amaki W, Suzuki S (1987) In vitro propagation of *Nephrolepis cordifolia* Prsel. Sci Hortic 32(1):105–113. doi:10.1016/0304-4238(87)90021-5

Jones DL (1987) Encyclopaedia of ferns. Timber press, Portland

Li T, Xu L, Li Z, Panis B (2013) Cryopreservation of *Neottopteris nidus* prothallus and green globular bodies by droplet-vitrification. Cryoletters 34(5):481–489

Li X, Fang YH, Han JD, Bai SN, Rao GY (2015) Isolation and characterization of a novel somatic embryogenesis receptor kinase gene expressed in the fern *Adiantum capillus-veneris* during shoot regeneration in vitro. Plant Mol Biol Rep 33(3):638–647. doi:10.1007/s11105-014-0769-2

Liao YK, Wu YH (2011) In vitro propagation of *Platycerium bifurcatum* (Cav.) C. Chr. via green globular body initiation. Bot Stud 52:455–463

Malabadi RB, Mulgund GS, Nataraja K (2004) Efficient regeneration of *Vanda coerulea*, an endangered orchid using thidiazuron. Plant Cell Tissue Organ Cult 76(3):289–293. doi:10.1023/B:TICU.0000009255.69476.b7

Martin KP, Sini S, Zhang CL, Slater A, Madhusoodanan P (2006) Efficient induction of apospory and apogamy in vitro in silver fern (*Pityrogramma calomelanos* L.). Plant Cell Rep 25(12):1300–1307. doi:10.1007/s00299-006-0215-5

Mikuła A, Pożoga M, Tomiczak K, Rybczyński J (2015a) Somatic embryogenesis in ferns: a new experimental system. Plant Cell Rep 34(5):783–794. doi:10.1007/s00299-015-1741-9

Mikuła A, Pożoga M, Grzyb M, Rybczyński J (2015b) An unique system of somatic embryogenesis in the tree fern *Cyathea delgadii* Sternb.: the importance of explant type, and physical and chemical factors. Plant Cell Tissue Organ Cult 123(3):467–478. doi:10.1007/s11240-015-0850-z

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Ng CY, Saleh NM (2011) In vitro propagation of *Paphiopedilum* orchid through formation of protocorm-like bodies. Plant Cell Tissue Organ Cult 105(2):193–202. doi:10.1007/s11240-010-9851-0

Park S, Murthy H, Paek K (2003) Protocorm-like body induction and subsequent plant regeneration from root tip cultures of *Doritaenopsis*. Plant Sci 164(6):919–923. doi:10.1016/S0168-9452(03)00019-0

Pence VC (2015) Propagation and cryopreservation of *Asplenium scolopendrium var. americanum*, the American hart's-tongue fern. Am Fern J 105(3):211–225. doi:10.1640/0002-8444-105.3.211

Skała E, Grąbkowska R, Sitarek P, Kuźma Ł, Błauż A, Wysokińska H (2015) *Rhaponticum carthamoides* regeneration through direct and indirect organogenesis, molecular profiles and secondary metabolite production. Plant Cell Tissue Organ Cult 123(1):83–98. doi:10.1007/s11240-015-0816-1

Teixeira da Silva JA (2013) The role of thin cell layers in regeneration and transformation in orchids. Plant Cell, Tissue and Organ Cult 113(2):149–161. doi:10.1007/s11240-012-0274

Teng WL (1997) Activated charcoal affects morphogenesis and enhances sporophyte regeneration during leaf cell suspension culture of *Platycerium bifurcatum*. Plant Cell Rep 17(2):77–83. doi:10.1007/s002990050356

Teng WL, Teng MC (1997) In vitro regeneration patterns of *Platycerium bifurcatum* leaf cell suspension culture. Plant Cell Rep 16(12):820–824. doi:10.1007/s002990050327

Thakur RC, Hosoi Y, Ishii K (1998) Rapid in vitro propagation of *Matteuccia struthiopteris* (L.) Todaro—an edible fern. Plant Cell Rep 18:203–208. doi:10.1007/s002990050557

Thomas TD (2008) The role of activated charcoal in plant tissue culture. Biotechnol Adv 26(6):618–631. doi:10.1016/j.biotechadv.2008.08.003

Winarto B, Teixeira da Silva JA (2012) Improved micropropagation protocol for leatherleaf fern (*Rumohra adiantiformis*) using rhizomes as donor explant. Sci Hortic 140:74–80. doi:10.1016/j.scienta.2012.03.017

Wu Q, Yang XW (2009) The constituents of *Cibotium barometz* and their permeability in the human Caco-2 monolayer cell model. J Ethnopharmacol 125(3):417–422. doi:10.1016/j.jep.2009.07.017

Zhang Z, Finer JJ (2015) Sunflower (*Helianthus annuus* L.) organogenesis from primary leaves of young seedlings preconditioned by cytokinin. Plant Cell Tissue Organ Cult 123(3):645–655. doi:10.1007/s11240-015-0867-3

Zhang XC, Jia JS, Zhang G (2002) Survey and evaluation of the natural resources of *Cibotium barometz* (L.) J. Smith in China, with reference to the implementation of the CITES convention. Fern Gazette 16(6):383–387

Zhao X, Wu ZX, Zhang Y, Yan YB, He Q, Cao PC, Lei W (2011) Anti-osteoporosis activity of *Cibotium barometz* extract on ovariectomy-induced bone loss in rats. J Ethnopharmacol 137(3):1083–1088. doi:10.1016/j.jep.2011.07.017

Zhou X, Gui M, Zhao D, Chen M, Ju S, Li S, Lu Z, Mo X, Wang J (2013) Study on reproductive barriers in 4x–2x crosses in *Dianthus caryophyllus* L. Euphytica 189(3):471–483. doi:10.1007/s10681-012-0819-5