Chapter 10
Tree Ferns Biotechnology: From Spores to Sporophytes

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10.1 Introduction

Tree ferns are typical for rain forest of tropical and subtropical climate and play a very crucial role in the ecology of lands of origin and some of the species are economically very important due to various utilization by autochthons or citizens.

Majority of tree ferns are illegally collected from natural sites, which has resulted in the decrease of their population, though they are listed on both the National and International Red Books and are protected by CITES (Convention on International Trade in Endangered Species).

Very reach references concerning fern biology bring various definitions of alternation between two independent generations: haploid gametophyte and diploid sporophyte. These two generations have the same genome but are fundamentally different organisms, however, both are photosynthetically independent and are very strong autotrophs for the majority of their life.

Gametophyte life starts when spores uptake water, their coat breaks and unequal first cell division takes place, and prothallial and rhizoidal initials are formed. This generation lacks the typical vascular character with limited number of meristematically active cells (1–4) in notch, which are responsible for its somatic growth, produce tiny size with fast growth and with limited life span. The major function of this generation is to generate and to comfort next generation, it means, sporophyte. Due to these facts, the formation of the sexual organs, both archegonia and antheridia, occurs on the bottom surface of the body. Such location of sexual organs helps to carry on the "water path" for sperms to the egg cells to make fertilization. In nature, gametophyte biological function is limited and completed when the first leaf of sporophyte starts photosynthetic activity. Until now, there is only limited information concerning perennial growth of gametophyte through vegetative proliferation, which increased its life span and formation of both sex organs, when met with favorable conditions for life (Khare et al. 2005).

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H. Fernández et al. (eds.), *Working with Ferns: Issues and Applications*,
DOI 10.1007/978-1-4419-7162-3_10, © Springer Science+Business Media, LLC 2011

Generally, two methods of fern propagation have been developed: sexually and asexually by offshoots. The first one consists in sterile spores germination and consecutive culture of two next generations. Today, manipulation of plant cell is called plant cell biotechnology, however, the application of in vitro culture for fern spore germination, proliferating gametophyte culture, and sporophyte production have been carried out from the first part of twentieth century (Freer 1926). The first paper concerning biotechnology involvement in the scientific studies was published only 60 years later. There are number of papers describing gametophyte and sporophyte culture of various non-tree ferns having different aim of studies. The list includes: *Microgramma vacciniifolia* (Hirsch 1975), *Anemia phyllitidis* (L.) (Douglas and Sheffield 1990; Scheffield et al. 1997, 2001), *Pteridium aquilinum* (L.) (Douglas and Sheffield 1990; Scheffield et al. 1997, 2001), *Athyrium filix–femina* (Sheffield et al. 2001), *Dryopteris expansa* (Sheffield et al. 2001), *Platycerium coronarium* (Koenig) (Kwa et al.1995), *Asplenium trichomanes* (L), *Asplenium scolopendrium* (L.) (Pangua et al. 1994), *Schizaea pusilla* (Kiss and Swatzell 1996), *Microsorium punctatum* (Srivastava et al. 2008), *Dregaria fortunei* (Kunze) (Chang et al. 2007), *Osmunda regalis* (Fernández et al.1997), *Matteucia struthiopteris* (Zenkteler 2006), and numerous endangered serpentine fern species in Poland (Marszał and Kromer 2000; Kromer et al. 2006).

The aim of present chapter is to summarize only published results dealing with the application of biotechnology methods, which are restricted only to tree ferns originated from various climatic conditions, different continents and countries. According to our best knowledge, the number of studied species is limited to ca. 20. We have to stress that our interest on this group of ferns is connected with the founding of tree fern indoor collection to show botanical garden visitors the natural variation in the frame of the fern world. Tree ferns possessing trunks present a completely different appearance from Polish native ferns characterized by bushy type of growth. Unsuccessful ex vitro spore germination in greenhouse conditions of *Cyathea australis*, pioneer species in our studies, helped us to take the decision to employ biotechnological facilities for formation of numerous species gametophyte collection in jars and to have adults in greenhouse collection. Finally, 12 of 16 species reached plant stage, which after acclimatization to greenhouse condition have been performing very well until today (Goller and Rybczyński 1995). The progress of in vitro gametophyte culture resulted in the development of cryostorage strategy for tree ferns (Mikuła and Rybczyński 2006; Mikuła et al. 2009) (for details see Chap. 13).

In 1987, two papers from different laboratories concerning in vitro multiplication of tree ferns: *C. dregei* (Finnie and van Standen 1987) and *C. gigentia* (Padhya 1987) were published. In case of *C. gigentia* it is the only paper describing tree fern multiplication with help of in vitro method, which was based on the using of living explants: leaflet primordia and apical domes from garden grown plants. At the presence of modified Knudson medium supplemented with Kinetin, naphthalene acetic acid (NAA) and sucrose, each leaflet primordium were grown and finally developed into complete rooted frond. The culture of apical meristems required higher sucrose concentration and twice higher NAA concentration. All regenerated, later excised and cultured buds, developed in plantlets in the presence of the same medium (Padhya 1987). The experiments carried on *C. dregei* inscribe in a schema

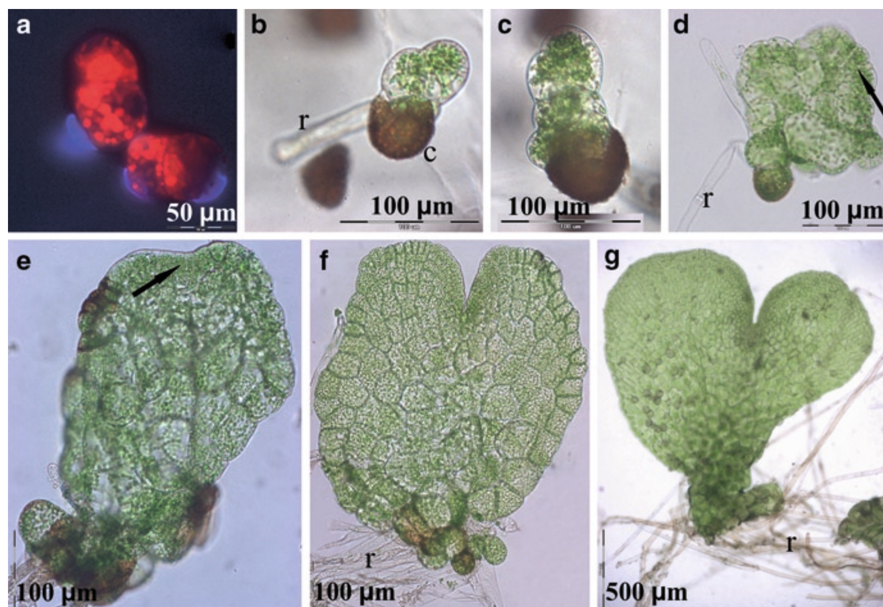

Fig. 10.1 *Cyathea australis* spore germination and prothallium formation on ½MS medium supplemented with 2% sucrose: (**a**) 9-day-old culture of two spores after the first division indicated by red autofluorescence of chlorophyll and blue autofluorescence of spore coat induced by blue-violet light (BV filter: 400–440 nm); (**b**) very young prothallium after two divisions about the 12th day after spore sowing (r – rhizoid; c – spore coat); (**c**) prothallium after several cell divisions; (**d–e**) initial stage of meristematic notch formation (*arrow*), (r – rhizoid); (**f**) heart shape formation by gametophyte; (**g**) well developed carrying heart-shaped gametophytes with numerous rhizoids (r)

(Fig. 10.5), which summarizes the consecutive stages of tree fern in vitro culture procedure presented by all cited papers of this chapter. Additionally, Fig. 10.1 visualizes what happens to the spore subjected to our in vitro culture system. Considering both visuals the chapter will analyze particular stages in vitro cultured events initiated by spores sowing in or on liquid or solidified media.

10.2 Media Most Often Used

It is very good to know that simple vascular plants have various usually not so high mineral salts demand than angio- or gymnospermous plants.

Due to different purposes of spore culture and gametophyte production various media with their physical status (solidified or liquid) were chosen. MS medium (Murashige and Skoog 1965) with broad spectrum of mineral salts dilutions of original concentrations is quite often used. Media used is to be solidified by agar and other gelling reagent for example Gellan Gum (Kuriyama et al. 2004). For mass culture manipulation of the encapsulated spores or a few cell old prothalia, liquid culture seem more useful due to easier possibilities to observe developmental changes of propagules (Mikuła and Rybczyński 2009). Some changes of particular salt

concentration modified the medium. Not always vitamin complex supplements medium. Sterile spores were sown in other nutrient media: Parker and Tompson (Khare et al. 2005), Knop (1865), Knudson (1946), Dyer (1979), Anderson (1984), and Klekowski medium (1969). Adjustment of pH of the media does not differ typically from other plant species.

10.3 Plant Growth Hormones

Fern tissue culture generally does not require special plant growth regulator treatments. In studies of plant hormones involvement in the fern sex determination, various forms of gibberellins were used. The antheridial development of protoplasts derived from young gametophyte cultured in the presence of GA_7 was generated (Treyes et al. 2001). Cytokinins especially 6-benzylaminopurine (BA) on morphogenesis in tree ferns have been studied a few times (Goller and Rybczyński 2007; Somer et al. 2010). It is necessary to say that non-sexual "apoconversions" require plant growth hormones application. Inodol-3-acetic acid (IAA) is the auxin which is present at 40 μM induced apogamy in *Platycerium coronarium* gametophytes growing in vitro and the total number of apogamous sporophytes per gametophyte clump was the highest. This very efficient system was reduced when ethylene was allowed to accumulate in the culture vessel (Kwa et al. 1995).

10.4 Origin of Spore

For culture initiation spores were originated from various sources. In majority fronds or their pieces were collected from sporophytes growing in nature or botanical gardens collections including outdoor and indoor for example greenhouse, however, other sources are mentioned, too. International Seeds Exchange Program helps to receive the required plant material. Fronds or their fragments were dried in room temperature or at 30°C during few days to induce dehiscence and later on to deliver spores. In some publications it is said that stored in various conditions spores were taken for experiment initiation. The temperature of spore storage was 4°C, 7°C, but at approximately 10°C the spore germination of *Dicksonia sellowiana* reached 82% (Fiilippini et al. 1999). The desiccation conditions is the way to carry on spore viability on the high level when they are kept in glass jars.

10.5 Spore Sterilization

The pieces of fronds released from sporangia spores are collected in paper bags and sterilized. After 70% alcohol treatment various sterilization agents are employed. Spores were treated with solution of commercial bleach or sodium (calcium)

hypochlorite of different concentrations for various times experimentally specified. Sometimes sterilization solutions are additionally supplemented with Tween 20. Change of sterile distilled water several times helps to ride off sterilization agent and to have spores ready for culture initiation.

10.6 Spore Germination

In majority of the cases, sterile spores were usually distributed on the surface of agar solidified medium. Spore development follows the *Cyatheace* type of development which consists of the first wall laid down parallel to the polar axis and the spore is divided into two unequal cells. The larger one gives prothalial initial and the smaller one rhizoidal initial. The rhizoidal initial is elongated and developed into an elongated, hyaline, unicellular rhizoid. The first cell division and elongation of the prothalial initial is in a plane parallel to the equatorial plane of the spore (Huckaby and Raghavan 1981; Khare et al. 2005). For easier manipulation of the huge sample of spores, the method of alginate encapsulation procedure was developed (Mikuła and Rybczyński 2006). This procedure is very convenient when cryopreservation experiments, and various methods of spore and early stages of prothalium pretreatments are undertaken. The method helps to synchronize the *C. australis* spore germination and early stage of its gametophyte development in huge mass (Mikuła and Rybczyński 2006). To initiate the pteridophytes culture, spores are usually sown in full or half strength of MS medium (Kuryiama et al. 2004). In cultures of *C. spinulosa* after 7 days of culture on Parker and Thompson medium more than 90% of spores germinated (Khare et al. 2005).

There are a lot of evidences that spore germination is controlled by light and type of their storage (Simabukuro et al. 1998a, b). Fern spore germination has been shown to be stimulated by red light, but blue light and far red light have no effect, delay germination, or inhibit it. It was proved that *D. sellowiana* and *C. delgadi* possess spores sensitive for light. Spores of *D. sellowiana* are positively photoblastic and 48 h of darkness and 1 min of red light photoinduction resulted in a greater percentage of spore germination (Randi and Felippe 1988). It was observed that the spores of *D. sellowiana* were able to germinate in a wide range of light intensities from 2% to 43% of full sunlight, but the higher percentages were seen for the intermediary light intensity and the lower germination for the higher and lower light intensities (Fiilippini et al. 1999). The lowest mean germination time of *D. sellowiana* spores was achieved under 5% and 20% of irradiance. Higher level of irradiance (50% and 36%) effected the delay of spore germination by 14 and 21 days respectively in contrast to 20% and 5% of irradiance (Renner and Randi 2004). In the case of *C. delgadii*, spore germination in the presence at 22% light intensity reached 76% and mean germination time 19.7 days, but at 5% light intensity germination achieved level of 83.5 % and mean germination time 20.16 days.

10.7 Gametophyte Growth and Development

Table 10.1 shows the list of tree fern species which, with the help of in vitro culture passed the first stage of development, its mean germination. The lower mean germination time was observed for spores cultivated under 5% and 20% of irradi-

Table 10.1 The list of tree fern species used for biotechnology experiments

No.	Name of species	Sp. ger.	Gam.	Spo.	References
1.	*Blechnum brasiliense* Desv.	+	+	ns	Hiendlmeyer and Randi (2007)
		+	+	+	Goller and Rybczyński (2007)
2.	*Cibotium glaucum* (Sm.) Hook. and ARn.	+	+	–	Goller and Rybczyński (2007)
3.	*Cibotium schiedei* Schltdl. and Cham	+	+	+	Goller and Rybczyński (2007)
4.	*Cyathea australis* (R.Br.) Domin.	+	ns	ns	Huckaby and Raghavan (1981)
		+	+	+	Goller and Rybczyński (1995)
		+	+	+	Goller and Rybczyński (2007)
		[a]+	+	+	Mikuła and Rybczyński (2006)
5.	*Cyathea brownie* Domin.	+	+	+	Goller and Rybczyński (2007)
6.	*Cyathea capensis* (L.f.) Sm.	+	+	+	Goller and Rybczyński (2007)
7.	*Cyathea contaminans* (Hook) Copel.	+	+	ns	Treyes et al. (2001)
8.	*Cyathea cooperi* (F. Muell.) Domin.	+	ns	ns	Huckaby and Raghavan (1981)
		+	+	+	Goller and Rybczyński (2007)
9.	*Cyathea dealbata* (D. Forest) Sw.	+	+	+	Goller and Rybczyński (2007)
10.	*Cyathea delgadii* Sternb.	+	+	ns	Hiendlmeyer and Randi (2007)
		+	ns	ns	Simabukuro et al. (1998b)
11.	*Cyathea dregei* Kunze.	+	+	–	Goller and Rybczyński (2007)
12.	*Cyathea lepifera* E. Copel.	+	+	+	Kuriyama et al. (2004)
13.	*Cyathea leichhardtiana* (F. Muell.) Copel.	+	+	+	Goller and Rybczyński (2007)
14.	*Cyathea robertsiana* (F. Muell) Domin.	+	+	+	Goller and Rybczyński (2007)
15.	*Cyathea schanschin* Mart.	+	+	+	Goller and Rybczyński (2007)
16.	*Cyathea smithii* Hook. f.	+	+	+	Goller and Rybczyński (2007)
17.	*Cyathea spinulosa* Wall. ex Hook.	+	+	+	Khare et al. (2005)
18.	*Dicksonia antarctica* Labill.	+	ns	ns	Huckaby and Raghavan (1981)
		+	+	+	Somer et al. (2009)
19.	*Dicksonia fibrosa* Colenso.	+	+	+	Goller and Rybczyński (2007)
20.	*Dicksonia sellowiana* Hook.	+	+	ns	Fiilippini et al. (1999)
		+	+	ns	Renner and Randi (2004)
		+	+	+	Goller and Rybczyński (2007)

[a]After spores and gametophytes cryopreservation experiments ns – non studied

ance. The physiological studies shown that the chlorophyll content of gametophytes cultured for 49 days under 20% and 5% of irradiance was the highest. This result reflects the highest soluble sugar content (Renner and Randi 2004). Young gametophytes of *D. sellowiana* grown under red and white light showed various responses expressed in their morphological pattern of shape. Young gametophytes growing under white light were bidimensional and plain while the ones treated by red light were filamentous (Fiilippini et al. 1999). Light plays an important role in the development of the ferns and 42% and 62% of light was the factor causing the death of young gametophyte of *Dicksonia delgadii*, after a few days of cultivation (Heidlemeyer and Randi 2007).

10.8 Gametophyte Multiplication

Since the in vitro culture of pteridophytes was developed and various propagules were used for multiplication, at least three methods of gametophyte multiplication were developed. Two of them exploring natural morphogenic potential of particular or complex of green vegetative cells of gametophyte (Fig. 10.2), and the third "mechanical" one disrupting biological value of gametophyte as the organ, to benefit non-defined its pieces. Among two ways of gametophyte multiplication, the first one consists in particular gametophyte cell proliferation, the second one goes via green centers formation which usually develop in the gemmae. Individual gametophyte excised from multigametophyte structure of *C. dealbata*, *C. leichhardtiana*, and *C. schanschin* maintained on half strength MS medium without sucrose, richly regenerated this structure (Goller and Rybczyński 2007). The third method was developed for tree fern *C. dregei* by Finnie and van Staden (1987). The method presented the most drastic procedure using blender to have homogenized very small pieces of gametophyte body to initiate new culture handle in liquid (better) or solidified medium. It was proved that homogenate cultures of *D. antarctica* gametophyte regenerated next gametophytes actively in all pieces, and the generation was able to form sporophytes (Somer et al. 2010).

Protoplasts as the smallest explants derived from antheridial primordial cells of *C. contaminans* young prothallia developed in the antheridia containing spermatocytes during approximately 2 weeks time in the presence of GA_7 (Treyes et al. 2001).

If the growth of biological material is expressed by fresh and dry weight, the gametophyte of *D. sellowiana* changed both parameters depending on the presence of sucrose concentration in Dyer and MS media. Dry mass was higher in 30-day-old gametophytes cultured in Dyer medium with the addition of 3–5% of sucrose and MS medium supplemented with 2% of sucrose, which was similar to Dyer medium with 4% of sucrose (Renner and Randi 2004).

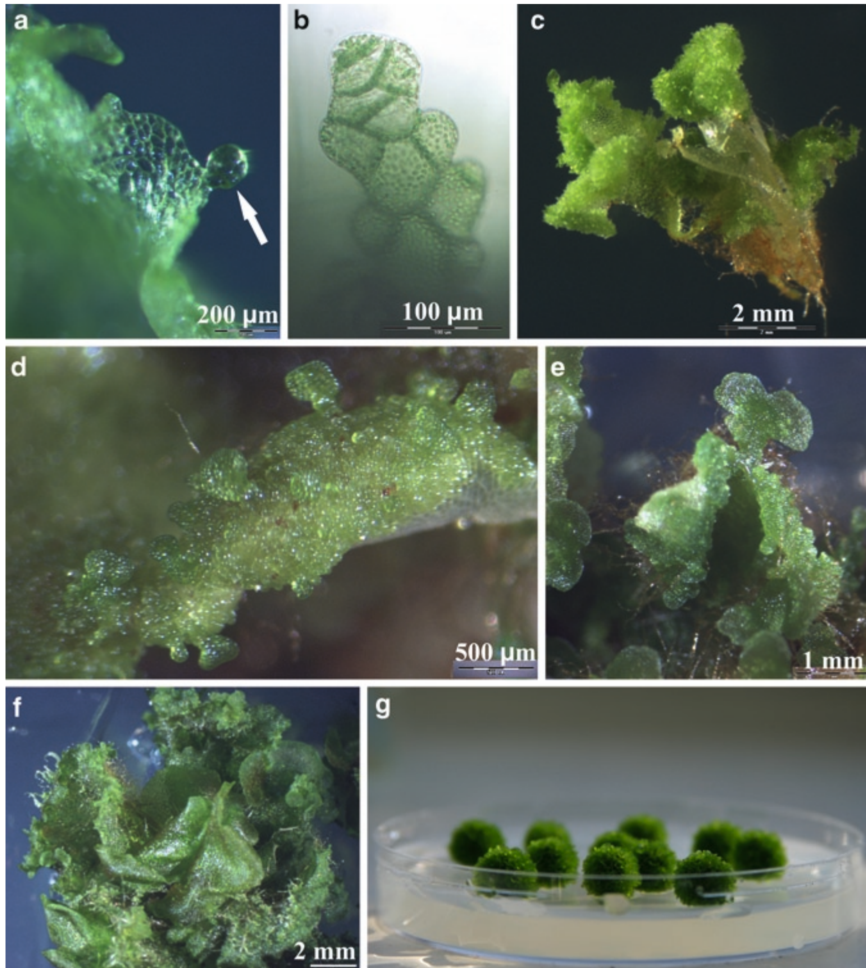

Fig. 10.2 Secondary gametophyte production on primary explants of various tree fern species: (**a**) the first stage of secondary prothallium proliferation on the *Cibotium glaucum* gametophyte (*arrow*); (**b**) specific cell divisions leading to young gametophyte formation; (**c**) multiplication of young gametophytes on the edge part of *Dicksonia fibrosa* gametophyte and (**d**) *Cyathea smithii* gametophyte; (**e**) secondary, heart-shape gametophyte of *Cyathea smithii*; (**f**) intensive proliferation of new gametophytes of *Cyathea australis*; (**g**) multigametophyte clumps in *Cyathea australis* culture

10.9 Sexual Determination of Gametophyte

Gametophytes of *D. antarctica* originated from homogenized gametophytes cultured on MS medium initially presented male sexual phenotype with well developed antheridia (Somer et al. 2010).

10.10 Sporophyte Production

Favorable growth conditions of gametophyte resulted in formation of generative organs (Fig. 10.3) via various pathways. As it was said, water helps male gamete to reach the archegonium for egg cell fertilization to produce polar embryo and sporophyte (Fig. 10.4). The alternative way shows the sporophyte formation without gametes involvement, called apogamy. Typical in vitro multiplication consists on the using of sporophyte explant. Very young leaves originated from axenic culture of *Platycerium bifurcatum* were used to develop the leaf cell suspension as the tool for sporophyte multiplication in the presence of activated charcoal, NAA, and BA (Teng 1997). For the propagation of sporophytic plants of *C. lepifera*, shoot tip with a few very young leaves was divided into two or three pieces and subcultured on fresh medium. Segments of rhizome and leaf were excised and cultured on MS medium with sucrose. The explants produced no adventitious shoots but only aposporus gametophytes (Kuriyama et al. 2004).

The stage of fern development is the objective of plant growth regulators studies, with attention paid for abscisic acid and gibberellins. It is worth to mention that

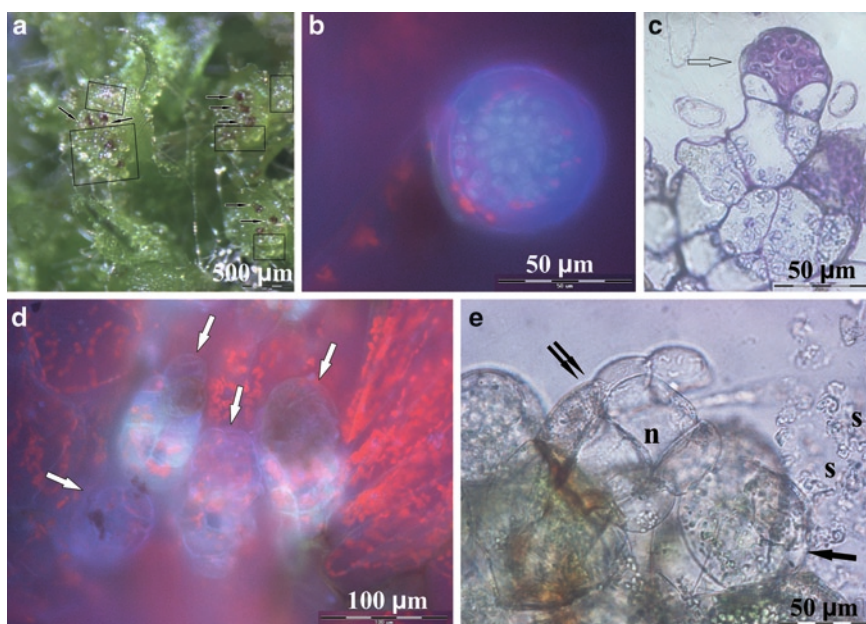

Fig. 10.3 Sexual organs of tree fern gametophytes cultured in vitro: (**a**) a few archegonia (*arrows*) and numerous antheridia (*in frames*) on the bottom side of gametophytes; (**b**) mature antheridium with male gametes in blue-violet light; (**c**) long section of mature antheridium (*arrow*) dyed with 0.1% toluidine blue; (**d**) blue autofluorescence of four mature archegonia (*arrows*) cell walls and red autofluorescence of chlorophyll in the background of the picture, induced by blue-violet light; (**e**) light micrograph of mature sexual organs of *Cyathea delgadii*; sperm cells (s) released from antheridium (*arrow*) and open archegonium (*double arrow*) with neck canal (n) ready for fertilization

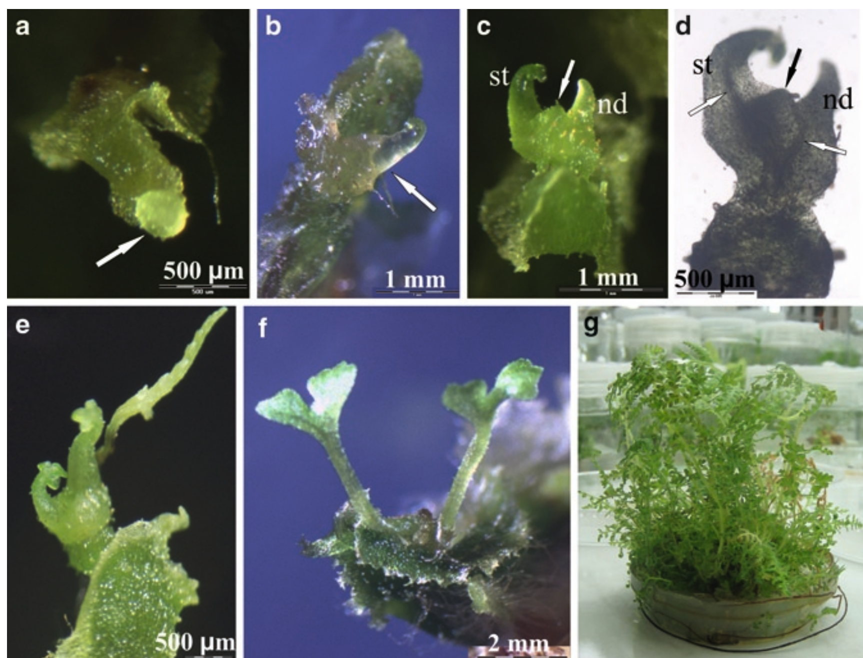

Fig. 10.4 Tree fern sporophytogenesis in vitro: (**a**) archegonium after fertilization with early embryo inside (*arrow*); (**b**) the first leaf primordium (*arrow*) of *Cyathea delgadii* embryo; (**c**) two leaf stage embryo of *Cyathea delgadii* (st – first and nd – second leaf primordiium; *arrow* show the apical meristem); (**d**) long section, non-stained specimen of two leaf stage embryo with bundle of vessel (*white arrows*), (st – first and nd – second leaf primordium, *black arrow* shows the apical meristem); (**e**) three leaf young sporophyte of *Cibotium schiedei*; (**f**) two young sporophytes of *Cyathea delgadii*; (**g**) numerous sporophytes in a few month old *Cyathea australis* in vitro culture

sporophytes originated from ex vitro growing of three tree fern species namely: *C. australis*, *Cibotium glaucum,* and *D. antarctica* were analyzed to identify and compare endogenous gibberellins with various position of hydroxylation (Yamane et al. 1985; Yamane et al.1988).

Sporophyte ex vitro adaptation requires thought to selection of proper stage of development express by the number of fronds. High surface of leaf, non-properly develop respiration system, and weak root system are the other significant reasons which should be take on consideration. It is necessary to pay attention for outside conditions like substrate for planting, light intensity, photoperiod, and air humidity, which limit plant adaptation (Goller and Rybczyński 1995).

10.11 Conclusion

Presented above is an overview of already published papers indicates possibilities to ascertain the procedure for getting the tree fern sporophyte starting with freshly collected or stored spore (Fig. 10.5). Even though the tree fern species are a significant

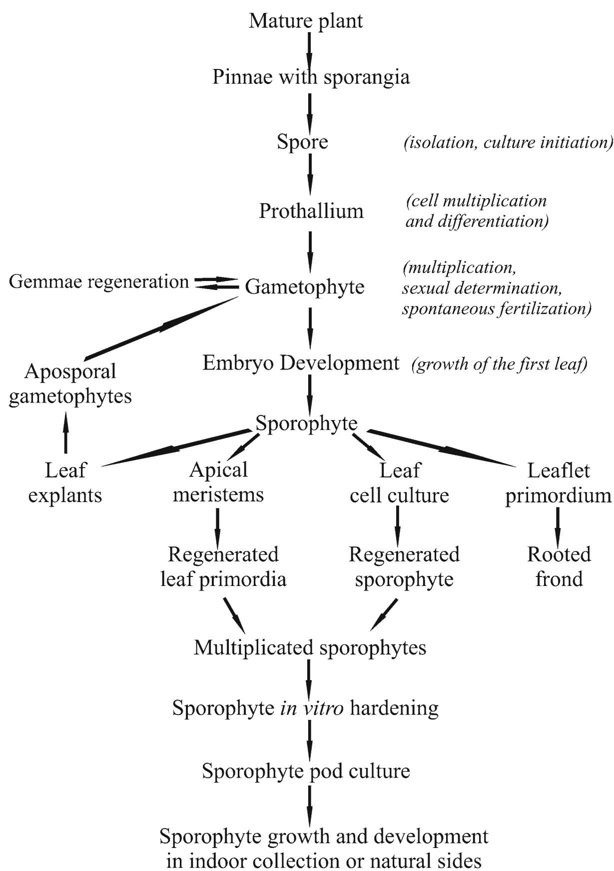

Fig. 10.5 Diagram summarizes the alternation of generation and developmental modification due to in vitro culture methods application in tree fern life cycle

component of tropical forest and play an important role in its protection and have very significant economical value, the present knowledge, subject of papers and number of already released publications, in comparison to other group of ferns is limited, if not very poor.

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