

GAMETOPHYTE AND SPOROPHYTE OF TREE FERNS IN VITRO CULTURE

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ABSTRACT

Experiments had been carried out on gametophytes and very young fronds of sporophytes with application of Murashige and Skoog (1962) medium. The paper described the results of 15 years in vitro experiments on 16 species of tree ferns belonging to various genera: *Blechnum*, *Cibotium*, *Cyathea* and *Dicksonia*. Genus *Cyathea* was represented by: *C. australis* (R.Br.) Domin., *C. capensis* (L.f.) Sm., *C. cooperi* (F.Muell.) Domin., *C. brownii* Domin., *C. dealbata* (G.Forest) Sw., *C. dregei* Kunze, *C. leichhardtiana* (F.Muell.) Copel., *C. robertsiana* (F.Muell.) Domin., *C. schanschin* Mart., *C. smithii* Hook.f. and *Cyathea* sp. In case of genus *Dicksonia* only two species were introduced into our experiments: *D. fibrosa* Colenso and *D. sellowiana* Hook.. Taxa *Blechnum* was presented by *B. brasiliense* Desv. and *Cibotium* by *C. glaucum* (Sm.) Hook. and Arn. and *C. schiedeii* Schldtl. and Cham..

The studied species presented various responses on culture conditions depending on the level of stage of development. Time required for spores germination differed between species and took from only a few to 16 weeks. Prothallium formations showed various types of growth presented by marginal meristems. For all investigated species long term gametophyte in vitro cultures was established. Mature gametophyte possessed functional antheridia and archegonia. Spontaneous fertilization helped to establish the culture of young sporophytes. For all species the *ex vitro* culture in greenhouse collection was established. Manipulation of sucrose content in the medium stimulated the multiplication of gametophytes, but its lack induced formation of gemmae. Apospory was observed when culture of very young fronds was extended for 6 months and new generation of gametophytes was developed. Finally, sporophytes of 12 species were obtained and they have been growing in our greenhouse.

KEY WORDS: apospory, gametophyte and sporophyte, gene bank, greenhouse collection, in vitro culture, multiplication, tree fern, spores.

INTRODUCTION

Tree ferns are typical for rain forest of tropical and subtropical climate. Because of their ornamental beauty they are usually used for out- and indoor decoration. The majority of tree ferns are illegally collected from natural sites, what resulted in decrease of their population, though they are listed on the International Red Book and protected by CITES (Etter 1993). They are excluded from official exchange programmes of plant material. This is the way of this plant species protection against total extinction (Tryon 1976). We estimate that the threats of the species are even higher; there are not many papers concerning vegetative propagation of the ferns over the world. However, we know that the gametophyte is the objective of studies conducted in the Institute of Forestry of Taiwan. Our involvement in the studies of tree fern multiplication dates back to the beginning of 90-ties of the twentieth century (Goller and Rybczyński 1995). In the meantime, with the application of in vitro culture of spores (obtained within the frame of the Botanical Garden Exchange Program) and consecutive stages

of development, the system of plant material multiplication was developed. Young plants could be reintroduced to natural conditions, too (Gomez 1983; Kramer 1990). Now, we possess the next ten species in our gametophyte bank, together giving us the biggest collection of tree fern in Poland, and we hope in Europe, too. Plants carried on in our tropical greenhouse collection already reached maturity and they can serve as the source of spores for next experiments. At present, a lot of efforts is done to develop the system of cryopreservation of spores and gametophytes.

In the paper we would like to describe the morphological events which were observed in long term cultures of gametophytes and very young sporophytes of tree ferns.

MATERIALS AND METHODS

Plant material

Experiments were carried out with application of various stage of development of four genera of tree ferns; *Blechnum*, *Cibotium*, *Cyathea* and *Dicksonia*. Genus *Cyathea*

was represented by: *C. australis*, *C. capensis*, *C. cooperi*, *C. brownii*, *C. dealbata*, *C. dregei*, *C. leichhardtiana*, *C. robertsiana*, *C. schanschin*, *C. smithii* and *Cyathea* sp. In case of genus *Dicksonia* only two species were introduced into our experiments: *D. sellowiana* and *D. fibrosa*. Taxa *Blechnum* was represented by *B. brasiliense* and *Cibotium* by *C. glaucum* and *C. schiedeii*.

Media used

Murashige and Skoog's (1962) medium in various mineral salt concentrations supplemented with or without sucrose was mainly used in our experiments.

Plant material sterilization

Spores and sporangia were sterilized with the application of 96% ethanol at 30 sec. and 3% chloramines during 10, min and at least three times washed with sterile distilled water.

Sporangium and spores culture initiation

The culture initiation consisted in sporangia and spores implantation on agar hormone-free 0.5 MS0 medium (half strength of mineral macroelements composition of medium). The culture was carried out with the help of sterile plastic Petri dishes in culture chamber with 16/8 hrs of day/light photoperiod and temperature $20 \pm 1^\circ\text{C}$.

Maintenance of gametophytes

For long term culture, gametophytes were implanted to the 200 ml jars with 40 ml of medium. The culture extension stimulated the multiplication of gametophytes. Subcultures of plant material were species depended. Gametophytes of some species were subcultures many times during several years. Gametophytes were used for numerous other experiments describing their morphogenetic potential in relation to the supplementation of culture medium with sucrose (Table 1).

Fertilization and sporophyte production

The temperature changes of culture chamber stimulated water condensation on gametophytes and stimulated spontaneous fertilization. After a few months the primary leaves of sporophyte (fronds) were raised up and after next few months plantlets were rooted and transferred to the ex vitro condition. This stage of sporophyte development appeared to be an excellent source of young leaves for other experiments.

Sporophytes hardening and their growth in greenhouse

Hardening of sporophyte was gradually done with slow acclimatization to various humidity and temperature. Plants of two fronds stages were transferred to the pots in greenhouse nursery and after next two years they were implanted to the final ground position in greenhouse collection.

Induction of the apospory

Primary 4 cm long leaves of sporophytes were excised with the help of a fine knife and implanted into Petri plates with 0.5 MS medium supplemented with or without sucrose. The culture was maintained in the presence of light conditions described above during 9 to 12 months (Table 1).

Methods of morphogenic events documentation

For light microscopy, the selected plant material was analyzed without staining, using "Olympus" microscopes. The

TABLE 1. Steps of tree fern culture with proper and manipulated development of gametophyte and sporophyte.

	Pinnae with Sporangia
	↓ Collection
	Spore
	↓ Isolation and germination
	Prothallium
	↓ Cell multiplication and differentiation
Gemmae	Gametophyte
	↓ Proliferation and multiplication Spontaneous fertilization
	Embryo development
	↓ Growth of first leaf
	Sporophyte
	↓ Growth in vitro culture
Apospory	Sporophyte leaves culture
	↓
	Sporophyte in vitro hardening
	↓
	Sporophyte ex vitro hardening
	↓
	Sporophyte pot culture
	↓
	Sporophyte soil culture and getting maturity

scanning electron microscope studies of gametophytes were performed with explants collected every seven days during consecutive 8 weeks. Cultures were fixed with 2.5% glutaraldehyde in 0.1 mol.l⁻¹ cacodylate buffer at pH=7.0 for 2 hrs and 1.0% OsO₄ in 0.2 M Na-cacodylate for 24 hrs. After four times washing of buffers: cacodylate and phosphate (15 min each), plant material was transferred to the series of alcohols from 10% to 100% for 15 min. for dehydration. Alcohol was substituted by acetone with the application of mixture of both substances in proportion 3:1, 1:1, 1:3 for 30 min for each washing. For dehydration, the samples were placed in microporus specimen capsules and dried with liquid CO₂ in a critical point dryer Type E 31000 Jumbo Series II, Polaron Equipment LID. Samples were coated with gold using a Coater "Jeol". Finally, the samples were analyzed by means of a scanning electron microscope model JSM-S1.

RESULTS AND DISCUSSION

Plant material disinfection and spore germination

For more than 10 years, since the first of our papers has been published (Goller and Ryczyński 1995), not much

TABLE 2. The list of species which gametophyte cultures have been carried on during last few years.

No.	Species	Time of germination (weeks)	Gametophyte (weeks)	Sporophyte (months)	Apospory	Ex vitro / / greenhouse collection
1.	<i>Blechnum brasiliense</i> Desv.	7	12	8	Not studied	+
2.	<i>Cibotium glaucum</i> (Sm.) Hook. and Arn.	4	12	–	–	–
3.	<i>Cibotium schiedei</i> Schldtl. and Cham.	5	8	6	Not studied	+
4.	<i>Cyathea australis</i> (R.Br.) Domin.	Dry – 16; Fresh – 4	16	12	Not studied	+
5.	<i>Cyathea brownii</i> Domin.	8	8	5	Not studied	+
6.	<i>Cyathea capensis</i> (L.f.) Sm.	14	20	14	–	–
7.	<i>Cyathea cooperi</i> (F.Muell.) Domin.	16	16	12	Not studied	+
8.	<i>Cyathea dealbata</i> (G.Forest) Sw.	4	8	6	+	–
9.	<i>Cyathea dregei</i> Kunze	8	12	–	–	–
10.	<i>Cyathea leichhardtiana</i> (F.Muell.) Copel.	6	8	6	+	+
11.	<i>Cyathea robertsiana</i> (F.Muell.) Domin.	5	8	5	Not studied	+
12.	<i>Cyathea schanschin</i> Mart.	8	8	5	+	+
13.	<i>Cyathea smithii</i> Hook. f.	8	8	4	+	+
14.	<i>Cyathea</i> sp.	12	16	4	Not studied	+
15.	<i>Dicksonia fibrosa</i> Colenso	8	8	5	+	+
16.	<i>Dicksonia sellowiana</i> Hook.	4	6	6	Not studied	+

publications concerning tissue culture of tree fern were released. Table 2 summarizes our experiments carried out on material originating from various sources – botanical gardens within the frame of the International Seeds Exchange Program. The response of spores for in vitro culture conditions resulted in prothallium production (Figs 1A-D). We observed that the time required for spore germination into prothallium depended on application of the disinfection solution, however, we are sure that the type of storage in institution of spores origin affected their viability and power germination, too. Domestos, commercial bleach, appeared more effective in sterilization and spore germination than the initially used chloramine. It was shown that the type of disinfection substance had a significant effect on *Cyathea delgadii* spore germination (Simabukuro et al. 1998). Dyer (1979) tested numerous sterilization solutions such like NaOCl, Ca (OCl)₂, 4H₂O, and concluded that the majority of disinfectors of fungi and bacteria extend the time required for spore germination and decrease their viability. The spore germination depended on species and viability of the received material. According to Page (1979) in the majority of Cyatheaceae species the spore germination abilities decrease after a few weeks of storage, what is typical for tropical plants. For example, *Cyathea delgadii* spores lost their viability for germination during two months at 12°C in the presence of low humidity (Randi and Felipe 1988), and the percent of germinated spores decreased during the next six months (Marcondes-Ferreira and Felipe 1984). Constantino et al. (2000) distinguished two types of tree fern spores: chlorophyll and non-chlorophyll. The chlorophyll ones did not show the state of spore dormancy, and germinated almost directly after 1.46 day and possessed viability for 48 days. Non-chlorophyll spores germinated after 9.5 days and their viability was carried out during 10-45 days. (Lloyd and Klekowski 1970). For *Cyathea caracasana* and *Dicksonia sellowiana*, non-chlorophyll spore species, the longest time of storage was completed during 11 and 13 months, respectively.

Freshly isolated spores collected from sporangia of *Cyathea australis*, growing in our greenhouse, directly implanted on the medium germinated during four and six weeks.

Gametophyte culture

Gametophyte overgrowth and development was initiated by spore germination and formation of multicellular prothallium structure (Figs 1A-D) on 0.5 MS medium supplemented with sucrose. In case of *C. robertsiana* sporangium implantation the spore germination response was noticed. As the effect of overgrowth, the primary shape of young gametophyte was reached (Figs 1E, G). Finally a heart-shaped gametophyte was formed as the result of edge meristem cells activity (Fig. 1F), expressed by increase of the cell number and the surface of that generation (Fig. 1G). The culture extension for next two months resulted in initiation of proliferation of gametophyte (Figs 1G, H). In the presence of 0.5 MS (1962) medium, the process of spontaneous gametophyte allowed to form a colony of multi and individual gametophytes (Figs 1H, I). The alternative system of gametophyte proliferation, to get their bulk production, could be obtained with the application of their immobilization (Douglas and Sheffield 1990). SEM analysis of *C. brownii* (Fig. 2) improved the involvement of the whole gametophyte in its spontaneous proliferation. Their maturity was expressed by sexual organs' formation, and Figure 3 presents rich formation of antheridia.

Gemmae formation

Individual gametophytes excised from multi-gametophyte structure of *C. dealbata*, *C. leichhardtiana*, *C. schanschin* and *C. smithii* derived from many year-old cultures were maintained on 0.5 MS0 medium without sucrose. During two months of culture on the edge of gametophytes numerous intensive green centers were formed as the result of meristematic activity of the marginal cells. These green centers called "gemmae" and developed in new generation of gametophyte when implanted on the medium supplemented with sucrose. Our results confirmed the earlier description of such structure in *Osmunda regalis* cultures (Fernandez et al. 1997). Figure 4 shows the initial and mature stages of gemmae with typical for this structure rich rhizoids. The obtained new generation of gametophytes presented sexual maturity, confirmed by formation of numerous antheridia and archegonia. With help of UV light

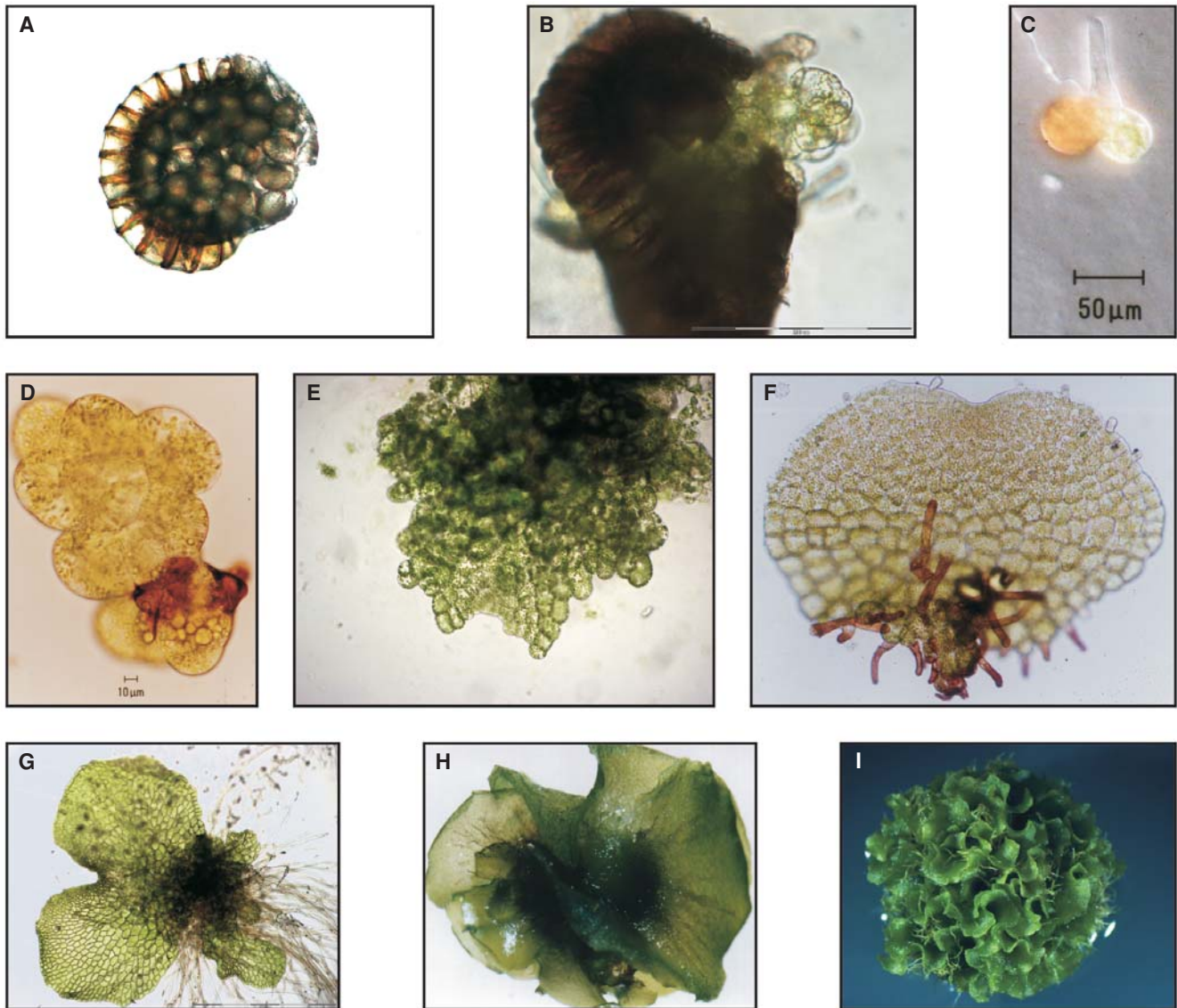


Fig. 1. Development, growth and vegetative multiplication of gametophyte of tree ferns on half strength of Murashige and Skoog (1962) medium + 3% sucrose. A – single sporangium with viable *C. robertsiana* spores; B – open sporangium with spreading *C. leichhardtiana* spores; C – initial stage of spore germination of *C. australis*; D – multicellular non-organized prothallium of *C. australis*; E – formation of edge meristem of *C. robertsiana* prothallium; F – heart stage of *C. australis* gametophyte; G – proliferation of *C. leichhardtiana* gametophyte; H – overgrowth of initially multiple gametophyte of *C. australis* after two months of culture; I – “Colony” of *C. dealbata* gametophyte after 6 months of culture

and special filters the structure of antheridia and mature sperm cells could be detected.

Apospory induction

The initial response of very young, primary leaves of *C. dealbata*, *C. leichhardtiana*, *C. schanschin*, *C. smithii* and *D. fibrosa*, on medium without sucrose, consisted in showing color changes from pale yellow to deep brown during four weeks of the culture. The extension of the culture for next two months did not change the response of explants. After next six months of culture only in case of *Cyathea* species about 50% of explants responded by the overgrowth of the basal part of leaves. Its overgrowth resulted in proliferation of green gametophytes, finally partly covering part of the explant (Fig. 5). Microscopic analysis revealed that initially the chloroplasts were intensively gathered in the ridge cells and later on the cell division of vegetative part of gametophyte, resulted in spontaneous gametophyte multiplication. The lack of sucrose in the medium

helped to induce apospory (Hirsch 1975; Zenkeler 1995). Hirsch (1975) described the response of young leaves of *Microgramma vacciniifolia* on sucrose concentration in the medium. The presence of 3% sucrose induced only sporophytes regeneration on the frond surface, however, its lack in the medium causes gametophytes and sporophytes occurrence on the edge of frond.

Young sporophyte hardening and greenhouse culture

In the jar conditions, because of high humidity, spontaneous fertilization took place as the result of independent translocation of sperm cells to archegonium. Figure 6A shows the intensity of sporophyte production by the colony of gametophyte. One month of the culture resulted in formation of new plantlets with 3-4 fronds. Usually such plantlets possessed a few roots (Fig. 6B), and transferred to the perlite with liquid medium, developed a good enough root system (Goller and Rybczyński 1995). Gradual decreasing of humidity of the culture played an important role in plant adaptation for

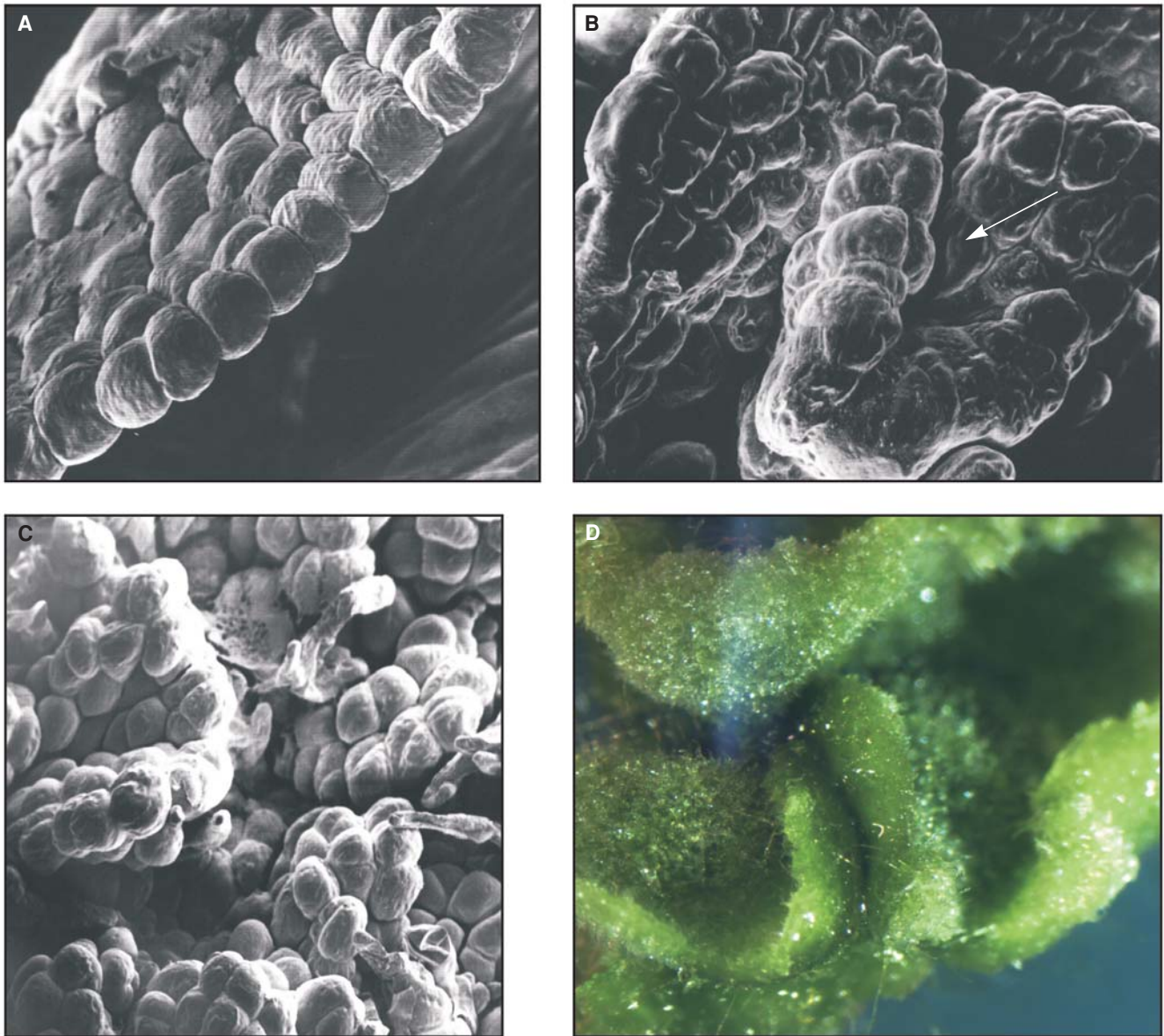


Fig. 2. Proliferation of *Cyathea brownii* gametophyte on half strength of Murashige and Skoog (1962) medium + 3% sucrose. A – typical one cell layer structure of the vegetative part of gametophyte (SEM); B – multicellular edge of gametophyte (SEM); C – SEM analysis of gametophyte proliferation and initial stage of multiplication of gametophyte; D – gametophyte cell proliferation in vitro

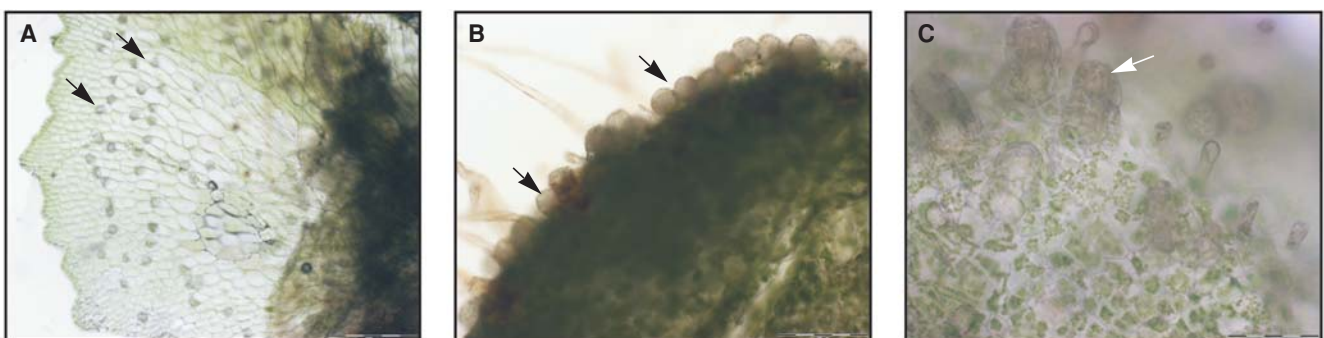


Fig. 3. Gametangium of tree ferns. A – general view of *C. leichhardtiana* gametophyte with two lines of antheridia (MS + 3% sucrose); B – numerous antheridia on the down side of *C. schanschin* gametophyte; C – single antheridium of *C. schanschin*

ex vitro conditions. Figures 6C, D presents particular stages of adaptation of plantlets from in vitro conditions up to growing mature plant in the greenhouse.

In conclusion, we can stress that gametophyte of tree ferns, can be used for studies of morphogenic events of ha-

ploidal single cell layer, and utilized for multiplication of lower vascular plants since in vitro spontaneous fertilization and sporophytes development takes place.

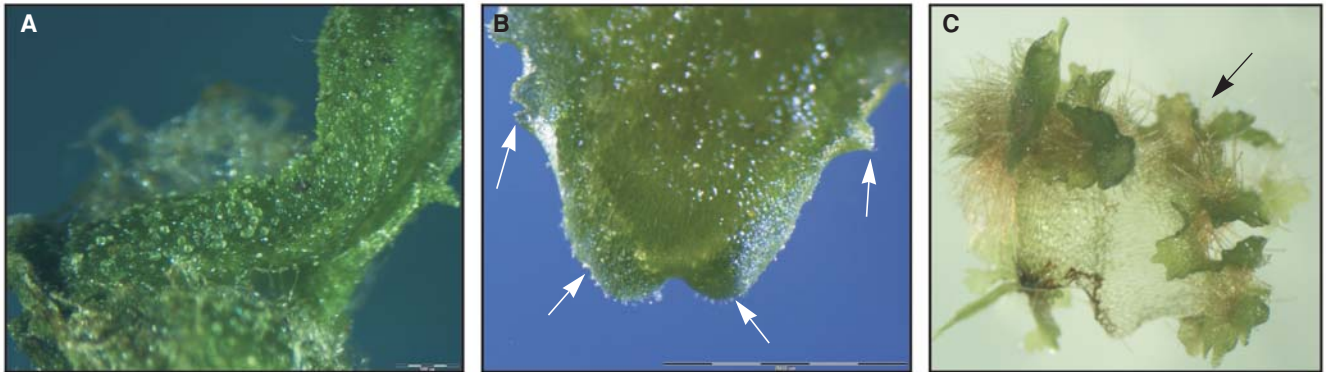


Fig. 4. Gametophyte response on 0.5 MS (1962) without sucrose medium. A – initial cell proliferation for “gemmae” formation of *C. schanschin*; B – organization of green edge cell centers of *C. schanschin*; C – mature “gemmae” on overgrown gametophyte of *C. dealbata*

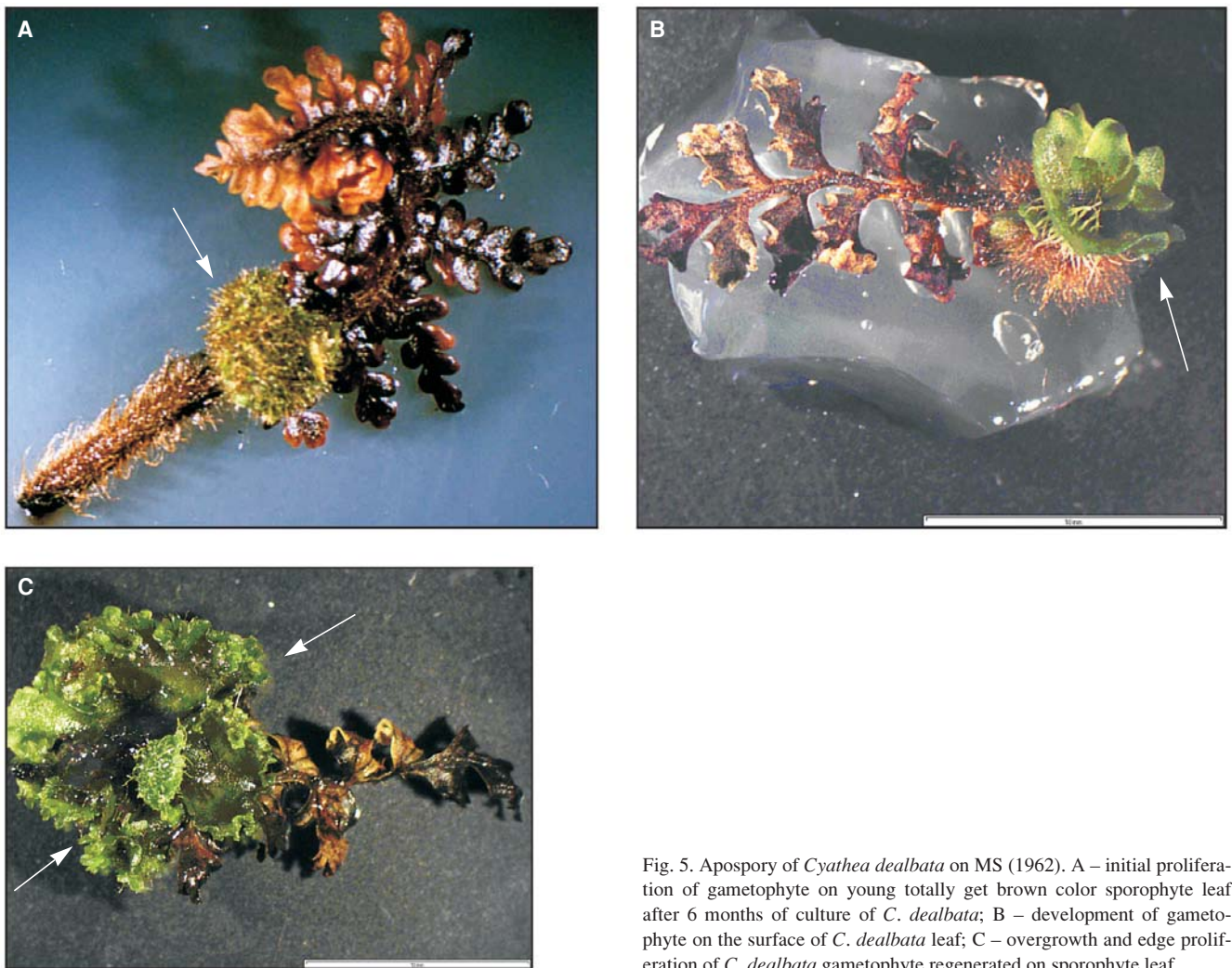


Fig. 5. Apospory of *Cyathea dealbata* on MS (1962). A – initial proliferation of gametophyte on young totally get brown color sporophyte leaf after 6 months of culture of *C. dealbata*; B – development of gametophyte on the surface of *C. dealbata* leaf; C – overgrowth and edge proliferation of *C. dealbata* gametophyte regenerated on sporophyte leaf

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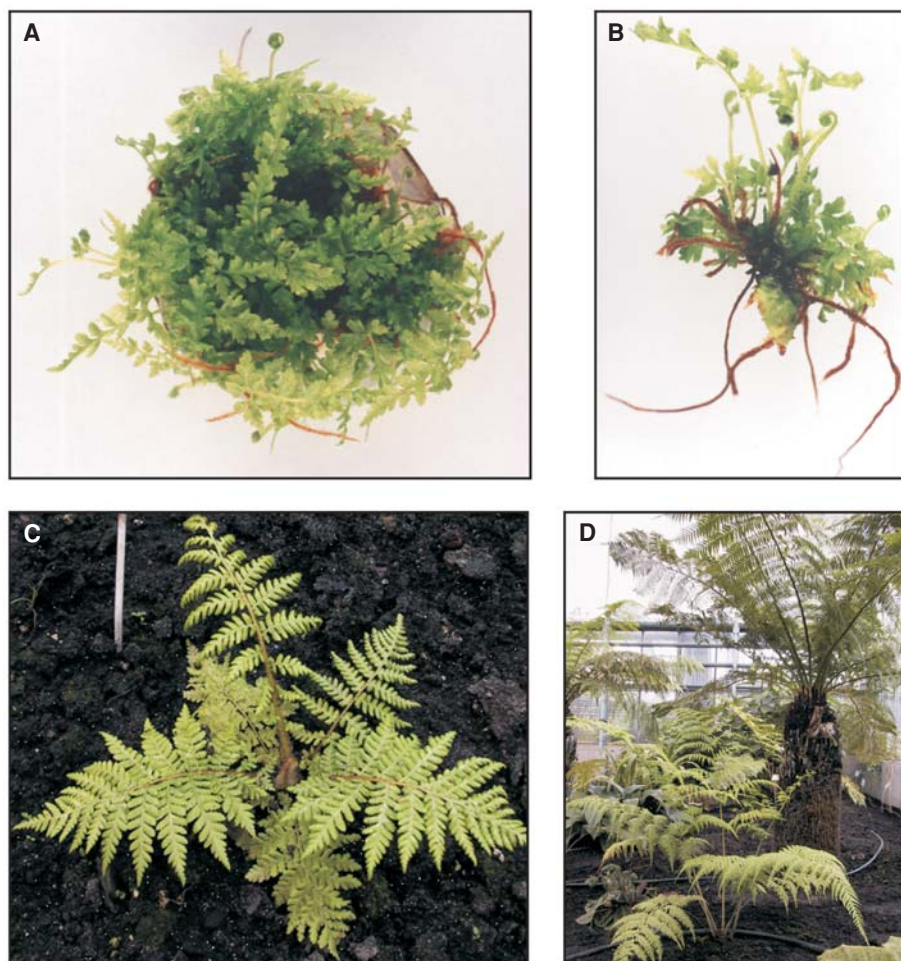


Fig. 6. Stages of sporophyte development. A – the effect of spontaneous fertilization and production numerous very young leaf in *Cibotium schiedei*; B – single young sporophyte of *Cibotium schiedei* with leaves; C – young *C. australis* in greenhouse collection in soil; D – old and young tree ferns in our tropical greenhouse

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