Helena Fernández · Ashwani Kumar
Maria Angeles Revilla *Editors*

Working with Ferns

Issues and Applications

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Preface

In the 1970s, Rolla and Alice Tryon of Harvard organized an annual fern conference at Harvard Forest every spring. The mission of the conference was to bring together researchers from across a broad spectrum of biology whose common link was that they either studied ferns per se or utilized ferns in their experiments. Talks ranged from classical pteridological subjects such as taxonomy, paleobotany and morphology, to experimental areas such as ecology, physiology, development, genetics, and biochemistry. For the participants, of whom I was lucky to be one, it was an intellectually exhilarating experience. We all learned new things, and the cross fertilization of different subjects and research approaches led to new ideas and a better understanding of ferns as organisms. The present volume, *Working with Ferns: Issues and Applications*, edited by Helena Fernández, Ashwani Kumar and Maria Angeles Revilla, carries the Harvard Forest Fern Conference philosophy into the twenty-first century.

The editors have assembled a truly remarkable array of contributions dealing with fern biology. Starting with researches utilizing *Ceratopteris*, a fern whose speedy life cycle I discovered during my doctoral research at the Royal Botanic Gardens, Kew more than 40 years ago, and ending with a study of *Pteridium aquilinum*, a cosmopolitan species taxonomically revised by Professor Rolla Tryon, the papers in *Working with Ferns: Issues and Applications* offer a look at what is new in pteridology. In this very catholic compendium, contributions include studies of development, propagation, conservation, environmental biotechnology, and medicinal applications. *Working with Ferns: Issues and Applications* is nominally about ferns, but it is more than that. For students and researchers these papers are seminal as they review an international scientific literature for areas of botany outside the normal purview of mainstream "angiosperm-oriented" plant biologists.

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Ed Klekowski

Preface

The great success of the giant Ferns on earth is already passed, they did not have the smash hit of the movie industry as happened with the giant reptiles, the Dinosaurs, of which we know only the fossils left behind, but ferns apart from the marvelous fossils found in Carboniferous rocks they are still with us, not covering the whole Earth but adapted to live and survive in particular ecological niches.

The study of ferns has not attracted as many researchers as those devoted to the rest of the plant kingdom; however, an upsurge of interest has been evident lately as ferns can become useful for man as beautiful ornamentals, or producers of secondary metabolites with interesting pharmacological properties, or can be even used as food. But the interest in the study of ferns can be considered under a more basic point of view, there are processes not very well known in the life of ferns as propagation or sex differentiation, all of which is reflected in the increasing number of published papers devoted to their study and that is what this book is about.

Each chapter reflects the individual approach and personal style of the authors. Nevertheless, each serves to introduce a particular field of study to a wide audience and to indicate the most active line of current research. The barriers to immediate progress be they the needs for better experimental systems or for advance in culture technique emerge very clearly.

The arrival of new and innovative techniques applied to the study of ferns has permitted a more widespread use of sophisticated instrumentation opening up new horizons in the cellular and molecular biology approach to the study of ferns as a result. But there are no less important developments, however, in other areas of study, particularly in *in vitro* culture or cryopreservation and maintenance of germplasm on the laboratory and global scale that are also essential ingredients to the new realm of fern biotechnology

Such a wide range of authoritative review papers each presenting new and often controversial ideas should prove valuable material for seminar discussions with university students in all branches of plant science. At the very last, we feel that it should provide a source of background information and references to both students and researches alike who wish to initiate or broaden their knowledge of ferns.

I hope that the outcome of this volume will be an increased awareness of the fact that there is still a wide field of study of ferns, these very old organisms still remaining with us.

Emeritus Professor of Plant Physiology Ricardo Sánchez-Tamés
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About the Book

Ferns existing today represent a genetic inheritance of great value as they include species of ancient vascular plants, which have direct connection with the crucial steps done in the past for settling life on Earth. Their life cycle is an example of alternation of generations, in which both a multicellular diploid organism and a multicellular haploid organism occur and give rise to the other. Spore, gametophyte, and sporophyte have been studied both for basic and practical purposes. In the first case, they offer us simple and easy to grow experimental systems to investigate on plant development, and secondly, they are cultured by their ornamental appeal, their environmental benefit or as source of metabolites.

This timely volume brings a selection of chapters, each one composed by experts in their respective fields. The chapters included cover a broad range from the knowledge of its biology and contribution to understanding of plant development, useful protocols for propagation and conservation purposes, genetic variability as well as environmental and theurapeutical applications. This wide spectrum of the contributions gives the reader a rapid idea of the enormous potential of this plant group.

The originality of this book is to expose the most recent tendencies in their investigation, which is far from the traditional perspective usually followed. The collected articles in this volume incorporate most of novel techniques used nowadays routinely to resolve traditional questions.

Helena Fernández Associate Professor, Department of Biology of Organisms and Systems, Oviedo University, Spain. Her research focuses on micropropagation and reproduction in ferns during the last 2 decades. In 2002, she obtained the award "Ramón y Cajal" from The Ministry of Science and Technology and is engaged to the Oviedo University as tenurer, full time, in the area of Plant Physiology since then.

Ashwani Kumar Professor, Department of Botany, University of Rajasthan, Jaipur. The author's repertoire of published works spreads across 150 research articles in various national and international journals. He has an experience of over 3 decades in his field of research, namely, tissue culture and biochemistry, and was awarded with the prestigious V. Puri Medal in 2008 for his services to the advancement of Botany.

M. Angeles Revilla Plant Physiology Assistant Professor in the Biology Faculty at the Oviedo University (Spain) since 1987. She has 20 years experience in plant tissue culture. She has also worked in cryopreservation and genetic stability for the last 10 years, mainly in the development of protocols for in vitro shoot apices in agronomic species.

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Chapter 1
Introduction

H. Fernández

Some time ago, I read the following sentence in a book on ferns: "robot's eyes cannot see ferns" (Salvo 1990). It made me think that perhaps I was a strange woman as ferns had consumed a great part of my time and energies, and people are not used to paying attention to these type of plants. Certainly, in the plant research field, other species are considered as better candidates for one reason or another to justify the time we dedicate to them. Through this book, a not too small group of researchers, (I do not know if they are as weird as I am), challenge the readers to find out the enormous potential that ferns have, demanding to be explored and considered useful tools in plant research.

Undoubtedly, we need to take into account these plants to get a more complete view about what land plant evolution has meant. Ferns existing today represent a genetic inheritance of great value as they include species of ancient vascular plants, which have direct connection with crucial steps taken in the past for settling life on Earth. Ferns have been with us for more than 300 million years, and in that time, the diversification of their form has been phenomenal. The ferns were at the pinnacle during the Carboniferous Period (the age of ferns) as they formed the dominant part of the vegetation at that time. Most of the ferns of the Carboniferous age became extinct, but some of them later on evolved into the modern ferns. Ferns grow in many different habitats around the world, in all continents except Antarctica and most islands, favouring moist temperate and tropical regions.

The life cycle of the ferns has worked quite successfully for millions of years. Spores from the parent fall to the soil and with an enormous amount of luck (millions perish for every success) they will find suitable moisture and light. The minuscule single-celled organism starts to grow by cell division, giving place to a little gametophyte or prothallia, whose morphological appearance passes through different stages: filamentous, spatulate and heart shaped. Although mostly are heart shaped, tuberous, strap-like, and ribbon-like gametophytes are also found in some specific families. This is an independent plant with its own simple "root" system (rhizoids) to provide it with nutrients and water. The prothallium then grows antheridia or male organs and archegonia or female organs on its underside. The antheridium produces antheridia, which will swim via a droplet of water to the egg

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produced by the archegonium. The fertilized egg then begins to grow the sporophyte, the plant that we know as fern.

It is not the aim of this book to open a discussion about the best choice for giving a classification of seed-free vascular plants. We limit our role here to present a recently published classification by Smith et al. (2006) for extant ferns, which is based on both morphological and molecular data. In this report, a basal dichotomy within vascular plants separates the lycophytes (less than 1% of extant vascular plants) from the euphyllophytes, which comprises two major clades: the spermatophytes (seed plants), which are more than 260,000 species; and the monilophytes with about 9,000 species, including horsetails, whisk ferns and all eusporangiate and leptosporangiate ferns. Along this book, traditional and modern phylogenetic allusions are made indistinctly.

Spore, gametophyte and sporophyte have been cultured either for basic or practical purposes. First, they offer us simple and easy to grow experimental systems to investigate plant development, and second, they are cultured for their ornamental appeal, their environmental benefit or as sources of metabolites.

In our opinion, most of the published books until recently provide information about the existing biodiversity in ferns, referring to aspects such as their morphology, systematics, ecology, evolution, phylogenetics, with no explicit references to the latest advances in the research done in ferns. The originality of this book is to expose the most recent tendencies in their investigation, which is far from the traditional perspectives usually followed.

The content of this book has been divided into four major sections: (a) the contribution of ferns to the understanding of plant development, (b) propagation, conservation and control of genetic variability in ferns, (c) environmental biotechnology: ecotoxicology and bioremediation in ferns, and (d) therapeutical/medicinal applications.

The *first* section shows how ferns may be used as experimental models to gain insight on plant development, and also how these simple and easy to grow plant systems are being taken into account more and more to delve into the biological processes governing plant growth. *Ceratopteris richardii* is an aquatic fern that represents a useful experimental system for studies on plant development. Recent research documents the cellular, molecular and gene expression changes that occur in gametophytes during polarity development, and also in response to the environmental stimuli such as light and gravity. Molecular approaches are used to characterize proteins that are critically involved in mediating the coupling of light and gravity stimuli to morphogenic changes in plants. Different approaches to achieve genetic transformation in this fern is also considered.

The morphological simplicity and the lack of connections with the mother plant of gametophyte allow us to study the mechanisms involved in sexual reproduction. In *C. richardii* and *Blechnum spicant* sex determination is epigenetically controlled by the pheromone antheridiogen, which is secreted by hermaphrodites or females gametophytes, respectively, and directs male development of young, sexually undetermined gametophytes. The chemical nature of these compounds has been related to gibberellins, and details for its purification and isolation are given. In addition, the role played by the growth regulators cytokinins and gibberellins, on sexual

development is commented from both their exogenous effect on gametophytes cultured *in vitro*, and also by measuring their endogenous levels in male and female gametophtyes. Recently, new tools such as proteomics techniques to explore sex determination are being applied.

One of the processes involved in sexual development is antheridiogenesis, the formation of male sex organs or antheridia. In the fern *Anemia phyllitidis,* antheridiogenesis is a complex process and can be prematurely induced by gibberellic acid (GA_3), which imitates the action of antheridic acid, the main antheridiogen of that fern. This gibberellin induces many specific cytomorphological and metabolical features in young gametophytes of this species, during "induction" and "expression" phases of male sex determination, which allowed to describe the useful "three-zonal" model. Influence of GA_3 on dispersion of nuclear chromatin, induction of cell division cycle, sugar metabolism and cell wall structural reorganization leads to transverse cell growth and asymmetrical division of antheridial mother cells and to antheridia formation. These processes are modulated by ethylene, playing the role of secondary messenger.

One interesting question, which remains yet unresolved, is the understanding of the ontogeny of the alternation, i.e., the comprehension of how the same genetic constitution can produce plants of such morphological dissimilarities. In addition to the normal life cycle using meiosis to generate spores and sexual union to form zygotes, in nature, some fern species can switch from one generation to another by different pathways termed apospory and apogamy. In the first case, a gametophyte is generated from sporophytic cells without meiosis, and in the second one, a sporophyte is generated from gametophytic cells without fertilization. In the laboratory, it is possible to induce both pathways using specific culture conditions. The independence of the two generations in ferns and the ease of switching from one generation to the other through these pathways offer a system suitable for studying how each generation is initiated. This developmental plasticity of crossing generation barriers, i.e., meiosis and fertilization, is not unique to ferns and is manifested in the complex pathways leading to apomixis in some seed plants. The asexual pathways in ferns are compared and contrasted with the current understanding of apomixis in seed plants.

The sporophytes of the main living groups of seed-free plants exhibit significantly divergent morphologies, both between the different groups, and between those and the seed plants. In terms of vegetative features, such differences are seen in embryo structure and development, body plan, stele architecture, branching, leaf development and phyllotaxis, and rooting structures. A comparative survey of major vegetative features of sporophytes is presented, emphasizing the differences between the various living seed-free lineages and between those and seed plants, reviewing the state-of-the art knowledge of molecular genetic pathways that control the development of seed-free plant sporophytes. Results published to date point, in some cases, to highly conserved pathways, such as the one shared between the control of rhizoid development in bryophytes, and that of root hairs in flowering plants; this broad taxonomic range brackets phylogenetically all seed-free plant lineages which are hence hypothesized to share the same pathway. In other cases, such as leaf development, different lineages reveal complex mosaics of shared and divergent pathways. However, as molecular genetic studies of seed-free plants are

still in their infancy compared to those of seed plants, and especially angiosperms, most aspects of their vegetative sporophyte development have yet to be characterized from a molecular standpoint.

The *second* section provides useful protocols for propagation and conservation of those species interesting for one reason or another such as the necessity of conservation of threatened species, large-scale propagation of species having ornamental qualities, therapeutical properties, etc. *In vitro* culture techniques represent an excellent tool for reaching successful benefits of their applications. The first chapter of this section summarises the *in vitro* culture of ferns from spore to sporophyte, giving details of how to proceed for achieving optimal performance of each phase involved in the life cycle, defining culture requirements leading to a greater production of these plants. Besides, four examples of fern micropropagation are presented. The first one refers to the influence of initial explants, culture media, plant growth regulators, wounding, etc., on morphogenesis in the regeneration systems of *Platycerium.* The second one refers to the propagation of Marattioid species from stipules, which might be an easy and important way for ex situ conservation of endangered Marattioid species. The third proposal deals with the application of biotechnology methods to tree ferns originated from various climatic conditions, countries and continents. Finally, one chapter is dedicated to the application of *in vitro* culture techniques to micropropagate endangered species.

Ferns are a diverse and important group of plants, but diversity of species and populations are at risk from increasing social pressures, loss of habitat and climate change. Ex situ conservation is a useful strategy to limit decline in genetic diversity and requires technologies to preserve fern germplasm. This book includes an overview of the methods and techniques employed or under study for germplasm conservation currently. Fern spore storage and viability have received little research attention but, by analogy to seeds, may benefit from the extensive knowledge of seed storage gained during the last 50 years. Fern species produce either non green or green spores, which have been considered to exhibit storage physiologies similar to orthodox and recalcitrant seeds, respectively. On the other hand, cryopreservation of gametophytic tissue is fairly established for mosses and liverworts, but is rare for ferns. Methods for cryostorage of gametophytes from several species of tree ferns originating from either the tropics or areas of mild winters are given and these species' amenability to cryopreservation are also compared with herbaceous species that originate in areas where winters are harsher.

This section includes also traditional and modern techniques used to determine genetic variability in order to establish adequate protocols for the propagation and conservation of ferns. Technical innovations, such as enzyme gel electrophoresis, has lead to a significantly deeper and newer knowledge of fern population biology and genetics. There exist a plethora of publications dealing with important results of enzyme electrophoretic methods published between the 1960s and the end of the millennium. In the last decade, newly developed research fields of DNA analysis have been introduced and have, to a wide extent, replaced the enzyme electrophoresis. They have also brought new insights into population biology and genetics of ferns. In this regard, a comprehensive review of the advantages and disadvantages

of the selection of dominant markers as genetic tools when compared to other molecular techniques available is considered. Dominant markers fulfil most of the ideal characteristics of a fingerprinting technique as they are usually technically simple procedures, inexpensive and allow fast data acquisition. In addition, dominant markers are based on one of the fundamental characteristic of the DNA, the probability of sequence repetition due to the existence of only four nucleotides, so these techniques are easily transferable from one organism to another as no prior genetic knowledge of each species is needed. However, the increasing availability of sequenced data due to the relative price decrease and apparition of new sequencing techniques together with their drawbacks have forced us to ask: are dominant markers still useful? The authors of the chapter do not try here to give a definitive answer to this question. They just want to point out that there is no perfect fingerprinting technique. Its choice is often a compromise that depends on a number of material and species related factors. The existence of previous genetic data of the species, the knowledge of close relative, the complexity of the genome, are other factors that dramatically influence our selection. The resources of the laboratory, financial constraints, available expertise, time limitations, and more importantly, the research pursued usually define our opinion about dominant markers. The purpose in the present chapter is to provide a detailed review of strong points and drawbacks as well the areas where the application of dominant markers has succeeded in answering questions in the genetically complex pteridophytes.

After peering the above mentioned methods to determine genetic variability, the low sensitivity attributed to the allozyme electrophoresis analyses and the inability to reveal heterozygotes by the dominant nuclear genetic markers, constitute important obstacles for detailed studies on several life traits of ferns. Microsatellites, short tandem repeats of nucleotides present in most eukaryote genomes, can fill those gaps, thanks to their hypervariability and codominant inheritance. This PCR-based technology has been widely used to study vertebrates and angiosperms with great success, but only a few works applying it to ferns have been published in the last decade. These studies allow us a glimpse of the great promise that microsatellites hold to address multiple biological issues unable to be resolved by less sensitive markers, such as fine-scale genetic diversity and recent evolutionary history. Beyond the confusing factor that ancient polyploidy and genome duplication may represent for result's interpretation, the most notable drawback of microsatellites is that they are still expensive to develop, although costs are becoming progressively cheaper as more laboratories and companies provide this service. The peculiarities of fern life and evolutionary histories make microsatellites even more interesting markers, as they can be used to track mutations through haploid and diploid generations and to accurately disentangle the intricate interspecific relationships within polyploid complexes. Microsatellites are, therefore, shaping up as a powerful genetic marker for fern research which is well worth the investment.

The *third* section in this book is dedicated to ecotoxicology and bioremediation. One of the main concerns of current environmental toxicology is the low number of taxons used for standard bioassays. Ferns are the second largest group of vascular plants and are important components of numerous plant communities.

Fern spores and gametophytes have long been recognized as useful models for plant research since they constitute a naturally miniaturized and economic higher plant model. Mitochondria are the main energy source in eukaryotic cells and any toxic damage will affect the whole organism. The reduction of tetrazolium salts to water-insoluble coloured formazan salts by the NADH reductase complex (E.C.1.6.5.3) has been used for more than 50 years as a measure of cell mitochondrial activity and viability in eukaryotic organisms. Here, the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) by mitochondria is adapted and optimized to measure fern spore or gametophyte viability. This method constitutes a promising low cost bioassay for higher plant toxicity during development.

The measure of DNA in developing gametophytes is a biomarker of the disturbance that the toxicant provokes in gametophyte growth and development. The use of the fluorescent probe Hoechst for DNA quantification is a rapid, sensitive and reliable method frequently used in cell biology which has been successfully applied to spores and gametophytes. The natural autofluorescence of chlorophyll can also be used as a biomarker of the physiological state of the gametophyte. Both biomarkers can easily be measured in high number of samples using 96 multiwell plates and plate readers. The combined use of these biomarkers in chronic toxicity tests using developing gametophytes of the riparian *Polystichum setiferum* is yielding very satisfactory results and is a promising new model for ecotoxicology.

Concerning the role of ferns in phytoremediation, the contribution of *Pteris vittata*, an arsenic hyperaccumulator, is presented. Arsenic is a toxic metalloid that is widespread in the environment due to both man-made and natural causes. Soils, food and ground water contaminated with arsenic pose serious health risks to millions of people in different parts of the world. While engineering methods to remediate arsenic-contaminated environments are available, they are often prohibitively expensive and cumbersome. It was discovered about a decade ago that the Chinese brake fern (*P. vittata*) had an extraordinary ability to tolerate and hyperaccumulate arsenic, up to about 2% of dry weight in its fronds. This opened up new opportunities to develop the brake fern for a cost-effective green technology to remediate arsenic-contaminated environments. The author of this chapter highlights some of the salient findings on this and related ferns regarding their arsenic tolerance and hyperaccumulation traits. Investigations have shown that arsenic hyperaccumulation in brake fern has evolved as a defence against herbivory. Research employing molecular biology tools have identified some of the key genes and proteins important for arsenic metabolism in this species including genes for arsenic-induced oxidative stress. Comparative biochemistry of how organisms adapt to arsenic suggests that many other fern genes related to arsenic transport and metabolism are yet to be characterized in this fern. The brake fern could be a source of genes that could inform us about how plants adapt to abiotic stress factors such as high temperature, stress and drought that have oxidative stress as a component. Some of these genes can be expected to be valuable for improving crops for increased tolerance to stress.

The last chapter of this section represents a novelty in relation to the aerobiology of pteridophyta spores. Pteridophyte airborne spores are scarcely represented

worldwide compared to fungal spores or even to pollen grains. However, the levels of fern spores in the atmosphere are connected to the distribution and abundance of different pteridophyta species, with tropical and subtropical zones of Asia, America and Africa being the areas where fern spores are more abundant. Their seasonal distribution includes all the months in tropical zones, according to the continuous sporing process that usually occur because of the sequential development of sporangia in the different fern species. In temperate areas, the presence of airborne spores is located in late summer and early autumn. In addition, there are few studies reporting information about hourly distribution of fern spores in the atmosphere, in which bracken spores were mainly observed at midday when spore traps are located near to fern formations. Airborne spore concentrations are also higher in near zones of fern populations and lower when spore traps move away of these zones, being registered processes of transport.

The *fourth* section deals with medicinal and therapeutical applications ferns exhibit today. Many fern species are used in traditional medicine in many countries to treat different ailments. The first chapter pay attention to traditional Chinese medicine. Six different ferns i.e. *Drynaria fortunei*, *Pseudodrynaria coronans*, *Davallia divaricata*, *D. mariesii*, *D. solida* and *Humata griffithiana* are used as source of medicine commonly known as "Gu-Sui-Bu." These have been claimed to cure inflammation, cancer, ageing, blood stasis, physique ache and bone injuries. However, no scientific investigation has been carried out so far, to evaluate comparative values of these sources. These plants have been over exploited for their fleshy rhizome as a source of "Gu-Sui-Bu" from their natural habitat. Hence, it has become a necessity to preserve these species for future generations. Thus *in vitro* methodology could be the key to preserve these species. The comparative antioxidative potencies, scavenging activities against DPPH radical, reducing power and estimation of polyphenol contents in six sources of "Gu-Sui-Bu" and their conservation through *in vitro* methodologies have been described in detail.

The second chapter refers to phytoecdysteroids, plant analogues of insect moulting hormones, which were first discovered in a gymnosperm in 1966 and almost simultaneously in the fern *Polypodium vulgare* in 1967. Ecdysteroids are believed to contribute to phytophagous insect deterrency, but in addition they exert various pharmacological effects on mammals/humans, and extracts from several ferns containing large amounts of ecdysteroids are used in traditional medicines. Many fern species were investigated for ecdysteroid presence soon afterwards, and a large number of different ecdysteroids has been isolated, including several unique molecular structures. Ecdysteroids are not found in all fern families, but in some (e.g., Polypodiaceae) almost all investigated species contain these molecules, sometimes at very high concentrations. Ecdysteroid biosynthesis from sterols has been little investigated in plants, but available data indicate that it probably does not proceed by the same pathway as in insects. The accumulation of these compounds is precisely regulated, as shown with prothalli of *P. vulgare*.

In the third chapter, more information about the medicinal uses of ferns are provided. In this regard, ferns belonging to the *Microsorum* genus such as

"Metua pua'a" represent major medicinal plants in the pharmacopoeia of Pacific islands. These pharmacological effects concerns very different diseases, but major uses are as purgative, as antibacterial, for the treatment of gastric and kidney infections, as diuretic, pain killer (to treat headache, stomachache, gastrointestinal aches) and as anti-inflammatory preparations (to treat rheumatism). Several classes of phytochemicals have been investigated, including both large families of secondary metabolites (e.g., terpenoids, alkaloids or phenolics), as well as more specific compounds responsible for toxic/carcinogenic effects. In practice, there are only a few examples where the active principles expected to be responsible for the pharmacological effects have been identified. The isolation and the identification of active principles require a combination of efficient separation methods and suitable bioassays and in this respect ferns should deserve more efforts in order to understand the chemical basis of their numerous pharmacological properties.

The section ends with two chapters, which give opposite impressions about fern effects. On one hand, a warm approach to the ferns and fern allies is done as they present important benefits for a healthy life style, possessing useful economical and natural materials used in manufacturing various functional life goods and natural air purifiers. Ferns and fern allies would be sources of amazing natural healthy products, allowing people enjoy their healthy and eco-friendly life. On the other hand, the last chapter shows the enormous noxious potential of *Pteridium aquilinum*, due to his great toxicological effects, based on its characteristic chemical composition. The plant has secondary plant metabolites of potentially high biological activities, which can cause serious health problems to animals and humans.

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Part I
Contribution of Ferns to Understanding of Plant Development

Chapter 2
Cellular, Molecular, and Genetic Changes During the Development of *Ceratopteris richardii* Gametophytes

Mari L. Salmi, Thomas Bushart, and Stanley J. Roux

2.1 Introduction

The aquatic fern *Ceratopteris richardii* has been used to decorate fish tanks and as a food source, but arguably its more significant value has been as a research tool. It has been a key model organism for many different laboratories to investigate basic research questions in genetics, developmental biology, and signal transduction. It is classified as a member of the Ceratopteridaceae family (historically, also listed in the Parkeriaceae and Pteridaceae), and is closely related to three other *Ceratopteris* species, *cornuta, pteridoides*, and *thalictroides.* It flourishes in tropical and subtropical regions throughout the world, including Africa, Asia, Australia, and North and South America. The last comprehensive review of research on this fern was a decade ago (Chatterjee and Roux 2000). This chapter will summarize some of the earlier works, but focus primarily on research published in the last 10 years, and primarily on studies that used the gametophyte stage of the fern.

The initial impetus to use *C. richardii* as a favored subject for diverse biological investigations came from publications of the laboratory of Leslie Hickok (University of Tennessee) on the genetics and biology of this fern. In addition to their research papers, the Hickok laboratory provided spores and technical assistance to many research groups and established the C-Fern website (http://www.c-fern.org/), which is still a valuable resource for educators, students, and fern specialists. Based mainly on the work of Hickok's laboratory and some seminal genetic studies by Jo Ann Banks' group, Chasan wrote a review in 1992 arguing for the value of *Ceratopteris* as model organism for genetic and other studies (Chasan 1992), and the many subsequent publications utilizing this fern amply support those arguments.

Key characteristics of *C. richardii* that make it especially attractive for research are its ease of culture and its relatively short life cycle. Typically, within less than 4 months *C. richardii* can complete both its gametophytic and sporophytic stages,

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and a mature sporophyte can produce over a million spores in about a month. These spores can then be stored and remain viable for years at room temperature. Methods of axenically culturing the spores to investigate cellular and developmental phenomena are simple and inexpensive (http://www.c-fern.org/).

C. richardii gametophytes have been used primarily for genetic and signal transduction studies and as teaching tools for laboratory courses. The genetic studies have been focused largely on the genes involved in sexual reproduction (Wen et al. 1999; Strain et al. 2001), and on mutations that confer salt tolerance (Warne et al. 1999). Signaling studies have taken advantage of the fact that the single-celled spore is responsive to both light (Cooke et al. 1995) and gravity (Edwards and Roux 1998), and its polarity development and germination are regulated by trans-cell calcium currents (Chatterjee et al. 2000; Bushart et al. 2010). The multicellular gametophyte also responds to diverse signals; e.g., its sex determination and sexual maturation, which occur within 10–12 days after spore germination, are initiated and are regulated by both light (Kamachi et al. 2007) and antheridiogens (Wen et al. 1999). The use of *C. richardii* gametophytes as an educational tool was originally inspired by publications of Hickok's group (Renzaglia et al. 1995), but this initiative is still very active (Spiro and Knisely 2008).

The value of *Ceratopteris* as a research tool has been further enhanced by recent transcriptomic studies. Methods of extracting RNA and generating cDNA libraries from spores are straightforward, and extensive sequence information from these libraries is available online (Salmi et al. 2005). Such data have been especially valuable for evolutionary (Hodgins-Davis and Townsend 2009), genomic (Nakazato et al. 2006) and computational (Yao et al. 2008) analyses. Deep sequencing (Roche 454) assays of gametophytic transcripts have been completed (Bushart, Roux, and Porterfield, unpublished, and Banks, unpublished) and data from these runs should be available online by December 2010. Transient transformations have been achieved (Stout et al. 2003; Rutherford et al. 2004) but, as yet, there are no methods for stable transformation. This technical barrier will have to be overcome before the advantages of *C. richardii* as a model system can be fully exploited.

Because of space limitations this chapter cannot comprehensively cover all the literature on *C. richardii*. Hopefully the selected topics that are critically reviewed in the following sections will convince the reader that this humble plant, not known for its landscape beauty, deserves to be ranked as a top model organism for fern studies generally, and, more specifically, for basic biological studies on the molecular mechanisms that control calcium signaling, the transition from dormancy to germination, polarity development, and sex determination.

2.2 Regulation of Differentiation in Multicellular Gametophytes

As with most ferns, *C. richardii* spores are the resistant state of the haploid gametophyte, and they remain dormant until imbibed and induced to germinate by exposure to light. Spore germination is a red-light induced, far-red light inhibited

photoreversible process (Cooke et al. 1987) presumably mediated by phytochrome, although phytochrome genes have yet to be cloned and characterized in *C. richardii*. Upon exposure to white or red light, spores begin a series of developmental steps that result in germination as defined by emergence of the primary rhizoid of the gametophyte.

Although light responses in another Pteridaceae family member, *Adiantum capillus-veneris*, have been more extensively studied (Wada et al. 1983; Nozue et al. 1998; Imaizumi et al. 2000; Kawai et al. 2003; Kodama et al. 2008), there have been recent findings in the light signaling pathways of *C. richardii* (Kamachi et al. 2004). Reverse genetics approaches used to study mutagenized spores have found several distinct classes of light-response mutants (Scott and Hickok 1991; Kamachi et al. 2004). Particularly interesting and well studied, the dark-germinating 1 (*dkg1*) mutants have pleiotropic defects throughout the life of the *C. richardii* gametophyte. Kamachi et al. (2004) have identified a gene whose product is very closely regulated by phytochrome in *C. richardii*. Cloning and characterization of this gene may provide insights on phytochrome-regulated signaling in many plant lineages.

C. richardii is a homosporous fern, and it usually develops as a hermaphrodite. However, the sex of its gametophyte can be determined epigenetically by a signaling pathway induced by a pheromone, antheridiogen. This regulatory chemical is secreted by the hermaphroditic gametophyte typically between 10 and 12 days after the spore has been induced to germinate (Banks et al. 1993), and it functions both to inhibit the development of female traits, which include a multicellular meristem and archegonia, and to promote the differentiation of the primary male trait, antheridia. An overview of this and related topics was published in a 1999 review (Banks 1999). Since then more recent findings have added depth to our understanding of sex determination in *C. richardii.*

Strain et al. (2001) used a genetic approach to discover new genes that regulate sex development in *C. richardii*. They mutagenized spores with EMS and screened for mutants that feminized gametophytes treated with antheridiogen. They identified 16 such mutants, 7 of which were linked to the *FEM1* gene locus that was already known to be a negative regulator of female traits. However, nine other mutants were at a new locus the authors termed "Notchless" because several of them were missing a meristem notch. The authors' evidence supported a model in which *FEM1* induces *NOT1* gene expression, which, in turn, functions as a repressor of the *TRA* genes that are positive inducers of feminine traits. Without the *NOT1* gene expression, antheridiogen cannot suppress the *TRA* genes, and this allows feminine traits to develop in the presence of the pheromone. Tanurdzic and Banks (2004) discuss this *NOT1-TRA* antagonism further and compare the genetic sex determining pathways in *C. richardii* to those in the Fly *Drosophila melanogaster* and the Nematode *Caenorhabditis elegans.* Figure 2.1 depicts the currently known phenotypic classes of sex-determination mutants.

As noted above, light strongly influences spore germination. It can also influence sex development in gametophytes, but this influence becomes apparent only in mutant strains, as reported by Kamachi et al. (2007). *her1* mutants are insensitive to antheridiogen in darkness. Whereas the sensitivity of wild-type gametophytes to

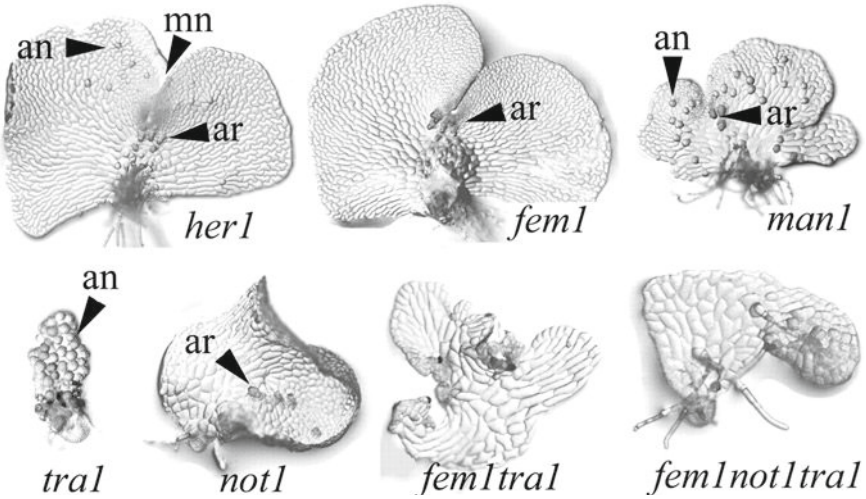

Fig. 2.1 Sex-determination mutants of *Ceratopteris richardii*. The *her1* (*hermaphroditic*) and the *tra1* (*transformer*) mutants are phenotypically normal for hermaphrodite and male wild-type plants respectively, but they are both insensitive to the absence or presence of the male-inducing antheridiogen hormone (A_{CE}). The *fem1* (*feminization*) mutant is also A_{CE}-insensitive. Antheridia development is another feature altered in these mutants. In comparison to wild-type hermaphrodite gametophytes, *fem1* mutants do not produce anteridia, *not1* (*notchless*) mutant rarely produces antheridia, and *man1* (*many antheridia*) mutant produces about ten times the number. The phenotypes of the double (*fem1 tra1*) and triple (*fem1 not1 tra1*) mutants suggests that the development of male and female characteristics are controlled by separate pathways influenced by the presence or absence of A_{CE}. Abbreviations: *an* antheridia, *ar* archegonia, *mn* meristem notch (Reproduced from (Tanurdzic and Banks 2004) with permission from The American Society of Plant Biologists)

antheridiogen for sex determination is unchanged in blue light, in *her1* mutants blue light promotes maleness. Red light, probably acting through the photoreceptor phytochrome, can cancel the effects of blue light, because simultaneous treatment of *her1* gametophytes with red and blue light does not promote male development (Kamachi et al. 2007).

Hormones that regulate growth and development in flowering plants also influence development in *C. richardii* gametophytes. Gregorich and Fisher (2006) reported that either of two synthetic auxins, 1-naphthalene acetic acid (NAA) and 2,4,5-trichlorophenoxyacetic acid (2,3,5-T) could suppress lateral meristem development and sex organ development. Moreover, the auxin antagonist *p*-chlorophenoxyisobutyric acid suppressed the development of both lateral and apical meristems, and the auxin transport inhibitor *n*-1-naphthylphthalamic acid induced the formation of a second lateral meristem.

In a related experiment, the effects of 1-naphthalene acetic acid (NAA) on the growth and differentiation of *C. richardii* gametophytes was further investigated (Stilts and Fisher 2007). When tested at 10^{-5} M, NAA significantly increased cell size and the length/width ratio, but decreased the overall length and width relative

to that of gametophytes cultured on basal medium alone (without NAA). Developmentally, NAA-treated gametophytes generate rhizoids along most of their periphery, whereas those grown without NAA produce rhizoids only at their base. These results are consistent with the hypothesis that endogenous auxins play an important role in the growth and development of *C. richardii* gametophytes. Measurements of auxin levels in gametophytes and molecular studies of auxin receptors and transport carriers would be needed to verify this idea.

There is evidence that cytokinins also influence the development of *C. richardii* gametophytes. Spiro et al. (2004) found that several different cytokinins could induce photomorphogenic changes in dark-grown gametophytes, even when applied at sub-nM concentrations. Thus, cytokinin treatment could substitute for both blue- and red-light stimuli in triggering changes of cell division, growth, and differentiation. However, the hormone could not induce the dark germination of spores or hermaphroditic sexual development, nor could it substitute for light treatments in inducing chlorophyll synthesis. These results suggest intriguing similarities and differences in the role of cytokinins in photomorphogenic responses in angiosperms and ferns. The form and level of native cytokinins in fern gametophytes are currently unknown, as are the molecular features of their cytokinin receptors. Filling in these information gaps will further clarify the role of cytokinins in the photomorphogenic development of gametophytes of *C. richardii* and other ferns.

2.3 Genes and Genomic Studies of *C. richardii* Spores

The diploid Hn-n strain of *C. richardii* has approximately 11 billion base pairs of DNA over a relatively large number of chromosomes ($n = 39$). High chromosome numbers are typically attributed to polyploidy and chromosomal duplication events. This could be problematic, but *C. richardii* appears to behave functionally as a diploid organism. This apparent contradiction is attributed to gene silencing, for which there is cDNA-DNA hybrid (McGrath et al. 1994), mutagenic (Hickok et al. 1995), and mapping (Nakazato et al. 2006) evidence. The "random" distribution of duplicate genes across linkage groups implies that small-scale, rather than whole genome, duplication was the main mechanism by which *C. richardii* accumulated its large genome.

Due to the highly defined alternation of generations that exists in ferns, it is easy to work separately with the diploid sporophytes or haploid gametophytes. As noted by Hickok et al. (1995), the ability for hermaphroditic gametophytes to self-cross also allows for the easy maintenance of genetically manipulated lines. A homozygous sporophyte can be formed in a single generation from a carefully conducted self-cross and this will produce genetically identical spores. Reproduction amongst these gametophytes will in turn produce genetically identical sporophytes in a process termed either "intergametophytic selfing" or "autogomy" (Hickok et al. 1995).

The *C. richardii* genome has not been completely sequenced. Its large size would make it rather expensive to perform a full sequence analysis at this time. This is unfortunate, since the large size and anticipated history of repeated partial duplication of the *C. richardii* genome would make it a highly valuable model for the study of how genomes evolve. Scott et al. (2007), for example, have characterized four additional members of the α-tubulin gene family for phylogenetic analysis of α-tubulin within and across species. Due to the limited sequence information on tubulins in *C. richardii*, the size of the gene family is difficult to define, although it appears likely to be significantly larger than the six copies known in *Arabidopsis thaliana*.

Technology continues to increase the speed and reduce the costs of sequencing projects, so it is likely only a matter of time before this fern joins the list of species with completely sequenced genomes. An indication of progress toward this goal is that the chromosomes have been linkage mapped (Nakazato et al. 2006) and there exists a growing amount of available sequence data. As of April 2010 there are 100 submitted gene sequences, 5,133 Expressed Sequence Tags, and 1,154 Genomic Survey Sequences in the public GenBank database (Pruitt et al. 2007). The labs of Jody Banks and Stanley Roux are also examining the genes expressed at different developmental time points of the gametophyte stage using "next generation" Roche 454 sequencing, which will add further genetic information. It is worth noting that expressed sequences are highly valuable even after obtaining a fully sequenced genome, again due to its extremely large size. The same features of size and gene duplication which make *C. richardii* an interesting subject for evolutionary studies also make it difficult to work with unless that information is combined with expression data.

Analysis of a current EST library showed the presence of 3,930 tentative unique genes (TUGs) being expressed 20 h after exposure of *C. richardii* spores to light (Salmi et al. 2005). Two thousand seven hundred and ten TUGs were found to have significant similarity (E value $\leq 1.0 \times 10^{-10}$) to Arabidopsis ESTs, allowing for categorization of these similar sequences based on possible functions and localizations. Having this sort of information in hand has allowed for examination of similarities and the differences between the *C. richardii* germinating spores and other systems.

One specific comparison that was performed was between other germinating systems, namely, seeds and pollen of *Arabidopsis thaliana* seeds (Salmi et al., 2005). This comparison between seeds, pollen, and spores identified eight genes expressed in common among the three systems. Some of these, such as transcription factors and metabolic enzymes could be expected, while others have known important functions in the development of higher eukaryotes, but thus far have no described function in ferns (Mago nashi family proteins and a MIR-domain containing protein). These sorts of comparisons allow for the paring down of the thousands of genes that are expressed during the development of *C. richardii* gametophytes to hundreds or less that would be of particular interest to study, both from an evolutionary perspective and as a way of defining common biological pathways and species-specific deviations. The continued use of established alternative model systems like *C. richardii* is essential for identifying both broadly conserved and species-specific genes.

2.4 Spores as a Tool for the Study of Cellular Gravity Response

The *C. richardii* spore is a remarkably elegant system for studying the cellular mechanism of gravity perception. Spores are large, 150–300 μM diameter, so they can be easily observed under low magnification. The spore is a single cell which detects and responds to the vector of gravity prior to cell division. The first physiological event documented in the gametophyte's early development is an efflux of calcium from the top of the spore, which is concurrent with an influx of calcium at its bottom (Chatterjee et al. 2000). Initial studies of this calcium current done using a self-referencing ion-selective electrode have been verified and refined recently using a novel integrated real-time system on a bio-chip referred to as the cell electrophysiology lab-on-a-chip (Fig. 2.2), or CEL-C (ul Haque et al. 2006a; ul Haque et al. 2007). This calcium current is parallel to the vector of gravity, and when spores are rotated at a rate of 180° in 5 s the current is re-established in the new vector of gravity at the same magnitude within 24 s (ul Haque et al. 2006b).

The rapid rate of re-orientation of this calcium current indicates that it is most likely due to localized activation or inactivation of calcium channels and pumps. The more or less spherical spore can be oriented in any way with respect to gravity. It is highly unlikely that calcium channels and pumps that produce this directed, trans-cellular current can be moved across the cell periphery at a fast enough rate to account for the observed re-orientation. It is much more likely and in keeping with the models of all known plasma membrane ion channels and pumps that these transport molecules are located throughout the spore periphery, and are locally activated due to the force of gravity.

The first event in spore germination that can be observed under a microscope is nuclear migration. The spore nucleus moves downward in over 90% of spores kept in fixed orientation 24–30 h after the spore is exposed to light. The direction that this nuclear migration will take is determined 6–20 h prior to when it occurs (Edwards and Roux 1998). This indicates that within the first 18–24 h of development the spore has responded to the force of gravity by irreversibly fixing the polarity of its development. Following the nuclear migration, about 48 h after light exposure, there is an asymmetric cell division that results in a smaller cell that will develop into the primary rhizoid of the gametophyte, and a larger cell that will develop into the photosynthetic thallus. The developmental timing and physiology of *C. richardii* spores allows researchers to investigate the sub-cellular and molecular changes and perform drug treatments (both transient and long term) to evaluate cellular processes involved in gravity sensing and gravity-directed development.

The involvement of calcium in the establishment of spore polarity has led to studies to find downstream targets of calcium in this process. One obvious target would be calcium-dependent calcium pumps. The involvement of Ca^{2+}-ATPases has been investigated using plasma membrane and endomembrane specific

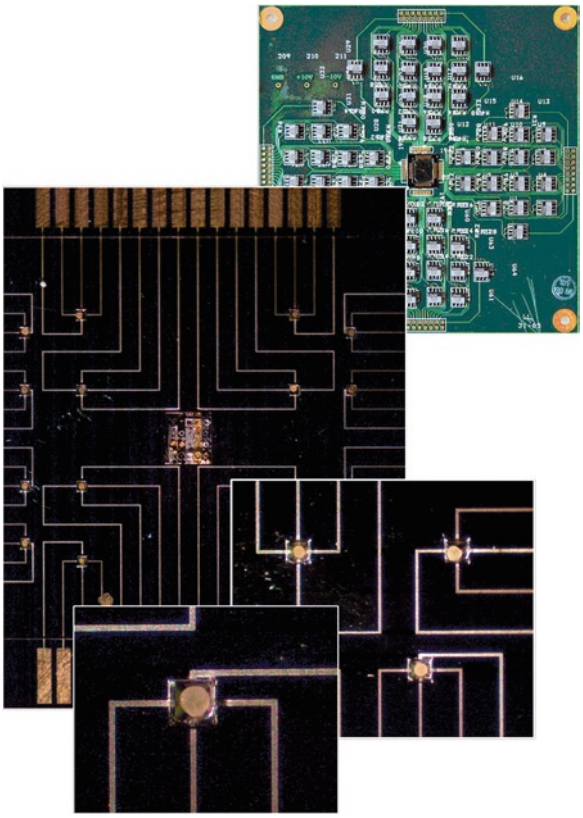

Fig. 2.2 CEL-C chip. One Ag/AgCl calcium specific electrode leads to each of the four positions (*top*, *bottom*, and *two sides*) around each well. The lowermost picture depicts a single well containing one *C. richardii* spore. For size reference in all photos, spores typically are 100–150 μm in diameter. Each CEL-C chip has a total of 16 wells (second photograph from *top*). The CEL-C chip is positioned in the center of the custom circuit board used for data collection (*top* photograph). One of the benefits of this system is that the entire circuit board may be repositioned in relation to the gravity vector and the spores monitored continuously during this time. An example set up is to have the board positioned vertically and then rotated 180°, such that the top and bottom electrodes measure changes in the trans-cellular calcium current before, during, and after the rotation

inhibitors of these transporters (Bushart et al. 2010). The results indicate that the plasma membrane pumps have negligible impacts on actual polarity alignment. However, plasma membrane pump inhibitors have strong effects on the tip growth of rhizoids, which would be expected based on similarities to other tip-growing systems (Bushart and Roux 2007). In contrast, inhibition of sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) during the window of polarity fixation was found to increase polar axis alignment (Fig. 2.3a). This supports a model for polarity fixation that predicts the influx of calcium into the spore via mechanosensitive channels and then its modulation by the SERCA pumps (Fig. 2.3b). A local

accumulation of Ca^{2+} at the bottom of the cell presumably leads to local activation of other downstream effects. Thus far, studies using nitric oxide inhibitors and donors have implicated both nitric oxide and cGMP as downstream targets of calcium in this signaling cascade (Salmi et al. 2007). There are many other known targets of calcium signaling, and future studies will hopefully elucidate the details of this process.

In an effort to document and evaluate changes in transcription that occur in spores during the time course of early development, cDNA microarrays were made using the products of the cDNA described previously (Stout et al. 2003; Salmi et al. 2005). Inhibiting transcription in spores by treatment with Actinomycin D delays germination, presumably until the light labile compound degenerates. This indicates that new transcripts are necessary for spores to germinate. Initial examinations focused on the germination aspects of breaking dormancy and preparation for cell division, but the fact that polarity fixation is known to occur within the first 24 h of light-induced germination makes time-based expression change data valuable for experimental design and future reference. In these experiments, roughly 26% of the genes expressed at the early 20-h stage of germination were evaluated. The relative abundance of more than 900 transcripts that change in some way during the first 48 h of spore development is publicly available. This EST library and microarray data are valuable resources for those studying ferns, cellular polarity, and bioinformatics (Nakazato et al. 2006; Bushart and Roux 2007; Scott et al. 2007; Yao et al. 2008; Alongi et al. 2009).

With the cut-back in Shuttle flights, experiments involving spaceflight plant material have been significantly reduced in recent years. However, in addition to the investigations noted above, *C. richardii* has also been used as one of the few model plants selected for studies in the microgravity environment of space (Kiss et al. 2009; Long et al. 2009; Matia et al. 2010; Millar et al. 2010). The *C. richardii* cDNA microarrays were used for valuable experiments comparing the gene expression of space flight flown samples with that of 1*g* ground-control samples (Salmi and Roux 2008). In this study, the expression of about 5% of transcripts evaluated had a reproducible difference in abundance between spaceflight and ground samples. Yet again, taking advantage of the power of comparison to narrow down thousands of possibilities to certain elements of unique interest, it was found that 37, 70, and 53 unique genes were upregulated while 25, 42, and 58 were downregulated in spaceflight conditions as compared to ground controls at 1, 8, and 20 h post light exposure. Comparisons of these changes to similar experiments done in *Arabidopsis* plants has identified 14 common genes that appear to be involved in gravity and/or mechanical stimulation responses. These data provide a logical basis for further evaluations of the role of many genes in the earliest cellular responses to gravity. More efficient tools for genetic manipulations of *C. richardii* spores are needed to further investigate the role of these candidate genes. The cDNA microarray comparisons of spaceflight and ground *C. richardii* spores should prove useful for comparisons to other organisms studied in simulated microgravity conditions.

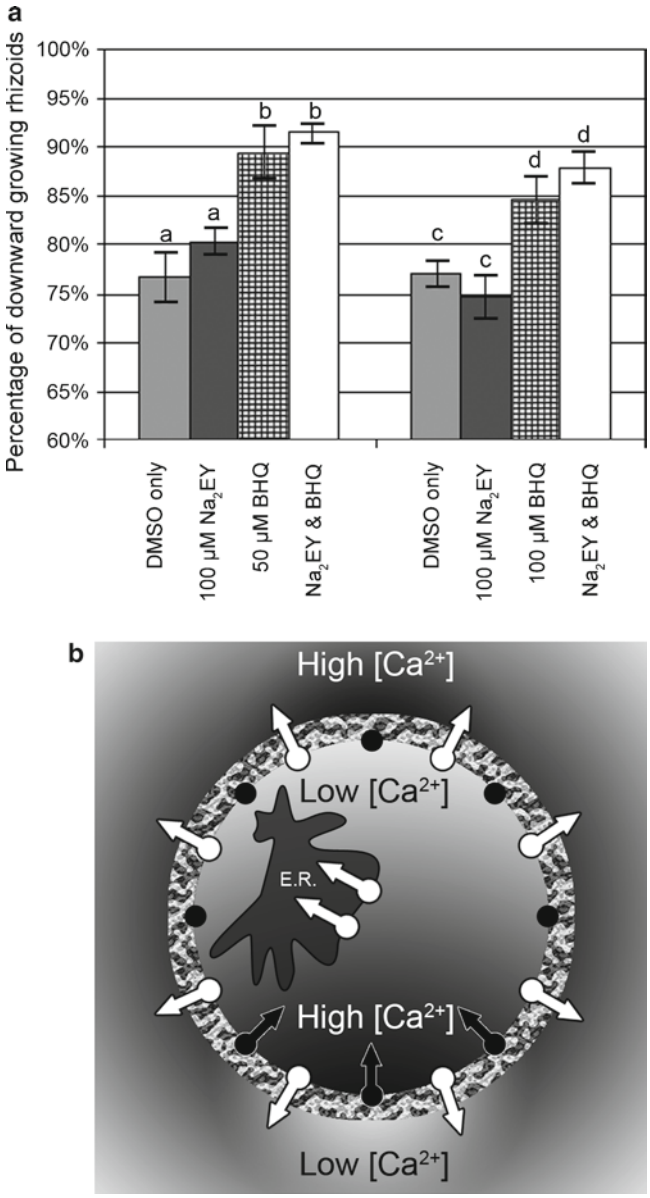

Fig. 2.3 Effects of Ca^{2+}-ATPase inhibitors on spore gravity response and a model for gravity-directed alterations of intracellular calcium concentrations. (**a)** Endomembrane pumps are important for spore polarity. Spores were exposed to inhibitors during the first 24 h of light induced germination, which coincides with the window for fixation of the gravity-directed spore polarity. The plasma membrane Ca^{2+}-ATPase inhibitor Eosin Yellow (Na_2EY) has no statistically significant alteration on spore polarity as measured by downward growing rhizoids. The presence of the SERCA pump inhibitor BHQ, however, increases the polar axis alignment of spores in a statistically significant manner (represented by different lower case letters). Error bars represent standard error

2.5 Resources Available and on the Horizon for Use in *C. richardii* Research

C. richardii has a number of previously discussed qualities that make it ideal for the study of features both in common with and unique from other plant systems. Because of these strengths, several valuable methods have been developed for genetic, signaling, and developmental studies, but the large genome size of this fern and the current lack of a method to stably transform it are currently limiting its usefulness as a model system.

In terms of what can be done, the system is amenable to general mutagenesis. As an example, further details of the feminization pathway in gametophytes were added by identifying antheridiogen sensitivity mutants generated by EMS mutagenesis (Banks 1994; Strain et al. 2001). Direct gene manipulations are, of course, more desirable than random mutation when working with identified genes or pathways. In this avenue, there has been success with transient gene expression manipulations via particle bombardment in the prothalli of *Adiantum* fern gametophytes (Kawai et al. 2003; Kagawa et al. 2004). Because technology developments are so rapid today, it would not be unreasonable to expect that it will just be a matter of modifying these protocols to work with the *C. richardii* system. Nucleotide interference manipulations also hold promise with RNAi applications in *C. richardii* (Stout et al. 2003; Rutherford et al. 2004), as well as DNAi in the gametophytes of *Adiantum* (Kawai-Toyooka et al. 2004). Moreover, it is thought that the *Agrobacterium* system commonly used with model systems like *Arabidopsis* could see success in fern systems (Wada 2007). Surely, as ease of transformation becomes a reality for *C. richardii* in the (hopefully not too distant) future, this fern will prove to be an even more valuable model system for solving diverse problems in biology.

Fig. 2.3 (continued) (Reproduced from (Bushart et al. 2010) with permission from the American Society of Gravitational and Space Biology). (**b)** A model based on external calcium flux measurements and inhibitor effects. Data suggests alteration of channel and pump activities rather than a change in localization cause the rapid re-orientation of a trans-cellular calcium current. Therefore, it is assumed that mechanosensitive channels (black circles) and Ca^{2+}-ATPase pumps (white circles) are distributed around the spore perimeter. Channels would be active (represented by arrows) along the bottom of the spore and inactive along the top. Plasma membrane Ca^{2+}-ATPases would be active throughout the cell periphery as they are Ca^{2+}-CaM regulated. SERCA pumps would be active as well. The net calcium concentrations externally and internally are then due to the local summative action of the channels and pumps, specifically resulting in the measured top-high/bottom-low external concentrations and an anticipated high concentration of calcium localized at the bottom of the spore

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Chapter 3
Laboratory-Induced Apogamy and Apospory in *Ceratopteris richardii*

Angela R. Cordle, Linh Thuy Bui, Erin E. Irish, and Chi-Lien Cheng

3.1 Introduction

A life cycle characterized by an alternation between two generations, each developing a multicellular body, is a feature unique to land plants (Embryophytes). In addition to the sexual life cycle defined by meiosis and fertilization, some species, widely distributed among the embryophytes, complete their life cycle asexually. The best-described variations of the asexual life cycle include apomixis in grasses and obligate apogamy in ferns. Recent studies using model plants to unravel the developmental program of the angiosperm female gametophyte at the molecular level have provided insights into the understanding of apomixis. This chapter begins with a review of the current view of how alternation of generations in the embryophytes evolved, then provides a description of apogamy and apospory in ferns and compares those alternatives to the sexual life cycle with apomixis in angiosperms. Finally, induced apogamy and apospory in the model fern *Ceratopteris richardii* are reviewed.

3.2 Alternation of Generations

All embryophytes progress through a life cycle that alternates between two generations, the haploid gametophyte and the diploid sporophyte. Unlike animals in which meiosis produces single-celled gametes directly, gametes of embryophytes arise from a multicellular entity, the gametophyte generation, which in turn develops from the products of meiosis, the spores. The dominant generation in the basal branch of the embryophytes, the bryophytes, is the gametophyte, which supports a minuscule sporophyte. Conversely, in the most advanced embryophytes, the angiosperms, the gametophyte generation is miniscule and deeply embedded in the

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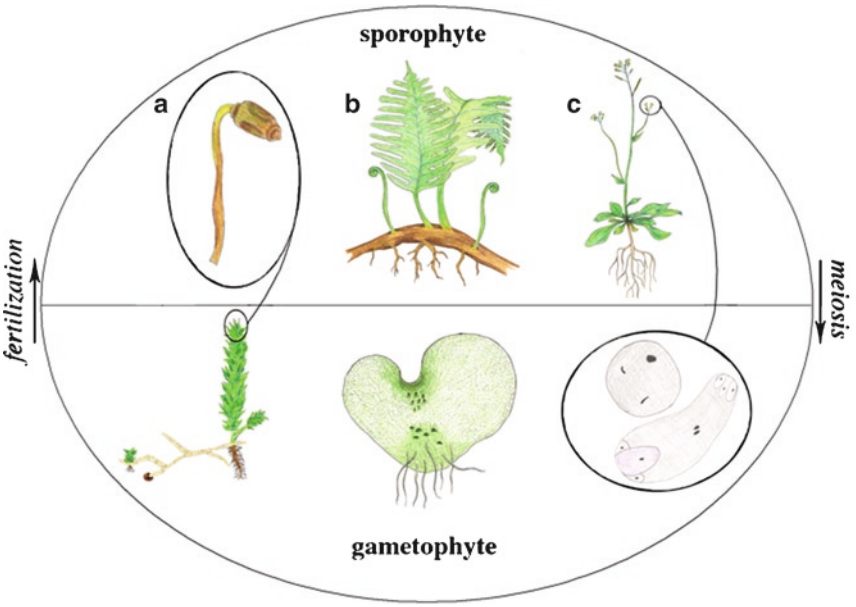

Fig. 3.1 Variation of alternation of generations in embryophytes. The three representative plants are a moss (**a**), a fern (**b**), and *Arabidopsis* (**c**); sporophytes are in the top panel and gametophytes are in the bottom panel

flowers of the sporophyte. Most deserving of the term "alternation of generations" is the life cycle of the seedless vascular plants, the lycophytes and monilophytes, whose two generations grow independently. Both gametophyte and sporophyte are photosynthetic in the monilophytes, the group that includes the ferns. The variation of alternation of generations among embryophytes is depicted in a cartoon (Fig. 3.1).

Although whether multicellularity arose first in the gametophyte or sporophyte in the ancestral green land plant can be argued either way, the current view of evolution of the two generations in land plants can be summarized as follows. Embryophytes belong to a monophyletic clade of photosynthetic eukaryotes that descended from a charophyte-like ancestor (McCourt et al. 2004; Becker and Marin 2009). All charophytes have a life cycle consisting of a single multicellular haploid generation – the only diploid cell is the zygote, which undergoes meiosis immediately (Bateman et al. 1998). Thus, an alternation between two multicellular generations in land plants is a derived condition (Niklas and Kutschera 2010). The ancestral land plants most likely possessed a dominant gametophyte generation that restricted them to dwelling in moist environments. The optimal reproduction and dispersal strategy for early terrestrial plants was via the production of spores, rather than gametes. The multicellular sporophyte, arising from a delay of meiosis, was thought to be the first adaptation to terrestrial life (Becker and Marin 2009). During the Devonian period (approximately 420–350 million years ago), an

explosion of morphological diversification occurred in the elaboration of the sporophyte, such as deep roots and strong vascular supports, allowing the sporophytes with these adaptations to colonize drier lands. Gametophytes, on the other hand, became both smaller and dependent upon the sporophyte generation for growth and development. Eventually the seed habit evolved, permitting the further colonization of new environments (Bateman et al. 1998; Dolan 2009).

Molecular evolution analyses of gene families encoding transcription factors have provided invaluable information for investigating the evolution of homology and novelty in developmental patterns and the complex regulatory circuitry inherited from a common ancestor (Shubin et al. 2009). Some gene families encoding evolutionarily conserved transcription factors important in angiosperm development have been identified. They have shown dynamic changes across the green plant phylogeny. Comparison of their expression patterns revealed that some family members function in development of the haploid reproductive structures in algae, indicating an ancient origin. Over evolutionary time these genes were redeployed for sporophyte development in lower embryophytes and later for the elaboration of more complex sporophytes in angiosperms (Niklas and Kutschera 2009). Take, for example, the *FLORICAULA/LEAFY* (*FLO/LFY*) regulation of the MIKC-type MADS-box transcription factors in angiosperms. In Arabidopsis, MIKC-type genes are positively regulated in the floral meristem once it has been converted from the vegetative state by the action of *FLO/LFY* transcription factors (Weigel et al. 1992). In the fern *Ceratopteris richardii,* which like all seedless vascular plants lacks floral meristems, the expression of *FLO/LFY,* and MIKC-type orthologs is not correlated, suggesting transcription of the MIKC-type genes is not yet under the control of *FLO/LFY* (Himi et al. 2001). In the moss *Physcomitrella patens*, *FLO/LFY* paralogs are required instead for the first division of the zygote and early embryogenesis (Henschel et al. 2002; Tanahashi et al. 2005). In the Charophyte *Chara globularis*, the expression of MIKC-type is highest in gametangia (Tanabe et al. 2005). The dynamic expression pattern of the MIKC-type genes and their relationship with *FLO/LFY* illustrates the gene duplication, loss, and recruitment for expression that had occurred from algae to flowering plants.

Another example is the three amino acid loop extension (TALE) superfamily of homeodomain proteins. Homeodomains are among the most ancient of DNA-binding protein domains (Treisman et al. 1992; Derelle et al. 2007). Classes I and 2 knotted1-like homeobox (*KNOX)* genes and *BELLRINGER* (*BELL*) class genes are all members of this superfamily. In Arabidopsis, class 1 *KNOX* genes and the *BELL* genes interact to specify shoot meristem identity (Hake et al. 2004). In *C. richardii*, the expression of three class 1 *KNOX* genes are expressed in the shoot meristem, leaf primordia, vascular bundles, and leaf margins of the sporophyte. The single class 2 *KNOX* gene is expressed throughout the sporophyte, but no member of either class of the *KNOX* genes is expressed in gametophytes (Sano et al. 2005). Similarly, in *P. patens*, expression of all *KNOX* genes is restricted to sporophytic tissues. Knocking out the two class 1 *KNOX* genes or the single class 2 *KNOX* gene disrupts sporophyte development (Singer and Ashton 2007). Most interestingly, in

the green algae *Chlamydomonas reinhardii*, the *KNOX* ortholog Gsm1 and the BELL-like-protein gene Gsp1 are expressed in *minus* and *plus* vegetative cells, respectively. When these two proteins are brought together upon syngamy, they form heterodimers and translocate to the nucleus to turn on genes for the zygote developmental program. When either protein is ectopically expressed in the other type of vegetative cells, zygote-program genes are turned on (Zhao et al. 2001; Lee et al. 2008). This illustrates how deeply rooted the TALE genes are in "generation" dimorphism and the eventual diversification of the function of family members in flowering plants.

3.3 Apogamy and Apospory in Ferns

In addition to the normal land plant life cycle marked by meiosis and fertilization, some species of moss and fern undergo generational transitions asexually through apogamy or apospory. In these processes, the morphological development of one generation to another is uncoupled from the changing of ploidy during meiosis and gamete fusion. A long debated but still unresolved question concerning the evolution of the gametophyte and the sporophyte generations is whether the ancestral land plant possessed an isomorphic or dimorphic life cycle. The capacity for naturally occurring apogamy and apospory in some present-day mosses and ferns species suggests that both haploid and diploid genomes contain the information required for constructing the body plan of gametophyte and sporophyte. This argues for a land plant ancestry with an isomorphic life cycle (Niklas and Kutschera 2009).

In nature, many fern species are obligatorily agamosporous, so that they undergo alternation of generations asexually (Bell 1992). These ferns typically are sterile due to nonfunctional archegonia or antheridia, and without fertilization, sporophytes are regenerated from a vegetative cell of the gametophyte by apogamy (Smith 1979; White 1979). Such apogamous sporophytes possess the same chromosome numbers as the gametophytes. During sporogenesis, a compensatory mechanism acts, giving rise to diplospores that retain the same number of chromosomes as the apogamous sporophyte. In most sexually reproducing ferns, the initial archesporial cell divides four times mitotically, resulting in 16 spore mother cells (SMC). Each SMC then undergoes meiosis, generating a total of 64 spores in 16 tetrads. The two types of compensatory mechanisms that are recurrent, allowing the life cycle to be completed, are the Dopp-Manton and the Braithwaite schemes (Klekowski 1979; Walker 1979). In the Dopp-Manton scheme, most commonly, the archespore first undergoes three rounds of mitosis, generating eight cells. During the fourth mitosis, the chromosomes double without cytoplasmic division, thus resulting in restitution nuclei that contain twice the number of the chromosomes. Subsequent meiosis proceeds with the formation of bivalents and produces 32 diplospores as tetrads. In the Braithwaite scheme, the archespore undergoes four normal mitoses to give rise to 16 spore mother cells; subsequently, during meiosis

I, the chromosomes do not pair and the cytoplasm fails to divide, resulting in restitution nuclei with doubled chromosomes. Meiosis II proceeds normally to give rise to 32 diplospores in dyads. Gametophytes develop from these diplospores but are defective in reproductive functions, producing sporophytes apogamously to complete the asexual life cycle. Such a life cycle does not require a water film for sperm to swim for fertilization and is thought to have evolved as an adaptation to a drier environment (White 1979). Indeed many Cheilanthoid ferns use this strategy in order to persist in xeric environments (Mickel 1979).

In apospory, gametophytes are produced by somatic cells of the sporophyte without going through sporogenesis (Sheffield and Bell 1981; Raghavan 1989; Bell 1992). Apospory only occurs in nature sporadically (Walker 1979) but is easily induced in the laboratory. In contrast to induced apogamy, which typically produces abnormal sporophytes, aposporous gametophytes appear normal, heart-shaped, and with functional antheridia and archegonia (Walker 1979; Ambrozic-Dolinsek et al. 2002). However, induced apospory in a normally sexual species cannot be repeated indefinitely from generation to generation because the chromosome numbers would be doubled with each fertilization event (Walker 1979).

3.4 Comparison of Apogamy and Apospory in Ferns with Apomixis in Angiosperms

While the majority of flowering plants reproduce sexually to form seeds, some 400 species from 40 families of angiosperms have evolved pathways bypassing the gametophyte to produce seeds, collectively called apomixis (Nogler 1982). Ferns, while being seedless, are nonetheless embryophytes with an experimentally accessible gametophyte generation, making them relevant for comparing their alternate life cycles to apomixis in angiosperms.

A successfully developed apomictic seed requires the maturation of endosperm, derived with or without fertilization. Because ferns completely lack an endosperm, its importance will not be discussed here. The origins of the embryo in plants with one of the three types of apomixis have been reviewed recently (Ozias-Akins 2006; Tucker and Koltunow 2009) and are summarized below (and in Fig. 3.2). In adventitious embryony, asexual embryos may arise from diploid nucellar or integument cells adjacent to the sexual embryo. In diplospory, the megaspore mother cell (MMC) either fails to initiate or complete meiosis before the onset of mitosis, resulting in an embryo sac containing an egg-like cell that proceeds onto embryogenesis without fertilization. In apospory, the MMC may or may not complete meiosis; in either case, one or more somatic cells in close proximity to the MMC differentiate into aposporous initial cells that develop into asexual embryos. In all the three types of apomixis, neither fertilization nor meiotic recombination occurs during the production of the asexual embryo, thus giving rise to a diploid embryo genetically identical to the parent.

Obligate Apogamy in Ferns	Apomixis in Angiosperms
Direct generation of sporophytes from gametophyte prothallus cells 	*Aposporous embryo*: developed from a nucellus or integument cell autonomously 
No comparable variation in ferns	*Adventitious embryo*: developed from a nucellus or integument cell adjacent to the fertilized embryo 
Sporophytes produce restitution nuclei by abnormal sporogenesis to produce unreduced spores 	*Diplosporous embryo*: developed from a unreduced-egg-like cell from abnormal gametogenesis 

Fig. 3.2 Comparison of apogamy in ferns with apomixis in angiosperms. Bright green indicates diploid, pale green, haploid. Embryo sacs are outlined in red and red circles represent cells that will develop into embryos

In contrast to angiosperms, the production of spores and sexual gametes in ferns are partitioned in two independent plants, the sporophyte and the gametophyte, respectively. The interval between the two events is also much greater than in angiosperms, depending largely on a favorable environment for the spores to break their dormancy and germinate. During sporogenesis in apogamosporous ferns, mishaps in mitosis or meiosis give rise to restitution nuclei, which allow the plant to complete the asexual life cycle. This aberration may be compared to diplospory apomixis in angiosperms; the difference is, of course, the direct formation of the asexual embryonic sporophyte in angiosperms and the formation of a free-living gametophyte in ferns prior to formation of the new sporophyte by apogamy (Fig. 3.2). Although apomixis has not been observed in any model plant species, insight has been gained toward understanding the mechanism of this sexual bypass by studies of female gametophyte development in model plants. Which genes and how they may function in model angiosperms during MMC differentiation and the subsequent meiosis have been reviewed recently (Curtis and Grossniklauss 2008; Tucker and Koltunow 2009) and are summarized below. The *MULTIPLE SPOROCYTE (MSP1)* and *TAPETUM DETERMINANT (TPD1)* genes may function together as part of an intercellular signaling mechanism regulating sporogenic cell fate; *SPOROCYTELESS (SPL)*, *WUSCHEL (WUS),* and downstream genes act in coordination with an auxin gradient (Sundaresan and Alandete-Saez 2010) on MMC cell differentiation. The *MEIOSIS ARRESTED AT LEPTOTENEI (MEL1)* mutation, affecting one of the

ARGONAUTE (AGO) proteins, suggests a role in siRNA regulation of target mRNAs in young sporogenous tissues. The *DYAD/SWITCH1 (SWI1)* genes are required in the subsequent meiosis of the MMC for sister chromatid cohesion and centromere organization (Mercier et al. 2001). Interestingly, in the *dyad* mutant, diploid "dyad" cells form, and those that are located closest to the chalazal end of the ovule are the most likely to generate an unreduced embryo sac with an egg that upon fertilization gives rise to a triploid embryo (Koltunow and Tucker 2008; Ravi et al. 2008). Although fertilization is involved in producing the embryo, the successful development of a gamete with unreduced chromosome numbers after a failed meiosis is similar to that in displospory apomixis. In addition to *DYAD*, a triple mutant defective in *OMISSION OF SECOND DIVISION (OSD1)*, *ATSP11*, and *ATREC*, which are known to be required for meiosis, turn meiosis into mitosis and produce diploid gametes apomeiotically (d'Erfurth et al. 2009).

Obligatory apogamy occurs in nature in those fern species whose gametophytes lack functional archegonia or antheridia, making sexual reproduction impossible. Apogamy can be forced to occur by preventing fertilization in ferns that normally reproduce sexually (Lang 1898). During induction of apogamy in *C. richardii*, Cordle et al. observed that only hermaphrodites, and not male gametophytes, could be made to produce apogamous sporophytes. In light of these observations, it is tempting to compare the direct generation of a sporophyte from a gametophyte cell in ferns to that of an egg-like cell undergoing displospory in angiosperms (Fig. 3.2). It is worth noting that in obligate apogamy, as in displospory, spores with unreduced chromosomes are produced, whereas in the case of induced apogamy, spores with reduced chromosomes are produced through normal meiosis. Arabidopsis loss-of-function mutants in the genes *FERTILIZATION-INDEPENDENT SEED 2* (*FIS2*), *MEDEA* (*MEA*), and *MULTICOPY SUPPRESSOR OF IRA 1* (*MSI1*), all encoding members of the polycomb repressive complex 2 (PRC2) (Hsieh et al. 2003; Guitton and Berger 2005a), exhibit embryogenesis from unfertilized egg cells (Chaudhury et al. 1997; Guitton and Berger 2005b). The most telling results connecting apogamy and displospory come from *P. patens*: when the moss ortholog of the Arabidopsis *CURLY LEAF* (*CLF*)/*MEA*/*SWINGER* (*SWN*) gene, also encoding a component of the PRC2, is deleted, the moss becomes apogamic (Okano et al. 2009).

The process of apospory in ferns and apospory in angiosperms are quite different. The major difference is the type of somatic cells that undergo apospory. In ferns, cells that most readily give rise to gametophytes are cells from new leaves of a young sporophyte whereas in angiosperms, it is the somatic cells in proximity to the MMC, i.e., the nucellus or integument cells, that give rise to the apomictic embryo. How these somatic cells gain their ability to become embryogenic is not clear. What has been demonstrated in flowering plants is the triggering of embryogenesis in young *Arabidopsis* seedlings and roots by over-expression of transcription factors that play important roles in major developmental processes, such as *BABYBOOM (BBM), LEAFY COTYLEDON1 (LEC1), LEC2*, *WUS,* and *SOMATIC EMBRYOGENESIS RECEPTOR KINASE1 (SERK1)* (Curtis and Grossniklauss 2008). This phenomenology may be more comparable to apospory in ferns where

a somatic leaf cell gives rise to a gametophyte than in apospory of flowering plants because these genes, except *SERK1* (Hecht et al. 2001), do not appear to be expressed in egg cells or the zygote of wild-type Arabidopsis.

3.5 Induction of Apogamy and Apospory in *C. richardii*

The homosporous fern *C. richardii* has emerged as a productive model system for studying developmental processes (Hickok et al. 1995; Banks 1999; Chatterjee and Roux 2000). Two obligately apogamous laboratory strains of *Ceratopteris* have been described, both of which produce nonfunctional spermatozoids. One strain resulted from the aneuploid gametophyte of a triploid hybrid and another from a diploid hybrid between *C. richardii* and *C. pteridoides* (Hickok 1977; Hickok 1979). In order to take advantage of the model fern *C. richardii*, Cordle et al. (2007)) developed an experimental system with which apogamy can be induced. This process involves growing gametophytes on a high-sugar medium, while preventing fertilization and thus the production of zygote-derived sporophytes. Fertilization is prevented by physically removing male gametophytes from the medium and by inverting Petri dishes to minimize water condensation on the hermaphrodites. Mutants may also be used to facilitate this process. The *hermaphrodite 1* (*her1*) mutant does not produce male gametophytes (Banks 1994), eliminating the need for their physical removal. If *feminization 1 (fem1)* mutant is used, fertilization is not a concern because the *fem1* spores only produce females in the absence of antheridiogen (Banks 1997); any sporophytes produced from these gametophytes will be apogamous.

In classic studies, apogamy was readily induced in several species of homosporous ferns by culturing gametophytes on exogenous sugars (Whittier and Steeves 1962). In the case of *C. richardii*, apogamy can be induced using various concentrations of sucrose, glucose, or trehalose, but it was found that basal medium (1% agarose supplemented with 0.5 × Murashige and Skoog salts at pH 6) supplemented with 2.5% glucose was optimal for the induction of apogamy. Under this condition, the highest percentage of gametophytes produce apogamous sporophytes in the shortest period of time (Cordle et al. 2007). Grown on this medium, gametophytes become thickened and proliferate, growing an extensive net of rhizoids and clusters of antheridia on the margins of the prothalli. After approximately 25–30 days of growth, apogamous outgrowths begin to appear as isolated sporophyte-like organs, taking the shape of leaves, stems, or sometimes roots. These outgrowths have three dimensional structure, vascular tissue, and stomata, all features that are hallmarks of sporophytes and never present in gametophytes. DNA content and chromosome counts remain at haploid levels, indicating that they are apogamous. Similar to apogamous sporophytes in other sexual fern species (Lang 1898; Whittier and Steeves 1960), the apogamous outgrowths of *C. richardii* may persist, but do not mature or produce spores.

This induced-apogamy system was used to determine the minimum time required on glucose for gametophytes to commit to apogamous development. Spores were

germinated and grown on glucose-containing medium for various lengths of time and then moved to basal medium for the remaining time to observe and score for apogamous sporophytes. The first visible sign of sporophyte outgrowth occurred around day 25 and the numbers continued to increase until around 40 days after plating. It was found that from 10 to 12 days on glucose, there was a fourfold increase in the percentage of gametophytes that produced apogamous sporophytes. Interestingly, for gametophytes grown continuously on basal medium, a basal level of apogamy of up to 2% was observed. Whatever factor other than glucose in the experimental condition might be conducive for apogamy is unknown. What this "control" revealed is the plasticity of *C. richardii* development. This experimental system can be useful for isolating genes that are being turned on or off during the commitment period (day 12 versus day 10). It is also useful for examining cell-specific expression by in situ hybridization during apogamy commitment for genes identified this way and for other candidate genes, such as some of the genes described in this review.

It is interesting to note that day 12, when the gametophytes grown on inductive medium are becoming committed to apogamous development, is also the time hermaphroditic gametophytes are producing their first mature archegonia. Under this induction system, apogamy has never been observed from male or *tra1fem1* intersex gametophytes. The latter produce antheridia and non-functional archegonia due to interactions between the signaling pathways involving the *TRA* and *FEM* genes (Eberle and Banks 1996). Combining these observations, we suspect that the trigger of sporophyte development is conferred from mature and functional archegonia.

Apospory, the formation of gametophytes from vegetative tissue of sporophyte leaves, can be induced readily in many ferns by culturing sporophyte leaves on medium without or with low concentrations of added sugars (Hirsch 1975; Raghavan 1989; DeYoung et al. 1997). Because aposporous gametophytes are fertile and they have the same chromosome number as the parental sporophytic tissue, they provide a means of inducing polyploidy series (Walker 1979). DeYoung et al. (1997) used induced apospory in *C. richardii* to generate autotetraploid sporophytes and used this system in genetic analyses of mutations affecting gametophyte development. With the goal of identifying genes that play a role in apospory, we set out to optimize the conditions for apospory induction in *C. richardii*. Apospory could be induced using detached leaves on various concentrations of sucrose, glucose, or trehalose. It was found that basal medium (0.8% agarose supplemented with 0.5 × Murashige and Skoog salts at pH 6) supplemented with 0.01% sucrose was optimal for the induction of apospory. Assays using leaves of various ages from young plants revealed that leaf 1 from 7-day-old sporophytes was most likely to undergo apospory. In addition, removing ethylene released by the plants increased aposporous gametophytes by 15%. Under this condition, 35% of excised sporophyte leaves formed at least one aposporous gametophyte after 49 days. Such gametophytes are fertile and produced tetraploid progeny that survived to produce spores.

Endowed with two independent generations and now with the establishment of induced apogamy and apospory, *C. richardii* offers a versatile system to join other model plants for understanding the development and evolution of alternation of generations in embryophytes.

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Chapter 4
Sexual Reproduction in Ferns

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4.1 Introduction

Sex expression in plants is a fundamental process and it is particularly important for economic reasons, especially in the case of commercially important crops (Tanurdzic and Banks 2004). Throughout a sequence of events, gender of gametes is set up in the corresponding sexual organs or cells. Sexuality in plants ranges from hermaphrodite individuals, in which female and male sexual organs are in the same flower, until dioecious species, where male and female flowers are produced in separate individuals. A great part of plants are hermaphrodites, while dioecy is not very frequent.

Different sex-determining mechanisms have been found in plants. In *Marchantia polymorpha* and *Silene latifolia*, sexual phenotype depends on the presence of sex chromosomes. In other species such as *Zea mays* and *Cucumis sativa*, it occurs through hormonal regulation. In some ferns, the gender of gametophyte is influenced by the epigenetic action of chemical compounds named antheridiogens (Tanurdzic and Banks 2004). Subsequently, a brief reminder about some features of these sex determinants in some plant models are given. The role of sex chromosomes in sex determination have focused, basically, on the moss *Marchantia polymorpha* and the dioecious species *Silene latifolia.* In the moss, sex is determined by different sex chromosomes (Lorbeer 1934; Grant et al. 1994, Label-Hardenack and Grant 1997). Sex determination in plants may be controlled also by plant growth regulators. The role of plant growth regulators in sex expression has been demonstrated by two different means: (a) exogenous administration of growth regulators to the developing plants, and (b) analyses of endogenous phytohormones (Duran and Duran 1984; Chailkyan and Khryanin 1987). In the light of these data, gibberellins and cytokinins seem to be involved in the expression of male or female sex in the flowering plant species. In particular, the predominance of cytokinins favored female-sex expression and that of gibberellins contributed to male-expression (Khryanin 2002).

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The effect of plant growth regulators on sex determination has been deeply studied in maize. In this plant, male and female flowers are in different locations in the shoot: staminate flowers in the terminal inflorescence, and pistillate flowers developing in lateral inflorescences. Sex determination in maize is achieved by the selective abortion of pistil primordial in developing staminate flowers and of stamen primordial in pistillate flowers. It has been reported that abortion of these organs are regulated separately, being monoecy in this plant depended on the correct functioning of two independently operating genetic pathways (Irish 1999). Applications of gibberellins or changes of growth conditions that result in an increase of GAs levels, tend to feminize the male flowers (Phinney 1981; Phinney and Spray 1982), suggesting that GAs promote pistil development and suppress stamen development. A pair of antagonistic genes, Silkless1 and Tasselseed2, are responsible for feminisation and masculinisation, respectively. The female active Silkless1 produces a gibberellin-like, pistil-specific factor that inhibits stamen formation and promotes pistillate florets (Dellaporta and Calderon-Urrea 1994). Finally, epigenetics mechanisms, especially DNA methylation, are known to have an important role both in flower setting and in sex organ formation (Vyskot et al. 1993; Janoušek et al. 1996; Vyskot 1999; Spielman et al. 2001).

4.2 Looking for New Experimental Systems

Sex determination in plants is a result of the expression of the haploid gametophyte genome. In the most widely studied plants, seed plants, the haploid gametophytic phase of the plant life history is greatly reduced and completely dependent upon the diploid sporophytic phase. Analysis of gender determination in seed plants is therefore greatly complicated by sporophytic effects on the expression of the gametophyte genome. Goldberg et al. (1993) have suggested that there are about 25,000 genes expressed in a tobacco anther at the time the microspore nucleus begins to divide, of which about 10,000 are anther specific. Therefore, it is a very difficult task to elucidate which genes are gametophytic genes whose products are involved in gender determination and which are sporophytic genes whose products affect the gametophytic genes and genes products.

During the last century, several green organisms from algae to angiosperms, have been proposed as experimental models to carry out studies in fields such as physiology, biochemistry, genetics, or developmental biology (Pryer et al. 2002). Among the features that make candidates adequate for these purposes are short generation times, small size, large number of offspring, cross ability, and relative ease of manipulation in laboratory, greenhouse, or field conditions. The haploid phase of ferns has shown some important advantages for use in biological studies such as to be easy of handling in "tissue culture," ready availability of material, easy of microscopic observations, very simple growth and differentiation compared with other photosynthetic eukaryotes, making gametophyte suitable for leading studies in areas such as developmental control of sexual reproduction as it has been proposed here. During the last decade, a

certain degree of knowledge has been accumulated about how to proceed to culture *in vitro* these plants for different purposes (Fernández and Revilla 2003).

The fern *Ceratopteris richardii* represents a useful experimental system for carrying out studies in different processes of plant development (Hickok et al. 1995). In this species, sex determination is epigenetically controlled by the pheromone antheridiogen, which is secreted by the hermaphrodite and straights male development of young, sexually undetermined gametophytes. The separation of male and female individuals in this species has contributed greatly to analyze the genes that control maleness and femaleness under the action of antheridiogen (Banks 1999).

4.3 Sexual Reproduction in Pteridophyta

When the formation of new individuals occurs by sexual mechanisms, the formation of sexual organs: female or archegonium and male or antheridia, takes place in the gametophyte. Two major categories of mating systems may occur in the ferns, intergametophytic and intragametophytic (Klekowski 1969). Intergametophytic mating is the fusion of gametes from two separate gametophytes, which may have been arisen from the same sporophyte or different individuals. Intragametophytic mating is the fusion of gametes produced by a single gametophyte, which results obligatorily in a completely homozygous zygote.

Although current evidence suggests that the majority of homosporous ferns will undergo intragametophytic selfing, there seem to be gametophytic adaptations which increase or decrease the probability of this phenomenon. According to Klekowski (1969), these adaptations may be morphological, populational, and genetical. Among the morphological it is the sequence of the ontogeny of gametangia. The initial formation of antheridia followed by the attainment of a prolonged hermaphroditic phase is an adaptation for intragametophytic selfing and it is probably the most frequent sequence found in these plants. Other possible sequences increase the probability for intergametophytic mating such as the formation of archegonia first and the later attainment of hermaphroditism in many of the studied species in the Blechnaceae (Klekowski 1969), among others.

In all homosporous pteridophytes, the lability of sex expression is considered the rule, Korpelainen (1998). A typical example of how sex is determined by environmental conditions is the epigenetic action exerted by the antheridiogen compounds, which promote also intergametophytic matings and the maintenance of genetic variation.

4.3.1 Antheridiogens

The choice between sex expressions may be determined by the presence of the gibberellin-like hormone antheridiogen secreted by neighboring gametophytes.

Since the initial discovery by Döpp (1950) of an antheridiogen system controlling sex expression in the gametophytes of *Pteridium aquilinum*, the majority of homosporous pteridophytes tested have been found to produce and to respond to antheridiogens (for reviews, see Voeller 1964; Näf et al. 1975; Yamane 1998). All the antheridiogens characterized so far are derived from Schizaeaceous ferns, they are all gibberellin-related compounds, and include GA_{109} (Wynne et al. 1998), GA_{73} methyl ester (Takeno et al. 1989; Yamauchi et al. 1996), antheridic acid (Yamauchi et al. 1991), 3-epi-GA_{63}, and 3α-hydroxi-9,15-cycle-GA_9 (Yamauchi et al. 1995). Partial characterization of an antheridiogen of *Ceratopteris* indicated that it is a small hydrophobic molecule with a molecular mass of 300 D (Koitabashi 1996), similar to other GAs.

Identification of the chemical nature of antheridiogens starting either from prothallia or culture medium, involves a sequence of steps. In case of starting from prothallia, they are homogenized and extracted with methanol. The methanol extract is concentrated under reduced pressure, and the resultant aqueous residue is fractionated to give an acidic and neutral fractions (Yamauchi et al. 1997). Both of them are purified and fractionated using high performance liquid chromatography (HPLC), antheridiogen activity of fractions evaluated, and finally analyzed by mass spectrometry.

In the lack of these substances, the fastest-growing gametophytes become hermaphroditic (*Ceratopteris*) or female (*Blechnum*), and they will produce and excrete the pheromone to the culture medium, which will act on the slower ones, that will be antheridial. Antheridiogen makes a situation in which many male gametophytes exist around a small number of female prothallia; that is, the biological role of antheridiogens in homosporous ferns is to minimize intragametophytic selfing, which would increase genetic risk and to favor intergametophytic mating (Takeno and Furuya 1987). On the other hand, gametophytes carrying archegonia may increase their fitness through the secretion of antheridiogens by increasing the pool of available males from which a mate may be chosen (Wilson 1981). Besides, antheridiogens may be important also for intra- and inter-specific competition (Schneller et al. 1990). Large gametophytes are more likely to bear zygotes and support young sporophytes, and through the production of antheridiogens may inhibit potential competitors. Finally, the response to antheridiogens enhances the reproductive success of small gametophytes by allowing for sex expression (maleness), which would not occur otherwise (Hamilton and Lloyd 1991).

4.3.2 Plant Growth Regulators and Sex Determination in Blechnum spicant L

B. spicant belongs to one of the most ancient groups of ferns. Sexual development in the gametophyte of this species is carried out through the epigenetic action of the unknown antheridium-inducing substances. When cultured *in vitro*, initially the gametophyte develops female sexual organs or archegonia, and produces and

excretes antheridiogens into the medium, which induce the formation of male sexual organs or antheridia in the youngest gametophytes, which are filamentous or spatulate-shaped (Klekowski 1969; Cousens 1979; Fernández et al. 1997).

The chemical nature of antheridiogen in this species is under investigation. Until now, the main antheridiogen activity present in the culture medium, which has supported growth of gametophytes for 3 months, was found in a fraction which included the most apolar compounds present in the extract (Fernández et al. 1999). As it was commented before, the chemical nature of antheridiogens has been related to gibberellins and the role of gibberellins in determining sex in the gametophyte of this species was studied through the effect of exogenous GA_3 and GA_{4+7}, by quantitation of the endogenous levels of GA_1, GA_3, GA_4, GA_7, GA_9, and GA_{20} in male and female gametophytes, and, finally, through the effect of flurprimidol, a GAs biosynthesis inhibitor of the steps of oxidation of ent-kaureno to ent-kaurenoic acid (Menéndez et al. 2006a). The results showed that GA_{4+7} had a slight effect of inducing either male and female sexual organs, antheridia and archegonia, respectively; the endogenous GAs content was not significantly different between sexes; and neither antheridiogen biosynthesis nor antheridia formation was inhibited by flurprimidol. Furthermore, it seems that GAs do not have a significant role in determining sex or in the biosynthesis of the antheridiogen in *B. spicant*. Banks (1999) had paid attention to the fact that antheridiogen signaling pathway would be defined by more than five genes as saturation of this phenotype had not been achieved, which contrasted with the few genes in *Arabidopsis* that are thought to define the GA signaling pathway. The same author suggested that the pheromonal function of antheridiogen could be detached from the hormonal function of endogenous GAs. The results of reproductive responses by gametophytes of *B. spicant* reinforce this suggestion. Certainly, more research is needed to establish a good correlation between antheridiogen and gibberellins.

The role of cytokinins on sex expression was also studied in the gametophyte of *B. spicant* (Menéndez et al. 2006b). It has been reported by these authors that the cytokinin 6-benzylaminopurine (BA) is a strong inductor of maleness in homogenized cultures of gametophytes, which were entirely composed of small-size gametophytes full of antheridia. Analyses of endogenous levels of the cytokinins trans-zeatin (tZ), cis-zeatin (cZ), trans-zeatin-riboside (cZR), dyhidrozeatin (DHZ), dyhidrozeatin-riboside (DHZR), isopentenyladenine (iP), and isopentenyladenosine (iPA) were carried out in male and female gametophytes. The levels of tZ, cZ, cRZ, DHZ, iP, and iPR were higher in females gametophytes than in males, being especially high those of iP and iPR (Menéndez et al. 2009). In the scarce reports on the influence of particular cytokinin metabolites on sex expression, there are some works in which a correlation between cytokinins and gender is found. In the dioecious species *Leuceadendron rubrum*, the content of the cytokinin iP increases in female plants (Dekock et al. 1994). In the most extensively studied system *Mercurialis*, where a number of genes control sex expression, accumulation of the nucleotide iP and high levels of zeatin-free base was detected in females (Duran and Duran 1990). Recently, studies on over-expressed zeatin O-glucosilation genes demonstrated the formation of tassel seed in the homozygous transformants in

maize, revealing a link between cytokinins and sex-specific floral development in monocots (Pineda Rodó et al. 2008).

4.4 New Tools to Study the Molecular Basis of Sex Determination

Despite increasing research efforts on a number of different plants species, there is relatively low information available on the molecular basis of sex determination and it is even difficult to estimate the number of genes involved. Until recently, two main approaches have been adopted in attempts to isolate sex determining genes from plants: using homologues of genes known to be involved in flower development in hermaphroditic model plants such as *Arabidopsis* or *Anthirrhinum*, and using cloning strategies involving enrichment for sex chromosome sequences or enrichment for sex-linked transcripts (Ainsworth 2000). We are beginning to understand how these genes evolved and how reproductive organ development occurs in different groups of plants. Comparative studies will, in future, provide a new insight into mechanisms of sexual organ development.

An excellent genetic work was conducted during the last decade to identify genes that are involved in the sex-determining process in this species (Banks 1999). The obtained results have revealed several phenotypic classes of mutations that alter the sex of the gametophyte and a simple model of the sex-determining pathway associated with antheridiogen signaling in *Ceratopteris* has been proposed by this author. This model included two major classes of sex-determining genes, one that promotes maleness (the FEM gene) and another that promotes femaleness (the TRA genes). As each also repress, or mutually excludes the expression of the other, only one class of gene (FEM or TRA) can be expressed in an individual gametophyte. What determines which of these two predominates in the gametophyte depends on antheridiogen, which activate the HER genes (Banks 1999). Assuming the validity of these and other hypotheses to explain how male and female gametophytes are formed will require cloning and molecular analyses of the sex-determining genes.

During the past years, an explosion in the development of new techniques in cell biology as well as in Biomics has occurred. Biomics are defined by Chen (2008) to include genomics, proteomics, metabolomics, glycomics, glycoproteomics, and any other body-wide molecular study. Proteomics is slowly gaining some confidence among plant biology researchers (Rose et al. 2004; Carpentier et al. 2005, 2008; Jorrín-Novo et al. 2009). One reason is based on the notion that the proteome reflects the expression of the molecules that more directly influence cellular biochemistry. Besides, another considerable value is the ability to isolate subcellular protein fractions and gain insight into subcellular localisation or function, even isolate protein complexes whose constituent polypeptides can not be predicted from DNA sequences or mRNA abundances. To date, most of the plant proteomic studies can be divided in two basic categories. The first one is Descriptive proteomics, a protein profiling of biological material with the objective of separating, sequencing and cataloging as

many proteins as possible. Here a complementary strategy with the aim of reducing the protein complexity of a particular extract is to target subcellular proteomes. The second basic category can be termed Comparative Proteomics or Differential Expression Proteomics, where the objective is to characterize differences between different protein populations. In this category, similar to comparative DNA microarray profiling, we can include proteins from tissues at different developmental stages or following responses to external stimuli. Other different areas such as Posttranslational Modifications, Interactomics, and Proteinomics are being developed.

Therefore, in our lab we have first applied a Differential Expression Proteomics Strategy to study the sexual differentiation in the gametophyte of *B. spicant*, which is influenced epigenetically by antheridiogen as mentioned before. Once the experimental design is done, proteins of male and female gametophytes are separately extracted and the samples are prepared following a specific created extraction method. These steps of extraction and sample preparation are the most critical in any proteomic study, so that the protein extraction procedure is of great importance. The ideal extraction method should reproducibly capture the most comprehensive repertoire of protein possible and minimizing degradation and contamination by non-protein compounds. Plant tissues are not easy to work with, because plant cells have a relatively low amount of proteins and are rich in proteases and oxidative enzymes. Another problem is that plants produce a broad spectrum of secondary metabolites (phenolic compounds, starches, oils, wall polysaccharides…) that contaminate and interfere the protein extraction. Besides, the plant cell wall not only complicates cellular lysis, but also is made of polysaccharides in which a proportion of cellular proteins can be irreversibly lost from extracts during tissue homogenization.

Based on the experience gained in our laboratory, an extraction method has been developed, because the most common protein extraction protocol, based on precipitating proteins from homogenized tissue with TCA in acetone did not work appropriately. In this phenol-based method, gametophytic tissue is frozen and grinded into a fine powder. For optimal results, maximum homogenisation is needed and the tissues should be kept frozen during this step. The grinded tissue is suspended in extraction buffer and then phenol saturated is added. Compounds such as polysaccharides and other water-soluble contaminants remain in the aqueous phase or are centrifuged into a pellet, away from the protein-enriched phenolic layer. To precipitate the proteins, ammonium acetate is used and, after washing, the obtained pellet with methanol and acetone, the protein pellet, is air-dried and ready-to-use.

This phenol based method is more time consuming and laborious than other protocols used in plants, but is the only method that gave positive results in gametophytic tissues, perhaps due its potential for retaining salts and polysaccharides, generating high purity samples. The air-dried pellet is then solubilised in rehydration buffer and insoluble material was removed by centrifugation. The protein concentration can be determined using colorimetric assays (for example using Bradford Reagent with ovoalbumin as a standard). If samples are not going to be used immediately, they can be stored at −80°C.

The use of 2-Dimensional Gel Electrophoresis, in combination with appropriate software has given us a simple and reliable technique for finger-printing crude plant

extracts. In *B. spicant*, immobilized pH gradients (IPG) strips pH 3–11 NL were used for isoelectric focusing. The strips were rehydrated with the samples for 12 h and then loaded onto an Isoelectric Focusing System. This system passes an electric current through the polyacrilamide strip, creating a positive "anode" and a negative "cathode" end. A protein that is in a pH region below its isoelectric point (pI) will be positively charged and so will migrate towards the cathode. As it migrates through a gradient of increasing pH, the protein's overall charge will decrease until the protein reaches the pH region that corresponds to its pI. At this point, it has no net charge and migration ceases. As a result, the proteins become focused at a point in the pH gradient corresponding to its pI. The technique is capable of extremely high resolution with proteins differing by a single charge being fractionated into separate bands. Isoelectric focusing is the first step in two-dimensional gel electrophoresis. Further, proteins are separated by their molecular weight through second dimension SDS-PAGE. It was carried out with lab cast 1.5 mm SDS polyacrylamide gels (13%).

Gels were stained with Colloidal Coomassie Blue G-250 following the method described by Mathesius et al. (2001) and images were immediately acquired with a densitometer. Digitalised gel images need to be analyzed with adequate software, although a spot-by-spot visual validation of automated analysis is recommended to increase the reliability of the matching. pI and molecular weight of the proteins are calculated using standards (Chich et al. 2007).

Proteomic data are characterized by a high dimensionality because the great number of variables (proteins spots) and a high dispersion of the dataset due to the low number of replicates. At this end, the employment of statistical tools for improving the data analysis and validation of the results is very important.

After gel staining, imaging, and analysis, we have observed differentially expressed spots. Spots were manually excised from the gel and to identify the cognate proteins, mass spectrometry approaches are used, (MALDI-TOF) MS or (ESI)MS/MS depending on the situation (Chen 2008). The proteins resulting are expected to be useful to describe first time the main metabolic pathways dealing in the sex determination mediated by the pheromone antheridiogen in the fern *B. spicant*.

2-DE is by far the predominant separation technology and is continuously being improved. The potential of proteomics in plant biology research is far from being fully exploited compared to other systems. Only a little fraction of the cell proteome has been characterized so far and only for a few biological systems (human, fruit fly, *Arabidopsis*, rice) and even for them, the function of quite a number of proteins remains to be investigated. It is not uncommon to find low confidence protein identification in the literature, especially in the case of unsequenced "orphan" organisms (ferns are this type of organisms) and this is one of the most important challenges that face these technologies. At this moment, the use of gel-free second-generation proteomic techniques is still anecdotal in Plant Proteomics studies.

In any case, proteomic analysis need to be validated and compared with those obtained by using transcriptomics, cell biology, and classical physiological and biochemical approaches, especially when we are dealing with proteomic analyses

from nonmodel plants or with poorly characterized genomes. As a result of the combination on high throughput- omics and classical biochemical and cellular biology techniques, the coverage of the plant cell proteome and the plant biology knowledge is increasing. Plant proteomics is beginning to make some practical contributions to applied fields including biomedicine and agronomy. We hope Proteomic studies in ferns will help in the understanding of mechanisms involved in plant reproduction and could contribute to gain insight on plant development, either for basic and practical purposes, especially when we are dealing with proteomic analyses from non model plants or with poorly characterized genomes.

4.5 Sexuality in the Laboratory and in Nature

Because gametophytes of most species are easy to grow in culture, many studies have been conducted on cultured gametophytes about different topics such as the sexual expression/ontogeny (Klekowski 1969), antheridiogen production and response (Stevens and Werth 1999), etc., and then numerous studies have employed data from lab-cultured gametophytes to make inferences about mating systems operating in nature (Soltis and Soltis 1990a; Chiou et al. 1998; Li and Haufler 1999). Unfortunately, a few studies have shared genetic data from natural populations of sporophytes with laboratory data from cultured gametophytes to support the relationship between gametophytic features and levels and patterns of populational genetic variation.

Genetic diversity of two fern species, the outcrossing *B. spicant* and the apogamic *Dryopteris affinis* (Lowe) Fraser-Jenkins ssp. *affinis* was measured through AFLP (see chapter 16). In gametophytes of *B. spicant* cultured *in vitro* an antheridiogen system promoting outcrossing has been reported (Klekowski 1969; Cousens 1979; Fernández et al. 1997). In the field, it may reproduce both sexually and vegetatively, connecting individuals by rhizome (Korpelainen and Pietiläinen 2008). In turn, in the gametophyte of *D. affinis* the formation of sporophyte occurs via apogamy, i.e. without the intervention of sexual organs (Fernández et al. 1996; Menéndez et al. 2006c). For each species, plants growing in three separated sampling sites were analyzed (unpublished). To avoid problems of repetition of samples due to the possible re-sampling of the same individual (i.e. rhizome mediated asexual reproduction), a safety distance was kept between each selected individual. AFLP analysis was performed according to Vos et al. (1995) with some modifications. The AFLP protocol includes a double digestion with endonucleases, a ligation–digestion step, and two amplification cycles: pre-selective and selective. The genetic diversity observed in *D. affinis* was very low and medium level in *B. spicant*. Most of the genetic variance detected in *D. affinis* was attributed to differences among localities indicating high fixation of alleles within each locality. In *B. spicant* we did not detect any cluster structure suggesting a continuous genetic flow among the localities. The very low genetic variance detected in the populations of *D. affinis* correlates with the existence of an apogamic

process governing the formation of new sporophytes. Concerning *B. spicant*, levels of genetic variation within populations obtained by AFLP were similar to those derived from electroforetic analyses (Soltis and Soltis 1990b), and always intermedia between low genetic values (those corresponding to the apogamous species) and high genetic values (those similar to data obtained for seed plants). In the light of these data, we might suppose the existence of vegetative propagation in the field and not to discard mechanisms promoting outcrossing among gametophytes, as predicted by cultures done in the laboratory.

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Chapter 5
Gibberellic Acid and Ethylene Control Male Sex Determination and Development of *Anemia phyllitidis* Gametophytes

Andrzej Kaźmierczak

5.1 Main Aspects of *A. phyllitidis* Gametophyte Development

Anemia phyllitidis (L.) Sw. is an epiphytic homosporous fern, which is classified either under Schizaeceae (Mickel 1982) or under Anemiaceae family (forming schizaeoid branch of phylogenetical tree; Schuettpelz and Pryer 2007). Spores of *A. phyllitidis* (Fig. 5.1a) do not contain chlorophyll but contain lipid and protein storage grains (Rutter and Raghavan 1978; Raghavan 1993). Haploidic nuclei (1C) of gametophyte cells with 38 chromosomes (Mickel 1982; Fig. 5.2b) contain about 11.4 pg of DNA (Kaźmierczak 2004). According to "paleoploid theory" *A. phyllitidis* is a tetraploid (Mickel 1982) organism, however, genetic studies suggest that ferns with high chromosome numbers are diploid (Haufler and Soltis 1986; Pichersky et al. 1990). Microcytophotometry shows that in *A. phyllitidis* gametophytes formed from less than 100 cells, over 50% of them contain 1C DNA. The remainder cells contain 1–2C, 2C, and about 3% are endopolyploid (more than 2C DNA; Kaźmierczak 1998, 2003a, 2010). In gametophytes consisting of 200 (at male stage) and 300 cells (without sexual organs), about 4% and 10.5% of them are endopolyploid, respectively. Up till now, the highest detected endopolyploidy level in *A. phyllitidis* gametophytes has been 6C (Fig. 5.3h; Kaźmierczak 2010). Profile area (PA; surface of a median optical section through a cell) of 60–80% of cells is bigger than 1100 μm^2. This CPA (cell profile area) is a "critical cell size" because smaller cells can divide while bigger ones endoreduplicate. The correlation between cell size and its polyploidisation level was detected only for about 12% of the cells bigger than 1,100 μm^2. The biggest CPA is about 13,000 μm^2. Endoreduplication in *A. phyllitidis* gametophytes is a function of gametophyte age, size, and cell numbers as well as type of morphogenesis (Kaźmierczak 2010) and concerns changing the proliferation activity of cells into postmitotic differentiation in which the nuclei with functional copy genes are eliminated from gametophytes by programmed cell death (Kaźmierczak 2008, 2010).

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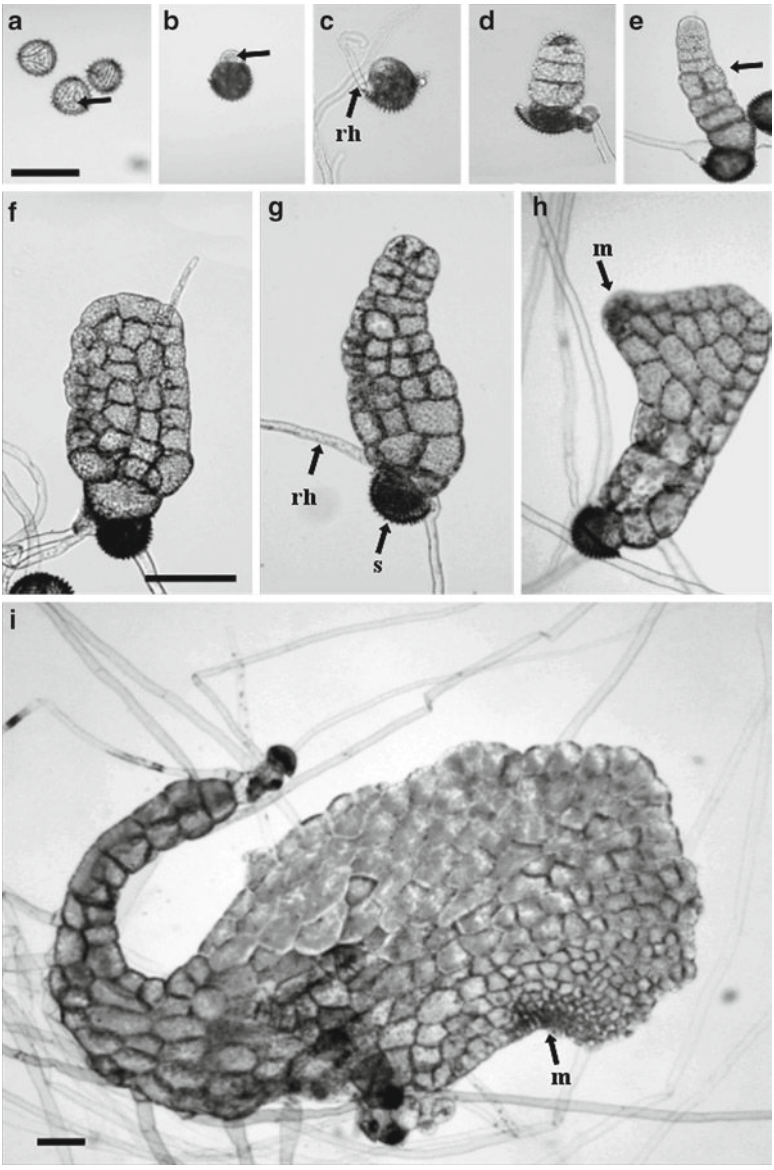

Fig. 5.1 Micrographs of *Anemia phyllitidis* spores before germination (**a**) and during germination with protonemal initial cell (**b**), during formation of initial rhizoid cell (**c**), during the first steps of filamentous growth (**d**), starting two-dimensional growth (**e**), and two-dimensionally growing (**f**, **g**) with lateral meristem (**h**) and in heart-shaped form (**i**). rh, rhizoid; m, meristem; s, spore. Unlabelled arrows indicate the structures mentioned in the text. Scale bars (100 μm) in (**a**) is applied to (**b–e**) while in (**f**) is applied to (**g**, **h**)

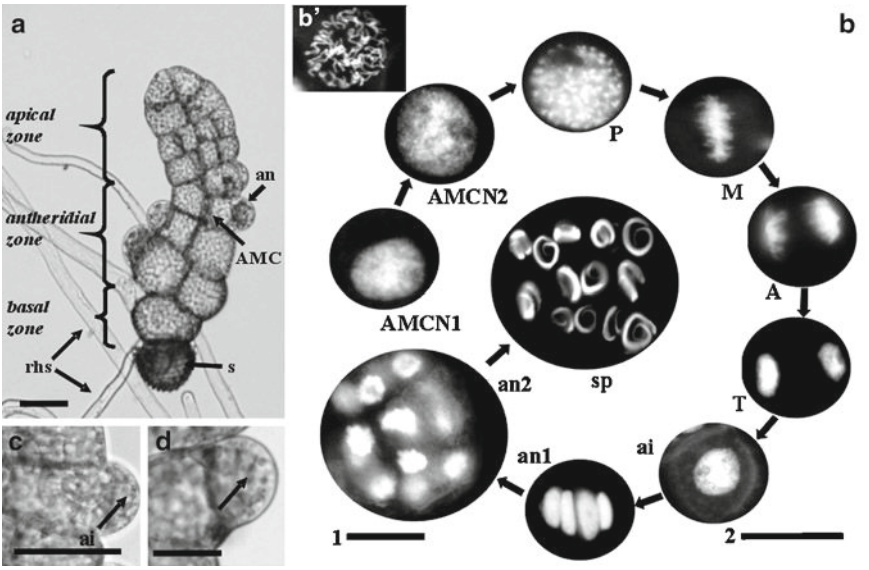

Fig. 5.2 "Three-zonal model" of *Anemia phyllitidis* gametophyte after 4-day treatment with GA_3 (**a**). Mitotic division (**b**) of nucleus of an antheridial mother cell (AMCN1, AMCN2) (**c**) with 38 chromosomes (**b'**) and four-celled stage antheridium (**d**). AMCN – nucleus of antheridial mother cells, *P* prophase, *M* metaphase, *A* anaphase, *T* telophase, *ai* antheridium initial, *an* antheridium, *an1* four-celled antheridium, *an2* antheridium in proliferation phase of spermatogenesis, *rhs* rhizoids, *s* spore, *sp* spermatozoids. Scale bars in (**a**, **c**) = 50 μm, in (**d**, **b**, **b'**) = 20 μm. The scale bar in (**b**) mark as "1" is applied to an2, sp while the "2" is applied to P, M, A, T, ai, and an1

5.2 Spore Germination, Growth, and Differentiation of *A. phyllitidis* Gametophytes

Spores of *A. phyllitidis* germinate in water breaking sporoderm in a triangle furrow aperture (Fig. 5.1a), according to "Anemia" type of germination (Raghavan 1989). The first, asymmetrical division perpendicular to the polar axis of the spore produces protonemal (Fig. 5.1b) and basal cell while the second division of the basal cell produces a rhizoid initial cell (Rutter and Raghavan 1978) and then a rhizoid (Fig. 5.1c). The divisions parallel to the first division of the spore produce a protonemal cell whose further divisions cause filamentous growth of a gametophyte (Fig. 5.1d; Näf et al. 1975; Kaźmierczak 2006). Perpendicular and symmetrical division of a singular gametophyte cell (Fig. 5.1e) induces the switch of gametophyte growth from filamentous to two-dimensional (Fig. 5.1f, g). This process is connected with the orientation of a cell plane of division which is in accordance with the concept of "minimal cell surface area," where the nonapical cells in filamentous gametophyte acquire length/width ratios below 1.0 (Cooke and Paolillo 1980). Then *A. phyllitidis* gametophytes grow and develop producing quickly growing meristematic asexual form (Fig. 5.1h) and then hermaphroditic or female

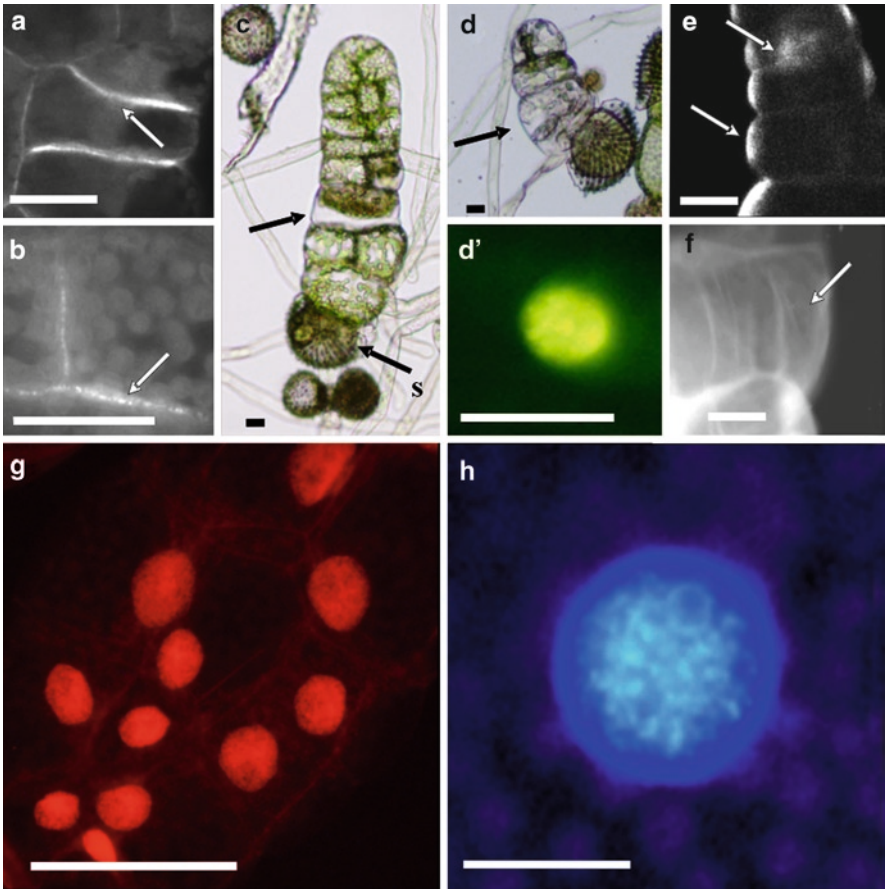

Fig. 5.3 Micrographs of *Anemia phyllitidis* gametophytes of divided (**a**) and differentiated (**b**) cells with fluorescently stained callose as well as gametophytes with one (**c**) or all (**d**) atrophied cells dying in programmed manner (**d'**). Cellulose spots (**e**) and microfibrils of cellulose arranged in perpendicular (**f**) direction in relation to the growth axis of antheridial mother cell as well as Feulgen- (**g**) and DAPI-stained (**h**) nuclei for microcytophotometrical determination of DNA content in *A. phyllitidis* gametophytes. Arrows indicate the structures mentioned in the text and in above figure description. Scale bars in (**a–f**), (**h**) = 20 μm and in (**g**) = 50 μm

and slowly growing ameristematic form (Näf et al. 1975). The lateral (Fig. 5.1h) and then notch meristem (Fig. 5.1i) form a heart-shaped gametophyte. Meristem cells differentiate into antheridia (male sex organs Fig. 5.2 c, d) producing spermatids (Fig. 5.2b, sp) and/or into archegonia (female sex organs) producing ovary cells (Kaźmierczak 2006). In the male gametophytes, nearly all their cells may produce antheridia (Näf et al. 1975). The number of each sexual type representatives in population of homosporous ferns depends on density of individuals in the culture (Warne and Lloyd 1986; Huang et al. 2004). High density (over 66 individuals cm^{-2}) promotes mostly asexual gametophytes, intermediate (12–41 individuals cm^{-2} or 6–37 individuals cm^{-2}) – mostly male or hermaphroditic, respectively, while low

density (1–3 individuals cm^{-2}) promotes female ones. Gametophyte size also depends on the sexual type. The female ones are bigger than hermaphroditic and male gametophytes while the asexual are the smallest ones (Huang et al. 2004). This clearly indicates that the sexual type of gametophytes in population is the simple function of accumulation of antheridiogens in the culture and it is also true for the development of *A. phyllitidis* gametophytes.

5.3 The Antheridiogens – Epigenetic Aspects of Development of *A. phyllitidis* Gametophytes

The most important group of factors controlling spore germination, development, and sex differentiation of fern gametophytes are antheridiogens (also named pheromones). These are endogenous substances secreted into a medium by quickly growing hermaphrodite and female gametophytes (Yamane 1998). These pheromones were identified or hypothesized in many ferns, including *Aspidiaceae*, *Blenchaceae*, *Parkeriaceae*, *Pteridaceae,* and *Schizaeaceae* (or *Anemiace and Lygodiaceae*) families (Miller 1968; Näf et al. 1975; Tanurdzic and Banks 2004). Antheridiogens show different species-specific ability to induce spore germination and male sex. This was used in the "cross reaction" (Näf et al. 1975; Chiou and Farrar 1997; Yamane 1998), and results of this cross reaction allow to classify them into three groups (Kaźmierczak 2006). (I) Highly species-specific antheridiogens, activated only in the gametophytes of their own species, e.g.: *Ceratopteris richardii*, *C. angustifolium, C. thalictroides, Lygodium japonicum,* and *Onoclea sensibilis.* (II) Antheridiogens with double species-specificity, biologically active towards their own and one other species (its name in brackets), e.g.: *Dryopteris filix-mas* (*P. aquilinum*), *Blechnum gibbum* (*O. sensibilis*), and *A. phyllitidis* (*L. japonicum*). (III) Antheridiogens of *Pteridium aquilinum* (A_{Pt}) and *Pteris vitatta* (A_{Ps}) are polyspecific, they induce antheridiogenesis in at least 38 fern species (Chiou and Farrar 1997), e.g.: *C. angustifolium*, *Ceratopteris phyllitidis*, *Lepisorus thunbergianus*, *Microgramma heterophylla*, *Phymatopsorus scolopendria*, *Polypodium pellucidum*, *O. sensibilis*, and *Phlebodium aureum.*

According to the results of many bio-tests with the use of gibberellin synthesis inhibitor (AMO-1618; Näf et al. 1975; Raghavan 1989; Yamane 1998; Tanurdzic and Banks 2004), of TLC (thin-layer chromatography), HPLC (high pressure liquid chromatography) and of full mass-spectrometry (Nakanishi et al. 1971; Yamane 1998), it seemed that all antheridiogens of homosporous ferns might belong to the big group of plant hormones, gibberellins (Miller 1968; Raghavan 1989; Yamane 1998; Tanurdzic and Banks 2004). However, the chemical structure has been described only for 12 antheridiogens (Yamane 1998). Antheridic acid (A_{An}), produced by *A. phyllitidis* gametophytes was the first chemically (Nakanishi et al. 1971; Corey et al. 1986) and biosynthetically characterized as well as synthesized (Corey and Myers 1985; Yamauchi et al. 1997). Antheridiogen is produced from GA_{103} through GA_{107}, Me-GA_{73}, $GA_{24,}$ and GA_{73} precursors (Näf et al. 1975) also in *A. rotundifolia*, *A. flexusa*, and *L. japonicum* (Yamane 1998). In

A. phyllitidis gametophytes the A_{An} is also produced with 3α-hydroxy-9, 15-cyclo-GA_9 and 3-epi-GA_{63} as precursors, which show slightly lower activity than A_{An}. Gametophytes of *A. mexicana* produce GA_{104} as the main antheridiogen in that fern while gametophytes of *L. japonicum, L. circinnatum*, and *L. flexuosum* also contain Me-GA_9 (methyl ester of GA_9) and Me-GA_{73} (Yamauchi et al. 1997) and derivatives of two above mentioned gibberellins (Yamane 1998; Mander 2003). Gametophytes of *A. phyllitidis* additionally contain GA_9, GA_{25} (Mander 2003), while *L. circinnatum* contain GA_9, GA_{20}, GA_{70}, GA_{88}, *3-epi*-GA_{88} (Yamauchi et al. 1996).

Activities of antheridic acid include: (1) mimicking the red light during induction of dark grown spore germination, (2) induction of male sex organ formation (antheridia) mimicking red and blue light and (3) inhibition of female sex formation (archegonia). In *A. phyllitidis* gametophytes antheridiogenesis-inducing activity of antheridiogens may be mimicked by gibberellins from higher plants (Schraudolf 1964; Raghavan 1989) and their activity of antheridia formation is as follows: $GA_7 = GA_4 > GA_1 > GA_3 > GA_9 = GA_5 = GA_8$ (Schraudolf 1964), while their activity of inhibition of female sex determination is: $GA_4 = GA_9 > GA_7 > GA_3 > GA_1 = GA_5 = GA_8$ (Raghavan 1989). Antheridiogenesis-inducing activity of natural gibberellins was observed only in fern species of *Anemia, Lygodium, Schizaea, Mohria*, and *Vittaria* (Yamane 1998). Gibberellic acid (GA_3) which is most similar in structure and functions to antheridic acid (Corey and Myers 1985; Corey et al. 1986; Yamane 1998) is frequently used to study sex determination and development of *A. phyllitidis* gametophytes (Schraudolf 1964, 1966; Yamane 1998; Kaźmierczak 2006).

5.4 Precocious, GA_3-induced, Antheridia Formation and the "Three-zonal Model" of Structure and Function in Development of *A. phyllitidis* Gametophytes

Gametophytes of *A. phyllitidis* are very useful to study the developmental concept of all plants. Using *A. phyllitidis* gametophytes, H. Schraudolf (1964) found that GA_3 induced antheridia formation and intensity of this process depended on developmental state and concentration of GA_3 and was independent of the growth rate (Schraudolf 1966). The level of GA_3-induced antheridiogenesis decreases proportionally to the number of gametophyte cells (Schraudolf 1964; Näf et al. 1975), so the sensitivity of gametophytes to GA_3 increases with the growing number of gametophyte cells. This relationship is characterized by a "critical cell number" (ccn; Schraudolf 1966). These results agree with the data obtained during permanent microscopic observations of gametophytes growing in the medium with 10^{-5} M of GA_3 concentration, in which the ccn value was 4.7 (Kaźmierczak and Maszewski 1998).

GA_3 induces antheridia and changes the number of cells as well as cell and gametophyte size (Schraudolf 1966; Kaźmierczak 1998, 2003a). These facts were the

basis to make an effort to study many aspects of precocious GA_3-induced antheridiogenesis in young *A. phyllitidis* gametophytes. Time-course experiments and morphometrical measurements show that in 12-days-old *A. phyllitidis* gametophytes 4-days GA_3 (30 μM) treatment induces 2.5–2.8 antheridia per thallus and changes cytomorphological parameters of growth leading to the formation of specific morphologically varied picture (Fig. 5.2a) in which: (1) cell number from 15 to 20; (2) thalli length from 240 μm to 370 μm and (3) cell size expressed in CPA (cell profile area) from 500 μm^2 to 900–2900 μm^2 increase (Kaźmierczak 2003a, 2006).

The morphological picture of *A. phyllitidis* gametophytes consists of three distinctive groups of similar cells. This picture was called a "three-zonal model" (Fig. 5.2a): "basal zone" – consists of approximately 20% of the cells which are involved in the formation of rhizoids and differ in growth rate and sensitivity to GA_3; "antheridial zone" – consists of 35% of the centrally located gametophyte cells, similar in size, responsible for growth of a young gametophyte. These cells are sensitive to GA_3, which induces their transverse growth, changing the width-to-length (W/L) ratio which reaches 1.6 value (Kaźmierczak 2003a) and then initial antheridial cells which transform into antheridia (Fig. 5.2a, b, c, d). "Apical zone" – containing 45% of small, actively dividing, and evenly growing gametophyte cells (Kaźmierczak 2003a, 2006).

Formation of GA_3-induced antheridia is the result of asymmetrical (Raghavan 1989; Kaźmierczak 2006; Fig. 5.2a, c, d) mitotic division (Fig. 5.2b – P, M, A, T) of an antheridial mother cell (AMC; Fig. 5.2a, b – AMCN1, AMCN2; Kaźmierczak 2004) leading to the formation of an antheridial initial (ai) cell (Fig. 5.2c, b). Division of an antheridial initial cell gives rise to a bottom and a top cell (Kaźmierczak 2006). The former differentiates into a basal shield cell while the latter divides producing a spermatocyte nucleus and a cell which subsequently divides into shield apical and ring cells. In *A. phyllitidis* gametophytes the first four-celled antheridia (an1) appear after 24 h of GA_3 treatment (Fig. 5.2b, d; Kaźmierczak 2006). Synchronous divisions of spermatocyte cells (an2; Fig. 5.2b') during proliferation phase of spermatogenesis lead to the establishment of a number of spermatids (sp; Fig. 5.2b', about 16–32). Spermiogenesis (the second phase of spermatogenesis) consists mainly in the reorganization of spermatozoid chromatin including exchange of histones into richer in arginine protamines (Reynolds and Wolfe 1984) and formation of numerous flagella and a spirally coiled nucleus (Wolniak et al. 2000).

5.5 ACCF and DDG Disturb GA_3-Induced Antheridiogenesis and Development of *A. phyllitidis* Gametophytes

Maturing male sex organs of *Chara tomentosa* contain low molecular (4.5 kDa) peptide "antheridial chromatin condensation factor" (ACCF) which is capable of inducing increased condensation of chromosomes and profound changes in the cell cycle progression (Kaźmierczak and Maszewski 1998). This factor changes development of GA_3-treated gametophytes lowering division of their cells leading to lower number of cells and antheridia (Kaźmierczak and Maszewski 1997). Comparative analyses indicate that the activity of ACCF may be interpreted as

inhibitory influence acting *via* repression or modification of the genetic device of the cells rather than a direct consequence of the retardation of cell division cycles (Kaźmierczak and Maszewski 1997, 1998).

Disorganization of plasmodesmata cell to cell communication systems (Lucas and Lee 2004; Kaźmierczak 2008) also present in ferns (Tilney et al. 1990) and of glucose metabolism and/or signaling pathways (Harthill et al. 2006) may also disturb development of *A. phyllitidis* gametophytes. Fluorescence microscopy of aniline blue staining gametophytes (Fig. 5.3a, b) with shows that DDG (2-deoxy-D-glucose; inhibitor of callose synthesis), decreasing callose amount, disturbs gametophyte development leading to cell atrophy (Fig. 5.3c) and cell death by the programmed cell death (Kaźmierczak 2008; Fig. 5.3d). This phenomenon is connected with the elimination of callose from cell plates of dividing cells and of callose granulations (Fig. 5.3b) from plasmodesmata. Thus, it disturbs the signaling pathways inhibiting cytokinesis, cell growth, and young gametophyte development leading to their atrophy (Fig. 5.3d) and disrupts cell and gametophyte differentiation as well as antheridiogenesis (Kaźmierczak 2008).

5.6 How Does Ethylene Participate in Development and Male Sex Determination in *A. phyllitidis* gametophytes?

GA_3-dependent male sex determination in *A. phyllitidis* gametophytes partially resembles the induction of male sex in *Cucumis sativus* plants in which gibberellin controls male sex while ethylene (plant growth regulator regarded as a sex hormone; Metzger 1995) is responsible for female sex (Yamasaki et al. 2000). Studies with *O. sensibilis* showed that treatment of spores with ethylene blocked DNA synthesis, inhibited movement of the spore nucleus (Fisher and Miller 1978) thus inhibiting spore (Fisher and Shropshire 1979) and rhizoid division whereas stimulated elongation of dark-grown gametophytes (Miller et al. 1970).

These facts inspired studies on the role of ethylene in development of *A. phyllitidis* gametophytes. Ethylene is produced from methionine *via* S-adenozylmethionine (SAM) and ACC (1-aminocyclopropane-1-carboxylic acid; Wang et al. 2002) by nearly all plant organisms: angiosperms, ferns (*P. aquilinum*, *Matteuccia strutiopteri,* and *Polystrichum munitum*; Tittle 1987), mosses (*Funaria hygrometrica*; Rohwer and Bopp 1984) and lichens and also by some microorganisms (Bleecker and Kende 2000).

To study ethylene participation as well as ethylene/gibberellic acid cooperation in development and morphogenesis of *A. phyllitidis* gametophytes in the different experimental arrangements, ACC and the precursor of ethylene synthesis (L-methionine, MET; Bleecker and Kende 2000), the inhibitors of ACC (AOA, aminooxyacetic acid; Wang et al. 2002) and ethylene ($CoCl_2$; Bleecker and Kende 2000) synthesis and ethylene perception (silver ions, Ag^+; 2,5-norbornadiene, NBD; Bleecker and Kende 2000) were used.

5.7 Regulation of Antheridia Formation

Regulation of transverse expansion of an antheridial mother cell is the crucial element of GA_3-induced antheridia formation. Antheridia induction takes place only in the presence of GA_3 and depends on transverse expansion of the antheridial mother cells with a stable growth index expressed as W/L ratio (≥1.6). According to this rule, the 10 μM ACC nearly doubles (from 2.8 to 5.7; Kaźmierczak 2003b) the number of GA_3-induced antheridia with reduction of gametophyte cell number in comparison to GA_3-treated gametophytes. MET and Co^{2+} ions with GA_3 increase the number of GA_3-induced antheridia by about 40% per gametophyte (Kaźmierczak 2005). AOA strongly inhibits GA_3-induced antheridiogenesis and fern gametophyte development. It is mainly due to the inhibition of elongation and transverse growth of the cells (Kaźmierczak 2004). Two of the ethylene perception inhibitors (Ag^+, NBD) have opposite effects on GA_3-induced antheridia formation. Ag^+ ions enhance the number of antheridia by about 40% per one gametophyte (Ag^+ alone does not induce antheridia) while NBD inhibits GA_3-induced antheridiogenesis completely (Kaźmierczak and Kaźmierczak 2009). The cell size and W/L ratio of individual antheridial mother cells are the same as in the GA_3-induced system, although statistically the numbers of formed antheridia are different because of the higher number of cells in the antheridial zone of gametophytes (Fig. 5.4a).

5.8 Regulation of Cell Growth and Cell Division Cycle during GA_3-induced Antheridiogenesis

Accumulating evidence suggests that plant cell size may be determined at the organ level through a total organ-size checkpoint, which balances cell growth and cell proliferation. Thus, decreased cell proliferation is compensated by increasing cell size to keep constant organ size. However, increase or decrease in cell proliferation seems not to influence cell size (Sugimoto-Shirasu and Roberts 2003).

Cell growth and expansion are mediated by plasma membrane-associated cortical microtubules (Nick 1999) which in cell walls, together with actin, determine orientation of the cellulose microfibrils in cell walls. Gibberellins and ethylene regulate both processes (Metzger 1995; Martin et al. 2001) by independent (Collett et al. 2000), or cooperative activities (Rijnders et al. 1997; Tadeo et al. 1997; Voesenek et al. 2003; Calvo et al. 2004). Arrangement of cellulose microfibrils in a cell wall reflects the direction of cell growth. Oblique orientation of cellulose microfibrils indicates that cells reach the end of elongation phase while modification from oblique to perpendicular orientation (in relation to the main axis) reflects a shift of cell expansion into transverse direction; while randomly organized cellulose shows inhibition of cell growth (Voesenek et al. 2003; Vriezen et al. 2003).

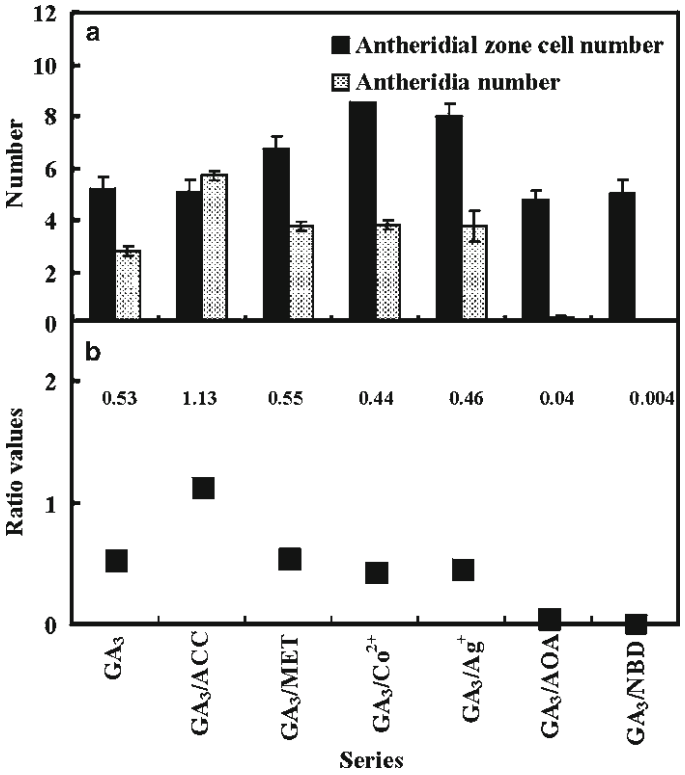

Fig. 5.4 Number of cells in antheridial zone of *Anemia phyllitidis* gametophyte and number of antheridia (**a**) and ratio between them (**b**)

Antheridiogenesis as well as development of *A. phyllitidis* gametophytes depend on specific regulation of cell expansion and their growth as well as cell division (Kaźmierczak 2006). Cell growth in the gametophytes treated with GA_3 alone as well as with GA_3/ACC, GA_3/MET, GA_3/Co^{2+}, GA_3/Ag^{+} is controlled similarly. Calcofluor White M2R staining shows that cellulose in cell walls is organized in clusters and spots. During GA_3-induced antheridiogenesis and that enhanced by ACC application together with GA_3 (GA_3/ACC series) cellulose transforms *via* dispersed (Fig. 5.3e) to fibrillar form (fibrills layered in oblique and perpendicular array in relation to the transverse direction of cell expansion; Fig. 5.3f Kaźmierczak 2004, 2007). The numbers of cells with perpendicular and oblique type of cellulose arrangement were similar to the average number of antheridia. In the series in which antheridiogenesis and development of fern gametophytes are inhibited, both random and longitudinal arrangements of cellulose microfibrils as well as oblique can be observed (Kaźmierczak 2004, 2007).

Changes in sugar metabolism are the background of induction of the transverse expansion of antheridial mother cells. Low level of free glucose and high of that released by α-amylase- and endoglucanase suggest a high ratio of energetical

turnover of simple sugars (Kaźmierczak 2007), synthesis of starch in a great number of chloroplasts (Kaźmierczak 1998) and/or relaxation of hemicellulose (xyloglucan-like or xylan-like) polymers (with small amount of xylose or cellobiose; Kaźmierczak 2007) by xyloglucan endotransglucosylases (XET) and endo-1,4-glucanase (KOR) responsible for tethering structural elements of cell wall (Bleecker and Kende 2000).

It seems, likely that in *A. phyllitidis* gametophytes, as in higher plants (Bleecker and Kende 2000), induction of transverse growth of cells depends on ethylene action while cell elongation is regulated by both ethylene and GA_3 (Kaźmierczak 2004, 2007).

The microcytophotometry of Feulgen- (Fig. 5.4g; Kaźmierczak 1998, 2003a) and DAPI (4,6-diamidinophenylindole, 1 $\mu g \cdot ml^{-1}$)-stained (Fig. 5.4h; Kaźmierczak 2010) nuclei shows that most (over 50%) of vegetative cells of *A. phyllitidis* gametophytes containing less than 100 cells are at the G1 phase of the cell cycle (Kaźmierczak 2003a, 2010). Nuclei of young (12- and 16-days-old) *A. phyllitidis* gametophyte cells show significant variety among particular phases of the cell cycle mainly in the antheridial and apical zones. In the apical zone the cells in G1, S, and G2 phases constitute 75%, 17%, and 8%, respectively, and in the antheridial zone 45%, 30%, and 25%, respectively, (Kaźmierczak 2003a).

GA_3 induces increase in the nuclei profile area (NPA; Kaźmierczak 1998, 2003a, b) which is a morphological sign of induction and continuation of transcription and/or replication (Marshall 2002). Changes of NPA observed after GA_3 application are the most intensive in cells becoming the antheridial mother cells (Fig. 5.2a, b – AMCN1, AMCN2; Kaźmierczak 2003a). GA_3 induces over 30% (in 3rd h) increase in the number of S-phase cells indicating the induction of mitotic division of antheridial cells (Kaźmierczak 2003a). ACC in GA_3-treated gametophytes leads to 40% (in 3rd h) and then to 60% increase (in 12th h) in the number of S-phase cells, together with reorganization of nuclear chromatin. This induction stimulates division of antheridial mother cells as well as enhancement of the number of G2-phase cells in gametophytes thus leading to two-fold increment of antheridia number (Kaźmierczak 2003b).

MET- as well as Co^{2+}- and Ag^{+}-enhanced number of GA_3-induced antheridia is partially reflected in changes of the mitotic indexes (141%, 72%, and 1.7%, respectively) which show the percentage of dividing cells. Because of the higher number of cells in the antheridial zone (Fig. 5.4a), the low rate of division in Ag^{+}/GA_3 series is high enough to increase the number of antheridia to 3.0 and 3.5 per gametophyte (Kaźmierczak 2005; Kaźmierczak and Kaźmierczak 2009).

AOA and NBD restrain antheridia formation as well as gametophyte development in GA_3-treated gametophytes *via* inhibition of cell divisions (mitotic index is about "0") with transient increase in number of S-phase cells, which is followed by the accumulation of G2-phase cells leading to the enhanced number of endopolyploid cells (Kaźmierczak 2004; Kaźmierczak and Kaźmierczak 2009). AOA inhibits cell division by inhibiting cell growth but not by inhibiting DNA synthesis promoted by GA_3. In GA_3/AOA-series gametophytes in antheridial zone contain 2, 3, 80, and 15% cells of G1-, S-, G2-, and endoploid cells, respectively (Kaźmierczak

2004). NBD inhibits cell division by inhibiting cell growth (Kaźmierczak and Kaźmierczak 2009) and GA_3-dependent DNA synthesis by arresting cells at the S-phase. In GA_3/NBD-series gametophytes in the antheridial zone contain 21, 67, 11, and 6% of G1-, S-, G2-, and endoploid cells, respectively (Kaźmierczak 2004). Thus, it seems that NBD is a more specific inhibitor.

The published results (Kaźmierczak 2003b, 2004, 2005; Kaźmierczak and Kaźmierczak 2009) indicate that ACC directly or indirectly, by ethylene, plays a regulatory role in GA_3-induced antheridiogenesis. The regulators of ethylene synthesis and perception directly influence cell growth and direction of cell expansion in the antheridial zone of gametophytes. Thus they limit cell division and suppress increase in the number of S-phase cells stimulated by GA_3 through induction cell division (Kaźmierczak 2003a, b).

5.9 *A. phyllitidis* Gametophytes Produce ACC

Detection of ACC in *A. phyllitidis* gametophytes might be the final explanation of the role of ethylene in antheridiogenesis. Indeed, capillary electrophoresis system shows the presence of ACC in *A. phyllitidis* gametophytes (Kaźmierczak and Kaźmierczak 2007). Analyses of ACC content in *A. phyllitidis* gametophytes in different experimental configurations: GA_3 (GA_3 series) and GA_3 completed with AOA (AOA/GA_3 series), $CoCl_2$ (Co^{2+}/GA_3 series), 2,5-norbornadiene (NBD/GA_3 series), or $AgNO_3$ (Ag^+/GA_3 series) show that most important changes in ACC content occur during the first 12 h of experiments. GA_3 induces ACC content increase in *A. phyllitidis* gametophytes with maximum in the 3rd and in the 12th h (Fig. 5.5). Interactions between the tested substances significantly change ACC content, which fluctuates similarly in AOA/GA_3 and NBD/GA_3 series with the maximum in the 6th h and in Co^{2+}/GA_3 and Ag^+/GA_3 series with the maximum in the 6th h and two minima in the 3rd and 9th h, respectively (Fig. 5.5). Thus the 3rd h seems to be an "antheridiogenesis induction hour" switching the development from vegetative to generative program while the 6th h can be "antheridiogenesis inhibition hour" (Kaźmierczak and Kaźmierczak 2009).

5.10 Main Aspects of Antheridiogenesis

It has been found out that:

1. *A. phyllitidis* gametophyte grows according to the "three-zonal model" which allows to study the GA_3-dependent physiological and metabolical aspects of development.
2. Antheridiogenesis in *A. phyllitidis* gametophytes consists of two phases: "induction" (0–12 h) and "expression" (24–96 h; Kaźmierczak 2007; Kaźmierczak and Kaźmierczak 2009). In the "induction phase" an antheridial mother cell

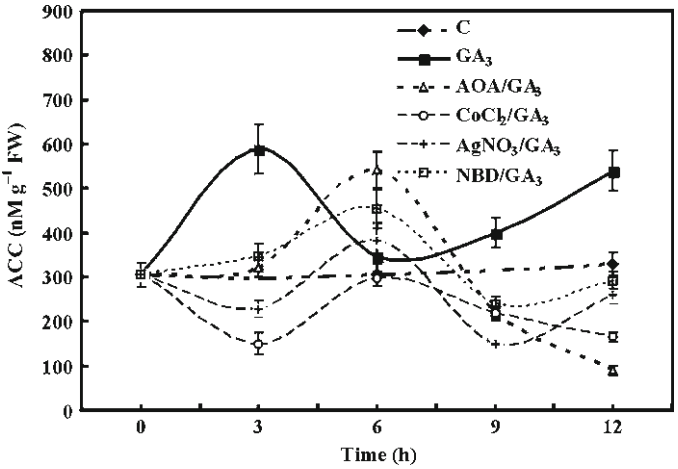

Fig. 5.5 Changes in ACC level (nM g^{-1} FW) during first 12 h of *Anemia phyllitidis* gametophytes culture in untreated series as well as treated with GA_3 (GA_3 series) or with GA_3 completed with 100 μM AOA (AOA/GA_3 series), 50 μM $CoCl_2$ (Co^{2+}/GA_3 series), 100 μM $AgNO_3$ (Ag^+/GA_3 series) or 10 μM NBD (NBD/GA_3 series)

differentiates (Fig. 5.2a, c, b') while in the "expression phase" antheridia differentiate (Kaźmierczak 2006).

3. In the "induction phase" of antheridiogenesis, the time course of ACC content elevation and DNA synthesis induction triggering cell cycle shows that regulation of antheridiogenesis in *A. phyllitidis* gametophytes is a time-dependent process. The 3rd h of the "induction phase" is the "hour of antheridiogenesis induction" while the 6th h is the "hour of antheridiogenesis inhibition" (Kaźmierczak and Kaźmierczak 2009).
4. On the basis of the number of cells in the antheridial zone (Fig. 5.4a) and the number of antheridia it is possible to form an antheridiogenesis index (Fig. 5.4b), which is the ratio between the number of antheridia and the number of cells in the antheridial zone of a gametophyte. The value of this index for GA_3/MET-, GA_3/Co^{2+}-, and GA_3/Ag^+-series is about 0.50 (Fig. 5.4b) and is similar to that for GA_3-series (0.53). This fact indicates that a gametophyte treated with GA_3 produces one antheridium per two antheridial mother cells. However, in GA_3/ACC-series, a gametophyte produces one antheridium per one antheridial mother cells (value of this ratio is 1.13; Fig. 5.4b). Thus, induction of male sex in the GA_3/MET-, GA_3/Co^{2+}-, and GA_3/Ag^+-series may be regulated by one while in GA_3/NBD- and GA_3/AOA-series and GA_3/ACC-series by other signaling and/or metabolic pathways (Kaźmierczak and Kaźmierczak 2009). These facts finally indicate that ethylene participates in male sex determination and controls the accompanying process.
5. Gibberellins and/or endogenous antheridiogens and ethylene cooperate in the induction of antheridia formation**.** Ethylene and gibberellin cooperation may depend on the regulation of their concentrations which is mediated by a

direct ethylene precursor, ACC (Kaneta et al. 1997; van der Knaap et al. 2000; Rijnders et al. 1997). ACC (or ethylene) may participate in antheridiogenesis and in development of *A. phyllitidis* gametophytes as the second messenger because without gibberellic acid no antheridia are formed (Kaźmierczak 2006; Kaźmierczak and Kaźmierczak 2009). GA_3 activates antheridiogenesis along two parallel pathways without or with cooperation with ethylene (Fig. 5.6). In the former, the cell cycle is directly induced by GA_3 activating specific CDK (cyclin dependent kinase) and cyclins (Kaźmierczak 2006), which probably stimulate antheridiogens synthesis thus (a) increasing PA of nuclei (Kaźmierczak 1998; 2003a, b, 2006); (b) inducing DNA synthesis thus switching the cell cycle (Kaźmierczak 2003a, b, 2004, 2008). In the latter pathway GA_3 induces elevation of ACC/ethylene amount, which regulates cell growth by (a) modulating sugar metabolism and energetical turnover (Kaźmierczak 2007, 2008); (b) changes in structural and functional reorganization of cell walls by changes in cellulose deposition

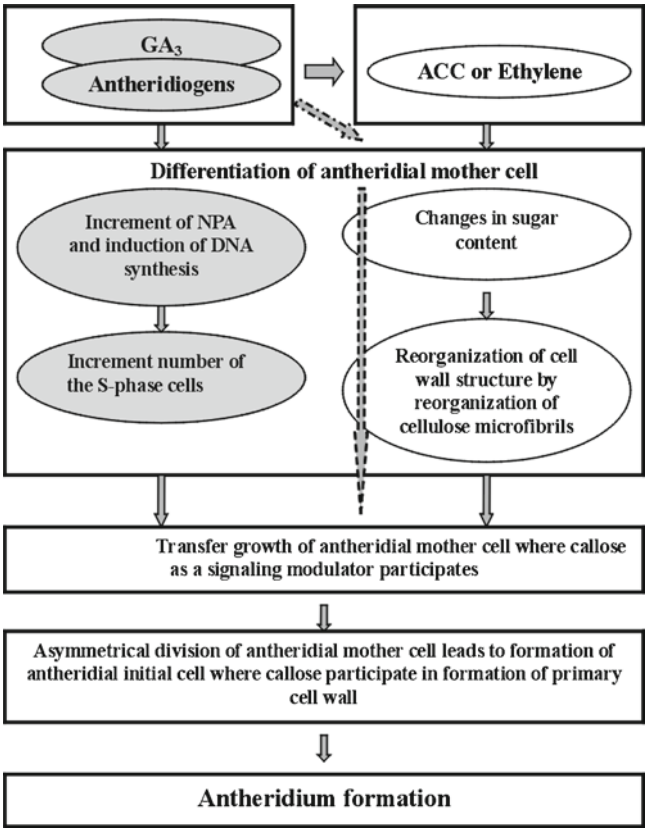

Fig. 5.6 Scheme of the regulation pathways of male sex determination in *Anemia phyllitidis* gametophytes

and in cellulose microfibrils arrangement (Kaźmierczak 2007); (c) transverse expansion of cells (Kaźmierczak 2003a, b, 2006, 2008). It is possible that GA_3 may independently of "ethylene pathway" change sugar metabolism and organization of cell walls (Fig. 5.6, doted line; Kaźmierczak 2004, 2007). The proper synchronization of both pathways finally leads to asymmetrical division of antheridial mother cells and formation of antheridial initial cells and then antheridium where the callose may influence modulation of gibberellin signal transduction and cytokinesis (Fig. 5.6).

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Chapter 6
The Sporophytes of Seed-Free Vascular Plants – Major Vegetative Developmental Features and Molecular Genetic Pathways

Alexandru M.F. Tomescu

6.1 Introduction

Seed-free vascular plants, collectively and informally referred to as pteridophytes, span most of the taxonomic breadth of vascular plant diversity above the order rank, yet all seed-free lineages taken together are considerably less speciose than the seed plants (spermatophytes). The latter are sharply distinct from the seed-free plants in sharing a unique fundamental sporophyte feature, a body plan dictated by two major developmental characteristics: bipolar growth that originates in a cotyledonary embryo, and axillary branching of the shoots. In contrast, seed-free plants encompass a much broader spectrum of sporophytic body plans and they are much more diverse from a developmental standpoint, a situation reflected in the breadth of their taxonomic span.

Numerous formal taxonomic schemes have been proposed in plant systematics (e.g., Judd et al. 2007; Cantino et al. 2007; Chase and Reveal 2009). They represent differing views and encompass a considerable range of differences in terms of the underlying systematic principles and nomenclatural rules they employ. This broad range of options can generate confusion regarding the legitimate formal name and rank of taxonomic entities above the genus rank. Since taxonomy is beyond the scope of the present volume, I opted for simplicity and equidistance, using a system of informal names, which do not carry any taxonomic rank significance, to designate the different groups of seed-free vascular plants, which are defined as follows. Lycopsids include the extant lycopodiales (club mosses and *Phylloglossum*), selaginellales (spike mosses) and *Isoetes* (quillworts), as well as several extinct lineages (protolepidodendrales, lepidodendrales, pleuromeiales); *Isoetes* and its closest fossil relatives, the lepidodendrales and pleuromeiales are grouped in the isoetalean clade (Stewart and Rothwell 1993). Psilotopsids comprise only two extant genera, *Psilotum* (whisk fern) and *Tmesipteris*. The pteropsids ("ferns") consist likely of three major lineages, of which two are extinct and one includes extant representatives.

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Extant pteropsids comprise the eusporangiate ophioglossales (*Ophioglossum*, *Botrychium*, *Helminthostachys*, *Mankyua*) and marattiales (*Marattia*, *Angiopteris*, *Archangiopteris*, *Christensenia*, *Danaea*), and the leptosporangiate filicales (with numerous genera) and hydropteridales (*Marsilea*, *Regnellidium*, *Pilularia*, *Salvinia*, *Azolla*). Sphenopsids are represented by only one living genus, *Equisetum* (horsetail), but have a rich fossil record including the sphenophyllales and calamitales.

A major divide in the phylogeny of vascular plants, recognized very early (Haeckel 1866), is generally well agreed-upon (Rothwell and Nixon 2006). It separates the lycopsids from a clade (the euphyllophytes) comprising all other pteridophytes and the seed plants; depending on whether their organography is considered highly simplified or plesiomophic (see discussion below), the psilotopsids are either included or excluded from the euphyllophyte clade, respectively. On the lycopsid side, monophyly of the group, as well as its close relationship with the zosterophylls (an extinct, probably paraphyletic group of vascular plants with simple organography), are strongly supported (Kenrick and Crane 1997). On the euphyllophyte side, the positions of deep internal nodes are disputed between two competing phylogenetic hypotheses. An apparently robust phylogeny based exclusively on extant taxa (Pryer et al. 2001) proposes that all extant seed-free euphyllophytes form a clade (the moniliformopses) that is sister to the spermatophyte clade. This widely popular hypothesis is apparently supported by the exclusively fossil-based phylogeny of Kenrick and Crane (1997).

However, on closer look, the analysis of Kenrick and Crane, which was the study that proposed the moniliformopses clade, is based on a very different taxon sampling compared to Pryer et al.'s (2001) study: the taxonomic affinities of the three fossils used by Kenrick and Crane as placeholders for pteropsids and sphenopsids are equivocal at best (Rothwell and Nixon 2006); and no seed plant is included in their analysis. Consequently, the apparent overlap between Kenrick and Crane's (1997) and Pryer et al.'s (2001) moniliformopses, and between the moniliformopses-spermatophyte clades found by the two studies, has no real support. Moreover, using a combination of extant and extinct taxa, and of molecular and morphological characters, Rothwell and Nixon (2006) showed that Pryer et al.'s (2001) moniliformopses is not a well-supported clade. In a competing hypothesis based on extinct and extant taxa and using morphological characters, Rothwell (1999) proposed that seed-free euphyllophytes form a paraphyletic grade at the base of the seed plants, with sphenopsids nested as the sister group of an exclusively extinct pteropsid clade. Thus, for the moment, as pointed out by Rothwell and Nixon (2006), the phylogenetic structure of the euphyllophyte tree remains controversial. A phylogeny based on the synthesis of phylogenetic trees supported in the analyses by Rothwell (1999) and Hilton and Bateman (2006) is presented by Tomescu (2009).

In this chapter, I present a comparative survey of vegetative sporophyte features emphasizing the differences between the various extant lineages of seed-free vascular plants and between those and seed plants, and I review the state-of-the-art knowledge of developmental genes in the sporophytes of the seed-free lineages of vascular plants. Evolution is the result of changes in developmental characteristics which are controlled by genes. Understanding evolution is therefore a matter of understanding the molecular genetic pathways that control development, and the

way these pathways have changed over time. The taxonomic patterns of distribution of shared and divergent developmental pathways point to phylogenetic relationships which reflect evolution. A deeper understanding of these pathways in seed-free plants is crucial for improved phylogenetic resolution, especially at deep nodes, and would, thus, greatly illuminate our understanding of evolution.

6.2 Six Sporophyte Body Plans

The seed plant sporophyte body plan originates in a cotyledonary embryo with two poles of growth, the radicle and the epicotyl. This polarization of the embryo results in a bipolar growth pattern that produces a positively geotropic root system, which branches laterally from the endodermis, and a negatively geotropic shoot system with axillary lateral branching. In contrast to the shared seed plant sporophyte body plan, growth patterns in the various groups of seed-free vascular plants produce six different sporophyte body plans. These are characteristic of the lycopodiales, selaginalleales, isoetaleans, psilotopsids, sphenopsids, and pteropsids. The body plans of these six groups are differentiated by organography, growth polarity, and branching characteristics (Table 6.1).

The sporophytes of seed-free vascular plants originate in noncotyledonary embryos, which may or may not (i.e., psilotopsids and isoetales) have an embryonic root pole. Even when present, the embryonic (primary) root is short-lived and growth of the mature sporophyte proceeds in a unipolar pattern exclusively from the shoot apical meristem which produces both positively geotropic roots (which are all adventitious) and negatively geotropic shoots. An exception from the unipolar growth pattern seen in the majority of seed-free plants is found in *Isoetes* and its fossil relatives (isoetaleans). Growth of isoetalean sporophytes is bipolar, with both poles of growth represented by shoots. Studies of living and fossil isoetaleans have shown that dichotomous branching of a unique embryonic pole of growth early in development produces the two poles of growth of the mature sporophyte. One of these two poles produces the shoot system, whereas the other produces a positively geotropic rooting system, the rhizomorph. The rhizomorph is, thus, a modified shoot bearing appendages ("rootlets") that are leaf homologs. The evidence leading to these interpretations has been assembled and discussed thoroughly by Rothwell and Erwin (1985). Interestingly, these rhizomorph appendages have co-opted some of the root developmental programs leading to the development of a root cap and apical dichotomous branching.

The selaginellales present another particular body plan that adds a fourth organ to the common list of three vegetative organs of the plant sporophyte (stem, leaf, and root). The rhizophore is a naked axis, which branches apically and dichotomously and mixes characteristics of roots and stems (Jernstedt et al. 1994; Lu and Jernstedt 1996; Imaichi 2008). Rhizophores originate exogenously from stem branching points, they lack a root cap and can sometimes produce leaves, all of which represent stem characteristics, but most usually they bear roots. In addition

Table 6.1 Seed-free vascular plants exhibit six different sporophyte body plans defined by organography, growth polarity, and the branching mode of shoots and roots (first four columns of the table). Other features that distinguish the different lineages are the structure of apical meristems and stele morphology. Based on Campbell (1911), Eames (1936), Paolillo (1963), Bierhorst (1971), Ogura (1972), Rothwell and Erwin (1985), Gifford and Foster (1989), Lu and Jernstedt (1996)

	Stem-leaf-root organography	Polarity of growth	Shoot branching	Root branching	Shoot apical meristem	Root apical meristem	Shoot/axis stele type
Lycopodiales	Present (primary embryonic root, then exclusively adventitious roots)	Unipolar	Apical dichotomous	Apical dichotomous	2–3 and sometimes multiple apical cells	Multiple apical cells	Protostele (actinostele, plectostele)
Selaginellales	Present (primary embryonic root, then exclusively adventitious roots) Rhizophore Ligulate leaves	Unipolar	Apical dichotomous	Apical dichotomous	Single apical cell in some species, 2 or more apical cells in others	Multiple apical cells (in root and rhizophore)	Protostele (actinostele, plectostele)
Isoetaleans	Present Roots absent Rhizomorph: shoot modified for rooting Ligulate leaves	Bipolar	Apical dichotomous	–	Single apical cell, but more often multiple apical cells	–	Protostele (medullated^{+})
Psilotopsids	Absent	Unipolar	Apical dichotomous axes	undifferentiated	Single apical cell		Protostele (medullated)
Sphenopsids	Present (primary embryonic root, then exclusively adventitious roots)	Unipolar	Lateral, non-axillary	Lateral	Single apical cell	Single apical cell	Equisetostele Protostele^{+}

Pteropsids	Present (primary embryonic root, then exclusively adventitious roots)	Unipolar	Apical dichotomous	Lateral	Single apical cell most often; some with multiple apical cells	Single apical cell most often; some with multiple apical cells	Protostele Siphonostele Eustele[+]
Spermatophytes	Present (primary embryonic root long lived)	Bipolar	Lateral, axillary	Lateral	Multiple apical cells, stratified apical meristem	Multiple apical cells	Eustele Protostele[+]

[+]Stele morphology known exclusively in extinct representatives of the lineage

to rhizophores, the selaginellales are also characterized by the presence of ligules on leaves, a feature that they share with plants in the isoetalean clade. The ligule is a specialized flap of tissue that develops on the adaxial side of each leaf. The function of the ligule has not been entirely elucidated, although it has been proposed that it keeps the leaf and sporangium primordia, as well as the shoot apical meristem, moist by secreting a mucilage that contains carbohydrates and proteins (Bilderback 1987; Gifford and Foster 1989).

The psilotopsids are characterized by a very simple body plan consisting of a system of undifferentiated branching axes bearing sporangia (grouped in synangia) and unvascularized (*Psilotum*) or vascularized (*Tmesipteris*) appendages that lack a definite arrangement. This body plan has been interpreted either as representing the plesiomorphic condition seen in the earliest vascular plants, or as an instance of extreme evolutionary simplification of the typical stem-leaf-root organography; the different competing interpretations are evaluated by Stewart and Rothwell (1993). The second interpretation received support from molecular phylogenetic studies (Pryer et al. 2001; Schneider et al. 2009) that have places psilotopsids among the seed-free moniliformopses (but see discussion of the validity of moniliformopses as a group above). Grounds for rejection of that interpretation are the complete absence of roots (even of an embryonic root pole) in *Psilotum* and *Tmesipteris*, and the lack of some or all defining leaf characters in the structures interpreted by some as reduced or modified leaves in *Tmesipteris* and *Psilotum*, respectively. Another intriguing side of the psilotopsid story is the complete absence of a fossil record for the group. This could be invoked in support of a secondarily derived simple organography, young age of the lineage and, hence, of the molecular phylogenetic placing of psilotopsids among crown-group euphyllophytes. However, such a placement would still imply anywhere between 136 and 56 million years of undocumented fossil history, based on the age of the oldest unequivocal fossils representing lineages proposed as sister groups to the psilotopsids in molecular phylogenetic studies – Early Cretaceous *Equisetum* (Stanich et al. 2009) and Late Paleocene *Botrychium* (Rothwell and Stockey 1989). Based on all the available evidence, I consider the organography of psilotopsids to reflect the same simple level of organization as that of the earliest vascular plants.

6.3 Embryogeny

Early work on seed-free plant embryogeny emphasized the orientation of the plane of zygotic division with respect to the archegonium, as well as cell lineage relationships between the two resulting cells, the octants of the early embryo, and the organs of the mature embryo and sporophyte. However, as early as the 1930s it became increasingly clear that such relationships are highly variable within major groups (as discussed by e.g., Wardlaw 1955; Johnson and Renzaglia 2009). A more interesting direction of focus in studies of embryogeny concerns the correlations between the polarity of the embryo and the polarity of the mature sporophyte.

In angiosperms, the division of the zygote is asymmetrical and produces a suspensor cell and a proembryo cell. A derivative of the suspensor cell, the hypophysis, subsequently gives rise to the radicle which later forms the root pole of the mature sporophyte. Derivatives of the proembryo produce the epicotyl which forms the shoot pole of the mature sporophyte. It can therefore be said that the fundamentally bipolar longitudinal patterning of the angiosperm sporophyte is determined by the first division of the sporophyte phase, the division of the zygote. If the same was true of seed-free plants, whose sporophytes exhibit a fundamentally unipolar structure, then we would expect the embryos of those plants to develop just one pole of growth in their earliest stage.

The embryos of all seed-free vascular plants have in common the foot, a temporary structure that is only indirectly involved in sporophyte growth by transferring nutrients from the maternal gametophyte to the embryo during early sporophyte development. Aside from the foot, the development of the embryo and the morphology resulting from it are highly variable among the different lineages, and even within lineages. Experiments on filicalean pteropsids have shown that embryos developed in incomplete archegonia or outside the archegonia fail to establish the normal set of embryonic organs (DeMaggio 1982). This suggests that the archegonium and the gametophyte provide cues crucial to polarity establishment in the embryo.

The psilotopsids, whose simple organography lacks roots altogether, conform to the concept of a unipolar embryo: their embryos have a foot and only one pole from which the axis grows. Interestingly, the embryo goes through a stage characterized by unipolar organization in the isoetaleans as well, although the mature body plan of those plants is determined by a bipolar growth pattern. As has been demonstrated in some fossil representatives (arborescent lepidodendrales), a unique embryonic pole of growth undergoes dichotomous division early in embryogeny to produce the two poles of growth that form the mature sporophyte body – the aerial shoot and the rhizomorph (Phillips 1979). Based on this, the isoetaleans can be regarded as primarily unipolar and only secondarily bipolar. However, early unipolar organization is not evident in *Isoetes* nor in some of the fossil lepidodendrales (reviewed by Stubblefield and Rothwell 1981), the embryos of which do not go through a unipolar stage and directly develop two poles of growth after the first leaf primordia (including those of leaf homologs for the rhizomorph pole) are produced. Given the embryogenetic pattern documented in the arborescent lepidodendrales, the embryogeny of *Isoetes* could reflect the fact that the unipolar stage of the embryo is skipped altogether as a result of extreme reduction in this genus, which undergoes very little elongation throughout its sporophytic life.

The lycopodiales, selaginellales, sphenopsids, and pteropsids, all of which have unipolar growth as mature sporophytes, have embryos that develop two poles of growth, a shoot pole and a root pole, aside from the foot. However, this lack of correlation between the polarity of the embryo (bipolar) and that of the mature sporophyte (unipolar) is only apparent. In these plants, the earliest polarity event, which precedes the establishment of the embryonic shoot and root poles, and sometimes occurs as early as the two-celled embryo stage, is the specification of two domains of which only one is involved in polar growth and generates the entire mature structure of the

sporophyte, while the other forms the foot. Thus, at this stage, the embryo is unipolar. In some groups (lycopodiales, selaginellales and some ophioglossalean and marattialean pteropsids), the division of the zygote specifies a suspensor cell and a proembryonic cell. Although this embryogenetic pattern seems similar to that of seed plants, the suspensor of seed-free plants has no direct developmental contribution to the polarity of the mature sporophyte. The proembryonic cell follows the typical developmental pattern involving specification of a foot (contiguous with the suspensor) and a polar growth domain. Therefore, the unipolar structure of the early seed-free plant embryo is not affected by the presence of a suspensor. Moreover, it is possible that the suspensor in seed-free plants is just a modified region of the foot (Johnson and Renzaglia 2009). Based on their taxonomic occurrence, the suspensors of lycopodiales + selaginellales, pteropsids, and seed plants have likely evolved independently.

In light of the primary unipolar condition of the embryo in lycopodiales, selaginellales, sphenopsids, and pteropsids, the presence of a shoot pole and a root pole in the later embryos of these lineages may not reflect bipolar organization; a transitional bipolar condition intercalated between the unipolar early embryo and the unipolar mature sporophyte would be difficult to justify developmentally. The most parsimonious interpretation is that the embryonic root pole that develops in the later embryo in these groups is the first adventitious root produced by the embryonic shoot pole, mirroring the pattern of development of the mature sporophyte. If that were the case, then specification of the embryonic shoot pole should precede that of the root pole. A review of embryogenetic patterns (Wardlaw 1955; Bierhorst 1971 and personal observations) shows that this is the case in lycopodiales, selaginellales, and *Equisetum*. The pteropsids, much more diverse taxonomically, exhibit a diversity of embryogenetic patterns. *Botrychium*, some marattiales and some filicales (*Actinostachys*, *Todea*) differentiate an embryonic shoot pole prior to the root pole, whereas the embryos of *Ophioglossum*, some marattiales and most filicales specify root and leaf primordia prior to the shoot pole. However, even in embryos of this latter category, the embryonic root is ephemeral. Furthermore, the early establishment of vascular tissue connecting the embryonic root and first leaf primordium mirrors the close developmental relationship seen between leaves and adventitious roots in these pteropsids.

Together, these lines of evidence support the hypothesis that the embryos of lycopodiales, selaginellales, sphenopsids, and pteropsids are unipolar, just like the adult sporophytes, and that the ephemeral embryonic root, when present, represents the first adventitious root produced by the embryonic shoot apex. A similar point of view was expressed by Kaplan and Groff (1995) who viewed the primordial embryonic body of pteropsids as a shoot meristem bearing the embryonic leaf and root primordia as laterally emergent organs.

6.4 Apical Meristem Structure

The growing tips of seed-free vascular plants are characterized by unstratified apical meristems in which one or several apical initials, present at the surface of the meristem, are responsible for tissue generation. This plesiomorphic vascular plant

character is contrasting with the stratified nature of most seed plant apical meristems, which are differentiated into regions distinguished by their mode of growth (predominantly anticlinal cell divisions in the tunica, and divisions occurring in various planes in the corpus); some gymnosperms represent exceptions with unstratified apical meristems. The classic, textbook example of seed-free plant apical meristem is the apical cell-based meristem, where a single apical cell, usually tetrahedral (three cutting faces), but sometimes wedge-shaped (two cutting faces), generates all the tissues of the axis. However, meristems with multiple apical initials are present in several seed-free lineages.

Meristems with a single apical cell are found in lycopsid stems (some *Selaginella* and *Isoetes* species), in the axes of psilotopsids and in the stems and roots of sphenopsids, and most pteropsids (Table 6.1). Apical meristems with more than one apical initial are more frequent than single cell meristems in the lycopsids: stems and roots of the lycopodiales have two to three or more apical initials; in *Selaginella*, rhizophores and roots have multiple apical initials, and the stems of most species have two or more apical cells; the shoot apical meristem has multiple initials in most *Isoetes* species and the linear rhizomorph meristem always consists of many initials. Among the pteropsids, apical meristems with multiple initials are found in the stems of some ophioglossales and in the stems and roots of older marattialean plants and of osmundaceous filicales. Thus, apical meristem structure shows a significant divide between lycopsids, characterized principally by meristems with multiple initials, and psilotopsids + seed-free euphyllophytes, in which single apical cell meristems are predominant. Given this divide between the two major clades of vascular plants, and the lack of information on the structure of apical meristems in early vascular plants, it is difficult to infer whether the common ancestor of vascular plants had meristems consisting of single or multiple initials at the tips of its undifferentiated dichotomous axes.

6.5 Branching

The mode of shoot branching sets apart the seed plants, characterized by axillary branching, from seed-free plants, most of which have apical dichotomous branching. An often overlooked aspect of this major divide is that axillary branching is inextricably connected to the presence of leaves, whereas apical branching is entirely independent of leaves. In seed plants, all branches (except for adventitious branches) occur in leaf axils where they develop from axillary meristems. The fact that axillary branching is highly canalized developmentally in this way suggests that it may be controlled by the same mechanism across all seed plants. In angiosperms, axillary branching is underwritten by a mechanism that shows strong association with the adaxial domain of leaf development and controls the retention of meristematic capacity in a group of cells located in the leaf axillary region, as they become detached from the shoot apical meristem. These cells may then assume a meristematic identity at a later moment, or they may never lose the meristematic identity conferred by origination at the shoot apical meristem (Bennett and Leyser

2006). Molecular genetic studies of gymnosperm branching have yet to confirm the degree of conservation of this mechanism. Apical branching, on the other hand, proceeds by direct dissection of the apical meristem, whether it is single-celled or it has multiple initials. In single-celled meristems, it can also arise by differentiation of the apical cell and dedifferentiation of two new apical cells from protodermal cells on either side of it (Kato and Imaichi 1997).

Axillary branching is also thought to occur in rhizomes of *Botryopteris* and *Helminthostachys* of the ophioglossales (Petry 1915; Kato et al. 1988). In these taxa, meristems derived directly from the shoot apical meristem are found in leaf axils, where, upon injury of the rhizome apex, they develop into branches connected to the rhizome stele by two vascular traces that diverge from the two sides of the leaf gap. It is unclear whether this mode of branching is controlled by the same mechanism documented in angiosperms, but current understanding of plant phylogeny suggests that rather than indicating a close relationship between ophioglossales and the extinct progymnosperms, as suggested by Kato et al. (1988), the similarity of the two branching modes is due to convergent evolution, and thus, does not imply homology.

In both apical and axillary branching, branches are specified at the shoot apical meristem and arise from the outermost cell layers (exogenous branching). In contrast, *Equisetum,* and possibly, its fossil sphenopsid relatives, exhibit a very different mode of branching. Branching in *Equisetum* is lateral (as opposed to apical) but nonaxillary; it is, nevertheless, correlated to leaf production in that branches occur at nodes and are, therefore, always associated with leaves. However, instead of developing in leaf axils, *Equisetum* branches are produced between the bases of adjacent leaves in a whorl. More importantly, in *Equisetum*, branches are not specified at the shoot apex and there is evidence (Stutzel and Jaedicke 2000) that their origin may be endogenous (i.e., from tissues beneath the outer layers).

Another type of branching, documented in extant and extinct filicalean pteropsids (Troop and Mickel 1968; Tomescu et al. 2008), is epihyllous branching. Sometimes referred to as epipetiolar branching, it consists of the production of stems on leaves, usually close to the base of the petiole, but not in the leaf axil. Epiphyllous branching is regarded more as a form of reiteration and, therefore, not included in general discussions of the main modes of shoot branching. However, its common occurrence in certain taxa (e.g., dennstaedtioids and the extinct *Botryopteris*, *Psalixochlaena*, *Kaplanopteris*, *Anachoropteris*), as well as the fixed positions in which it arises, suggest that epiphyllous branching is a constituent aspect of the regular developmental program of those taxa.

While the mode of shoot branching draws a sharp divide between seed plants and seed-free plants, branching in roots sets apart lycopsids from the euphyllophytes. In lycopsids (lycopodiales and selaginellales), root branching is apical dichotomous, from apices with multiple apical initials. In euphyllophytes, the roots branch exclusively laterally and branch roots are not produced directly by the apical meristem. Euphyllophyte root primordia arise endogenously, by dedifferentiation of one or several primary meristem or parenchyma cells of the parent root which form the apical meristem of the branch root. In seed plants and sphenopsids, the layer in

which branch roots originate is the pericycle, but in pteropsids branch roots originate from the endodermis (von Guttenberg 1966). Interestingly, despite the sharp divide in root branching mode between lycopsids and euphyllophytes, all root-bearing plants share the same endogenous mode of production of adventitious roots from the stem pericycle or endodermis. The only exception is *Selaginella* in which the roots are borne at the tips of rhizophores; there, they arise from inner cells of the rhizophore apical meristem (Lu and Jernstedt 1996; Kato and Imaichi 1997).

6.6 Radial Patterning of Sporophyte Axes

The stele of stems and roots consists of the primary vascular tissues (xylem and phloem) and associated ground tissues (pith, pericycle, leaf gaps, interfascicular regions) (Beck et al. 1982; these authors provided the latest in-depth discussion of the stele concept and classifications). Three main types of stele are traditionally distinguished based on the anatomy of mature tissues: protosteles, in which the vascular tissues form a central rod (these include actinosteles and plectosteles); siphonosteles, in which the vascular tissues form a hollow cylinder with gaps produced by leaf divergences (these include solenosteles, with no more than one leaf gap at any level, and dictyosteles, which can have two or more overlapping leaf gaps in some regions); and eusteles, consisting of discrete vascular bundles (sympodia), which can be more or less interconnected (the atactostele of monocot stems is included here).

Among extant vascular plants, seed plant shoots are characterized exclusively by eusteles, whereas seed-free plant shoots have protosteles (lycopsids, psilotopsids, some pteropsids) or siphonosteles (pteropsids) (Table 6.1). However, when fossils are taken into consideration, this distinction becomes blurred: some of the early seed plants (pteridosperms, e.g., *Elkinsia*; Rothwell et al. 1989) had protosteles and some seed-free plants (e.g., *Archaeopteris*; Beck 1960) had eusteles. Among sphenopsids, the extinct sphenophyllales had protosteles, but *Equisetum* and the extinct Calamitales have a unique stele morphology that is similar to a siphonostele at the nodes and to a eustele in the internodes, and for which a fourth stele type, the equisetostele, has been coined by Rothwell (1999). The roots of all plants have protosteles.

The anatomy of the mature sporophyte can be misleading in terms of stele morphology, when taken outside the context of development. In psilotopsids, for example, the anatomy of primary meristems at the apex of axes provides a better criterion that the anatomy of mature tissues for interpretation of the stele type. In these plants, the stele consists of a hollow, ribbed cylinder of vascular tissues, with pith-like nonvascular tissue at the center, and has the appearance of a siphonostele. However, a look at the arrangement of primary meristems at the tips of psilotopsid axes reveals that the center is occupied by procambium, the primary meristem from which vascular tissues differentiate; in psilotopsids, the procambium differentiates into vascular tissues around the periphery and into parenchyma at the center. Thus,

the "pith" of psilotopsid axes is, developmentally, vascular tissue "gone wrong" and the stele is a protostele (referred to as a "medullated protostele" to account for the presence of parenchymatous tissue at the center). Moreover, in the sporophytes of some pteropsids stele morphology is size- and age-dependent, as it transitions from an early protostelic state to a siphonostele (e.g., Thompson 1920). Also, although eusteles are thought to be absent in filicalean pteropsids, some siphonostelic taxa exhibit stele morphologies that resemble the eustelic condition quite closely (White and Weidlich 1995; Karafit et al. 2006).

These examples raise important questions not only about the way we classify stele morphologies but, more importantly, about our understanding of evolution as reflected in development, that such classifications should ultimately mirror. Furthermore, stele morphology is only the most prominent aspect, historically, of a more general feature of plants, the radial patterning of tissues in sporophyte axes (stems and roots). Since the radial patterning of tissues results from coordinated patterns of cell division and differentiation in the apical meristem, could studies of development help us reach a better understanding of commonalities and differences between vascular plant lineages and, ultimately, of phylogenetic relationships and evolution? Could we base our understanding and classification of stele morphology on the geometry of developing vascular tissues in apical meristems?

In the apical meristem region, the procambium forms a solid rod at the center of axes which have protosteles in their mature regions, and it forms a hollow cylinder (with ground meristem in the middle) in axes whose mature regions have siphonosteles. In the eustelic stems of angiosperms, provascular meristematic tissue forms a hollow cylinder (residual meristem) from which procambial strands of the stem sympodia and leaf traces differentiate (Esau 1977). The patterning of the vascular tissues of shoots is also influenced, to different degrees depending on the plant lineage, by leaves and their vascular supply (leaf traces). In the leafless psilotopsids, the vascular tissues are patterned exclusively by the apical meristem of the axis. In lycopsids, there seems to be little influence of leaves on the patterning of vascular tissues and the morphology of the stele, as suggested by the presence of provascular tissue above the level of the youngest leaf primordia (Gifford and Foster 1989). Pteropsids present a wide variety of conditions, as shown by experimental and comparative studies of the development of vascular tissues in shoot apices (Wardlaw 1944, 1946; White 1984) – there are species in which the leaves have a major influence on the establishment of the vascular pattern, while in other species the influence of leaves is less substantial. The eusteles of seed plants seem to be mostly an expression of the production of vascular tissues supplying the leaves, as suggested by experiments (summarized by Steeves and Sussex 1989) which showed that as a result of obliteration of leaf primordia, vascular tissue differentiation in shoot apices is arrested at the stage of hollow cylinder of provascular tissue (residual meristem). The relationship between development of the shoot apical meristem and the geometry of tissues in the shoots of *Equisetum* is still poorly understood, partly due to pronounced modularity, which makes it difficult to follow the differentiation of tissues longitudinally. All of these emphasize the need for renewed efforts in documenting the developmental anatomy of radial patterning in sporophytic axes.

Another aspect of radial patterning is the specification of a boundary layer that is continuous throughout the axes, where it forms the endodermis (in most plant roots and many seed-free plant stems) and starch sheath (in seed plant stems), and into the leaves, where it forms the vascular bundle sheaths (Tomescu 2008). The boundary layer separates the stele from the other tissues in the axes, and the leaf veins from the mesophyll. Beck et al. (1982) advocated the exclusion of the endodermis as a morphological criterion from treatments of stele morphology. However, the boundary layer separates clearly distinct domains in radial patterning and is, therefore, important in understanding this aspect of development and evolution. For instance, recent studies (Stewart and Tomescu 2009 and unpublished results) have shown that there are several patterns of endodermis development in terms of the origin of the endodermal layer (from procambial or ground meristem initials) and the identity of the sister layer produced by the periclinal division that generates the endodermis. The taxonomic distribution of the different patterns in the roots of euphyllophyte is congruent with phylogeny, providing support for some clades. Accumulating evidence suggests that a shared mechanism (the *SHR-SCR* positive feedback loop; Cui et al. 2007) is responsible for the specification of boundary layers throughout the plant body in seed plants (Tomescu 2008). Given the emerging diversity of endodermis development patterns present in seed-free plants, it will be interesting to see how these different patterns correlate with the molecular mechanisms that control boundary layer development in different sporophyte organs and in different vascular plant lineages.

Among the extant seed-free plant lineages, secondary growth is present only in *Isoetes*, in which a secondary meristem (cambium) produces secondary vascular tissue, consisting of both xylem and phloem cells, toward the center of the stem and rhizomorph, and parenchymatous secondary cortex toward the outside (Paolillo 1963; Gifford and Foster 1989). Secondary growth also characterized several extinct lineages, including the lepidodendralean isoetaleans, the sphenophyllalean and calamitalean sphenopsids, and the progymnospermous pteropsids. These represent independent evolutionary origins of secondary growth, and Rothwell et al. (2008) have provided evidence for parallel evolution of the regulation of secondary tissue production by polar auxin flow in isoetaleans, sphenopsids, and lignophytes (progymnosperms + seed plants). On the other hand, the idea that some ophioglossales may undergo secondary growth was dispelled by Rothwell and Karrfalt (2008), who showed that the radially aligned tracheids of *Botrychium* are the result of a particular type of primary growth rather than being produced by divisions of a secondary meristem.

6.7 Leaf Development

The leaves of plant sporophytes are lateral appendages that share four defining features: vascularization, determinate growth, bilateral symmetry (adaxial-abaxial polarity), and a definite arrangement (phyllotaxis). However, they are not homologous

across all vascular plants, a situation reflected in the early popular classification of leaves into microphylls (subsequently equated with lycopsid leaves) and megaphylls (later equated with euphyllophyte leaves). Modern phylogenies based on broad sampling of extant and extinct plant lineages (Rothwell 1999; Hilton and Bateman 2006; Rothwell and Nixon 2006; synthesized in Tomescu 2009) indicate that leaves may have evolved independently up to ten times in vascular plants – one origin for lycopsid leaves and up to nine among euphyllophytes –, supporting the abandonment of the simplistic microphyll-megaphyll dichotomy in favor of a more sophisticated approach for understanding the evolutionary origins of leaves (Tomescu 2009). Among extant euphyllophyte lineages, there is strong evidence for independent evolution of leaves in at least two lineages (seed plants and pteropsids + sphenopsids), according to the moniliformopses phylogenetic hypothesis (Pryer et al. 2001; Friedman et al. 2004). Phylogenies that include fossils support independent evolution of leaves in the seed plants, the sphenopsids, and the pteropsids. Although evidence for this has yet to be produced, differences in leaf morphology suggest that the ophioglossales, marattiales, and leptosporangiate pteropsids may represent three independent instances of leaf evolution.

Lycopsid leaves are usually small, with a simple lamina and a single vein, although extant plant diversity and the fossil record include exceptions to each of these three characteristics (Tomescu 2009); the selaginellales and isoetaleans share the presence of a ligule on the adaxial side of their leaves (discussed under Sect. 6.2). It is generally agreed, based on phylogeny and the fossil record, that lycopsid leaves share a common origin and are homologous (Kenrick and Crane 1997; Friedman et al. 2004), but the nature of the precursor structures and evolutionary processes that generated the lycopsid leaf is still debated. Three competing hypotheses propose that lycopsid leaves evolved either by vascularization of enations (small, lateral flaps of tissue on the undifferentiated axes of early vascular plants that lacked vascular tissues and a definite arrangement), by sterilization of sporangia, or by reduction of branching systems (Stewart and Rothwell 1993; Kenrick and Crane 1997), but none of these hypotheses has received unequivocal support to date.

In the absence of a demonstrated common mechanism of evolution, no defining characteristics are available presently to define the lycopsid leaf as a synapomorphy. Two features that may be synapomorphic are the mode of origination of leaf primordia and the relationships between stele architecture and phyllotaxis (Tomescu 2009). The leaves of *Selaginella kraussiana* have been shown to originate from a small number of initials specified in the outermost (protodermal) layer of the shoot apex (Harrison et al. 2007). This characteristic, although not documented exhaustively, has been nevertheless recorded in other lycopsids (von Guttenberg 1966) and differentiates the clade from pteropsid euphyllophytes, whose leaves originate from larger groups of primordium founder cells, which are usually specified in the protodermal, as well as underlying layers of the shoot apex. Another study (Gola et al. 2007) has shown uncoupling between stele architecture and phyllotaxis in *Lycopodium*. If documented in other lycopsids, this characteristic that contrasts the much tighter connections between stele architecture and phyllotaxis present in euphyllophytes, could represent another defining synapomorphy for lycopsid leaves.

In the psilotopsids, morphological evidence provides the strongest support for interpretation of organography as consisting of leafless undifferentiated axes, in spite of alternative interpretations that have been put forward. The two genera included in the group, *Psilotum* and *Tmesipteris*, have somewhat different morphologies. *Psilotum* sporophytes consist of a system of profusely but irregularly branched below-ground axes bearing rhizoids; some of the below-ground branches produce above-ground axes that exhibit regular, three-dimensional dichotomous branching and bear minute enations and synangia. Two alternative interpretations have been proposed to explain the organography of *Psilotum* as a derived state of a pteropsid-type body plan, a hypothesis which found support in the placement of psilotopsids among the hypothetical moniliformopses in "extant-only" phylogenies (Pryer et al. 2001; Schneider et al. 2009). In one of these interpretations, Bierhorst (1971, 1977) has argued that the above-ground axes are homologous to leaves (fronds) attached to a rootless rhizome represented by the below-ground axes. However, the absence of important leaf-defining features in the above-ground axes argues against this interpretation: they lack a definite arrangement (phyllotaxis) on the below-ground axes and they lack bilateral symmetry; additional grounds for rejection of this interpretation have been provided by Kaplan (1977). A more popular interpretation proposes that *Psilotum* axes are stems that bear highly reduced leaves represented by enations (Gifford and Foster 1989). However, the lack of any vascularization and of a definite arrangement in the enations of *Psilotum* make this interpretation untenable. In *Tmesipteris*, the above-ground axes bear appendages that have been interpreted as leaves because they are flattened in a vertical plane and, thus, exhibit apparent bilateral symmetry. However, unlike leaves, the appendages of *Tmesipteris* lack a definite arrangement and are vascularized by strands that are radially symmetrical, suggesting axial rather than leaf similarities.

The whorled leaves of *Equisetum* are highly reduced, single-veined, and fused at nodes, forming sheaths. There is good evidence in the fossil record that the leaves of *Equisetum* and, probably, of all the sphenopsids, have evolved by reduction of branching systems (Stewart and Rothwell 1993). Leaf development in *Equisetum*, like most other features of this genus, is unique. The shoot develops in a very regular pattern, by production of vertically stacked series of three derivatives, each cut from one of the three cutting faces of the apical cell. Each derivative produces a group of cells (merophyte) by transversal and vertical (periclinal and anticlinal) divisions. Each group of three merophytes, corresponding to a series of three derivatives of the apical cell, produces one node and one internode of the stem. The leaf initial cell is specified as the outer cell of the second highest cell tier of the merophyte, when the merophyte is four cells thick (vertically). When leaf growth is initiated, the leaf initial cell cuts off a leaf apical cell to the outside, by an oblique anticlinal wall (von Guttenberg 1966). Thus, like in the lycopsids, leaves originate in the outermost cell layer in *Equisetum*.

Most pteropsids have relatively large leaves with complex venation and, often, complex architecture, also known as fronds. The leaves originate from relatively large numbers of initials (except in taxa with diminutive mature sporophytes, such as *Azolla* or *Salvinia*) specified in the protoderm and underlying layers of the apical meristem, and phyllotaxis is strongly correlated with stele architecture.

All these features make pteropsid leaves similar to the leaves of seed plants, with which they have been traditionally and misleadingly grouped together under the now obsolete name of megaphylls. Today, there is general agreement based on the fossil record and phylogenetic analyses that pteropsid and seed plant leaves evolved independently (Friedman et al. 2004; Tomescu 2009). Moreover, an earlier hypothesis of partial homology of leaves in the two groups at the level of their hypothesized precursor structures, determinate lateral branching systems (Kenrick and Crane 1997), has become less tenable in light of a fossil study that demonstrated divergent pathways of acquisition of leaf-defining features, and therefore, independent evolution of leaf developmental mechanisms in pteropsids and seed plants. In the study, Sanders et al. (2009) compared an early filicalean pteropsid (*Psalixochlaena antiqua*) to an early seed plant (*Elkinsia polymorpha*) and showed that while the leaf precursor appendages of filicales acquired adaxial-abaxial polarity before determinacy, those of seed plants acquired determinacy prior to evolving adaxial-abaxial polarity.

The independent evolutionary origins of pteropsid and seed plant leaves are also reflected in the differences in developmental characteristics between their living representatives. Pteropsid leaves grow from an apical cell for a significant part of their development; their maturation is strictly acropetal and the apex retains a meristematic character for the entire duration of leaf development. In contrast, apical meristematic activity is much more limited in the leaves of seed plants, in which growth is much more diffuse throughout the leaf surface. Furthermore, leaf maturation in seed plants is acropetal in early stages, but once the tip matures, the final phases of tissue differentiation and maturation proceed basipetally (Kaplan and Groff 1995). Finally, the independent evolution of pteropsid and seed plant leaves is also supported by significant differences in the expression patterns of genes that control leaf development in the two groups (Tomescu 2009).

6.8 Developmental Genes

Few, if any, developmental processes in the seed-free plant sporophyte are understood at the genetic level. Homologs of genes involved in major developmental programs in seed plants, and especially angiosperms, have been nevertheless isolated in different seed-free vascular plants. However, the coverage of different lineages is very uneven (Table 6.2). The most studied species has been *Selaginella moellendorffii*, which is also the only seed-free plant with a completely sequenced genome to date. Several other *Selaginella* species, most prominently *Selaginella kraussiana*, have contributed data, and Floyd and Bowman's (2007) seminal work that sought to reconstruct the developmental tool kit of land plants and its evolution, considerably expanded the number of gene families isolated in *Selaginella*. The only other seed-free plant that has undergone significant study of developmental genes is the pteropsid, *Ceratopteris*. The lycopodiales, isoetaleans, psilotopsids, sphenopsids, and the eusporangiate (ophioglossales, marattiales) and hydropteridalean (marsileaceae, salviniaceae) pteropsids have received little attention. Analyses of expression patterns

Table 6.2 Developmental genes identified in seed-free plants

	KNOX	ARP	HD-ZIP	MADS-box	AP2/ANT	*FLO/LFY*	*CUC*	WOX	*CLV*	*PIN*	TCP	GRAS	EPFL	
Lycopodium				++										Svensson et al. (2000); Svensson and Engstrom (2002)
Selaginella	+++	♦	+++	++	+		+	+	+	+	+	+	+	Tanabe et al. (2003); Harrison et al. (2005); Floyd and Bowman (2006); Floyd et al. (2006); Prigge and Clark (2006); Floyd and Bowman (2007); Rychel et al. (2010)
Isoetes						+								Himi et al. (2001)
Psilotum			+			+								Himi et al. (2001); Floyd et al. (2006)
Ophioglossum				++										Munster et al. (2002)
Botrychium						+								Himi et al. (2001)
Angiopteris						+								Himi et al. (2001)
Osmunda	+++	+++												Harrison et al. (2005)
Ceratopteris	♦		+	+++	+	++								Kofuji and Yamaguchi (1997); Munster et al. (1997); Hasebe et al. (1998); Aso et al. (1999); Himi et al. (2001); Axtell and Bartel (2005); Sano et al. (2005); Floyd et al. (2006); Prigge and Clark (2006)
Anogramma	+++													Bharathan et al. (2002)
Matteucia						+								Himi et al. (2001)
Marsilea			+											Prigge and Clark (2006)
Equisetum						+								Himi et al. (2001)

+, presence of gene; ++, organ-level expression pattern characterized; +++, tissue-level expression pattern characterized; ♦, studies of gene function

are limited to a subset of the taxa and genes studied (Table 6.2). Most of these analyses only addressed expression patterns at the whole sporophyte level, seeking to characterize differences between organs, sometimes with added detail differentiating meristematic from mature organ segments. Although informative, data produced by such expression studies provide only circumstantial evidence for inferences on gene functions. However, a few studies using in situ hybridization documented tissue-level expression patterns, much more informative for functional interpretations, for several genes in *Selaginella* and pteropsids (*Ceratopteris*, *Osmunda*, and *Anogramma*). Gene functions have been addressed by only two studies to date, one using ectopic expression in *Arabidopsis* to characterize the functions of *Ceratopteris* class 1 KNOX (KNOX1) genes (Sano et al. 2005), and the other using complementation of *Arabidopsis* mutants to document the functions of an *AS1* ortholog (an ARP gene) in *Selaginella* (Harrison et al. 2005). Both studies also documented tissue-level expression patterns of the respective genes using in situ hybridization.

6.8.1 KNOX and ARP Genes

KNOX1 genes have been identified in *Selaginella kraussiana* and pteropsids (*Osmunda regalis*, *Ceratopteris richardii*, and *Anogramma chaeophylla*) (Table 6.2); at least one class 2 KNOX (KNOX2) gene is present in *Selaginella kraussiana* and in *Ceratopteris richardii*. In angiosperms, KNOX1 genes are involved in meristem formation and maintenance, positioning of leaf primordia within the shoot apical meristem, and internode elongation (Floyd and Bowman 2007). In *Selaginella*, two KNOX1 genes are expressed one in the shoot apical meristem (in the cells subtending the apical cells, but not in leaf primordium initials) and the other in internodal regions (Harrison et al. 2005). This expression pattern is also seen in some KNOX1 genes in angiosperms, in which it was suggested to have evolved independently of *Selaginella* (Harrison et al. 2005). As an alternative interpretation, Floyd and Bowman (2007) proposed that the common ancestor of extant vascular plants had one KNOX1 gene that covered both patterns of expression and which was duplicated independently in lycopsids and angiosperms, following divergence from their common ancestor; each of the two copies then became subfunctionalized for one or the other of the two functions independently in the two lineages.

KNOX1 genes have similar patterns of expression in the three pteropsids studied – they are expressed in the shoot apical meristem and in leaf primordia (Bharathan et al. 2002; Harrison et al. 2005; Sano et al. 2005). A study in *Ceratopteris* (Sano et al. 2005) showed that KNOX1 genes are also expressed in procambium at the shoot apex and along the margins of young developing leaves (at the tips and in associated provascular tissue of pinnules). These patterns of expression are largely consistent with those documented in seed plants for KNOX1 genes. Indeed, the results of overexpression of *Ceratopteris* KNOX1 genes in *Arabidopsis* suggest that the angiosperm

and pteropsid proteins have similar functions in meristem development and leaf architecture (Sano et al. 2005). The KNOX2 gene is expressed in all sporophyte tissues in *Ceratopteris*, like in the angiosperms (Sano et al. 2005).

ARP genes, expressed in the leaf primordia of all vascular plants studied to date, are thought to induce determinacy of growth by promoting cell fate determination. Among seed-free plants they have been identified in *Osmunda regalis*, *Selaginella kraussiana*, and *Selaginella viticulosa*. A *S. kraussiana* ARP gene has been shown to rescue the *Arabidopsis as1* phenotype (albeit in a dosage-dependent manner), which suggests conservation of function between lycopsids and angiosperms (Harrison et al. 2005).

The antagonistic interactions of KNOX1 and ARP genes are considered one of the major determinants of leaf development (Harrison et al. 2005). In angiosperms, KNOX1 genes are expressed in the shoot apical meristem, but are downregulated in the leaf primordium initials, where ARP genes are expressed. Once the leaf primordium is initiated, KNOX1 expression resumes in the primordia of species that produce compound leaves, but not in those of species with simple leaves. The patterns of expression of KNOX1 and ARP genes in pteropsids and *Selaginella* apparently fit the angiosperm model. KNOX1 genes are downregulated in the leaf primordia of *Selaginella*, and they are expressed in those of pteropsids, where they have been hypothesized to delay determinacy and, thus, promote complex architecture (Harrison et al. 2005). However, there are differences in both groups compared to the angiosperm model. In pteropsids, the transient downregulation of KNOX1 genes in leaf primordium initials is absent (this situation has also been documented in one angiosperm, *Medicago*; Di Giacomo et al. 2008). Furthermore, ARP genes, expressed only in the leaf primordia in angiosperms, are expressed in the leaf primordia, as well as the shoot apical meristem, where they are coexpressed with KNOX1 genes, in pteropsids (*Osmunda regalis*) and *Selaginella kraussiana* (Harrison et al. 2005).

These differences in the expression patterns of KNOX1 and ARP genes between major plant lineages suggest that vascular plants do not share a unique type of KNOX1-ARP antagonistic interactions. These interactions, although important in meristem patterning and leaf development, vary in their degree of antagonism and appear to be modulated differently in the different lineages, which is congruent with the multiple independent origins of leaves in vascular plants (Tomescu 2009). The expression of KNOX1 genes in the shoot apical meristems of selaginellales, pteropsids, and seed plants suggests that the function of these genes in meristem development may have been established early on in the common ancestor of all vascular plants. The fact that ARP genes are also expressed in the apical meristems of selaginellales and pteropsids suggests that this may be another plesiomorphic feature likely to have characterized the earliest vascular plants prior to the divergence of lycopsids and euphyllophytes. It will be interesting to characterize the functions of the KNOX1 and ARP genes in the seed-free plants and to find out whether they are shared between lineages.

6.8.2 HD-ZIP Genes

Class III HD-ZIP genes have been identified in *Selaginella kraussiana*, *Selaginella moellendorffii*, *Psilotum nudum*, and two pteropsids (*Ceratopteris richardii* and *Marsilea minuta*), but their expression patterns have been characterized only in *Selaginella* (Table 6.2). The two genes identified in *Selaginella* have different expression patterns (Floyd and Bowman 2006; Floyd et al. 2006). One is expressed in apical cells and below the shoot apical meristem at the center of provascular strands (which differentiates into xylem), as well as in differentiating protoxylem tracheids in both stem and leaves. The other HD-ZIP III gene is expressed on the adaxial side of expanding leaves where the ligule develops and conducting tissue first differentiates; its expression domain extends toward the leaf apex and into the stem procambium but is restricted to the outer layers of provascular tissue that differentiate into phloem and pericycle. These two patterns of expression seem to indicate complementary roles of the two genes in the patterning of the shoot apex and the vascular tissues of the stem and leaves (Floyd et al. 2006). These expression patterns have been partially confirmed by another study of *Selaginella* HD-ZIP III genes (Prigge and Clark 2006), which also found them expressed in regions of the shoot apical meristem associated with leaf initiation, suggesting a role in leaf initiation, as well as in the developing vascular tissue of roots.

In seed plants, HD-ZIP III genes are involved in shoot apical meristem formation and growth, vascular patterning and determination of leaf adaxial polarity (Floyd and Bowman 2007). The patterns of expression of HD-ZIP III genes in *Selaginella* and their apparently complementary roles in the development of the shoot apex and of tissues within vascular strands, suggest that stem and leaf vascularization are separated spatially and temporally, and are possibly initiated by two different genes (Floyd and Bowman 2006). These have potential implications for the evolution of development. Since *Selaginella* HD-ZIP III genes seem to not be involved in leaf polarity, but they are likely implicated in shoot apical meristem functioning and vascular tissue patterning, these two functions shared with the seed plants are probably plesiomorphic and were present in the common ancestor of all extant vascular plants (Floyd and Bowman 2007). The difference between lycopsids and seed plants, where HD-ZIP III genes are major players in the establishment of leaf adaxial-abaxial polarity, is congruent with the independent origins of leaves in the two lineages and may support the hypothesis of origination of lycopsid leaves from enations (Floyd and Bowman 2006).

Aside from HD-ZIP III genes, HD-ZIP genes belonging to subfamilies I, II, and IV have also been identified in *Ceratopteris richardii*, where they are expressed in both gametophytes and sporophytes, not associated with any sporophyte organ in particular, but expressed with different intensities in the different sporophyte parts (Aso et al. 1999). This is thought to indicate that those genes are not required for organ identity, but are likely involved in developmental and physiological processes common to the sporophyte and gametophyte phase.

6.8.3 MIKC-type MADS-box Genes

MADS-box genes have been identified in many eukaryotes (including plants, animals, and fungi) and MIKC-type MAD-box genes include most of the organ identity genes implicated in the development of the angiosperm flower. Among the seed-free vascular plants, MIKC-type MADS-box genes have been identified in lycopsids (*Lycopodium annotinum*, *Selaginella remotifolia*,and *Selaginella moellendorffii*) and pteropsids (*Ophioglossum pedunculosum* and *Ceratopteris richardii*) (Table 6.2), but none are orthologs of the angiosperm floral organ identity genes (Munster et al. 1997, 2002). Their patterns of expression have been documented at the organ level so only very general inferences have been made regarding potential functions, except for *Ceratopteris* in which tissue-level expression patterns have been reported.

In *Lycopodium*, several MADS-box genes of the LAMB2 group are expressed in all vegetative and reproductive parts of the sporophyte (with different levels of expression for different genes and tissues), whereas *LAMB1* is expressed exclusively in developing strobili (Svensson et al. 2000; Svensson and Engstrom 2002). In *Selaginella*, MADS-box genes grouped in the same clade with the *Lycopodium* LAMB2 genes are expressed in vegetative and reproductive sporophyte tissues except for the rhizophore and root. This has been interpreted as evidence for independent origins of roots in the two lineages (Tanabe et al. 2003).

Most of the *Ceratopteris* MADS-box genes are expressed in both gametophyte and sporophyte, but the levels of expression vary. In the sporophytes, they tend to be similarly expressed in both the vegetative and reproductive organs, with somewhat higher levels of expression in organ primordia and meristems. One gene has nevertheless been shown to be predominantly expressed in roots (Theissen et al. 2000). In situ hybridization studies by Hasebe et al. (1998) showed that *Ceratopteris* MADS-box genes are expressed in the procambium of stems, leaves and roots, as well as in the shoot and root apical meristems, leaf primordia, developing leaf tips and pinnule tips, and sporangium initials. These patterns of expression have led to the assumption that MADS-box genes are involved in cell division in the seed-free plants (Hasebe et al. 1998). MADS-box genes are also expressed relatively ubiquitously in *Ophioglossum*, where of the four genes identified three were expressed in both the trophophore (vegetative) and the sporophore (reproductive) segments of the leaves, and only one had sporophore-specific expression (Munster et al. 2002); the study did not check for expression in the rhizome and roots.

MADS-box gene phylogenies suggest that the last common ancestor of ferns and seed plants had at least two different MIKC-type MADS-box genes that were homologs, but not orthologs, of the floral homeotic genes (Munster et al. 1997). The largely ubiquitous patterns of expression documented in seed-free plants suggest that the functions of MADS-box genes are different from those of flowering plants and more general (Theissen et al. 2000), not exclusively associated with reproductive organs. However, the fact that one *Ophioglossum* MADS-box gene is expressed only in the sporophore segment of leaves, and that some genes are

expressed in *Ceratopteris* sporangium primordia, may indicate that some MADS-box genes with functions in reproduction evolved in pteropsids independently from those of seed plants.

6.8.4 AP2 and ANT Genes

In angiosperms, these genes are involved in flower development, floral organ identity and ovule development. AP2 genes of the euAP2 subfamily have been cloned from *Selaginella moellendorffii* and *Ceratopteris thalictroides*, and ANT genes from *Ceratopteris thalictroides*, but their expression patterns have not been documented (Table 6.2). Based on gene phylogenies and the functions of these genes in angiosperms, Floyd and Bowman (2007) have proposed that the ancestral function euAP2 genes may have been meristem cell maintenance and control of the transition from vegetative to reproductive meristematic development (i.e., repression of transition to a reproductive state). Interestingly, while *Ceratopteris* shares with the seed plants a miR172 binding site on the AP2 sequence, that binding site is absent from the *Selaginella* AP2 (Axtell and Bartel 2005), indicating that regulation of euAP2 expression by miR172 evolved in euphyllophytes after their divergence from the lycopsids (Floyd and Bowman 2007).

6.8.5 FLORICAULA/LEAFY

FLORICAULA and *LEAFY* are positive regulators of floral homeotic MADS-box genes in angiosperms. *FLO*/*LFY* homologs have been identified in *Isoetes asiatica*, *Psilotum nudum*, pteropsids (*Botrychium multifidum*, *Angiopteris lygodiifolia*, *Ceratopteris richardii*, and *Matteucia struthiopteris*) and *Equisetum arvense*. In *Ceratopteris*, they are expressed in vegetative and reproductive shoot tips and in circinate reproductive leaves, with strongest levels recorded in the reproductive shoot tips. Because this expression pattern is different from that of *Ceratopteris* MADS-box genes, *FLO*/*LFY* probably have different functions in pteropsids from those of angiosperms, and do not act as positive regulators of the MADS-box genes (Himi et al. 2001).

6.8.6 Other Genes

Floyd and Bowman (2007) identified a series of other developmental genes in *Selaginella*, but their expression patterns are not characterized. Among these are NAC family genes similar to *CUC*; considering the organ separation functions of *CUC* in angiosperms (leaf primordium and leaf lamina lobe delimitation), these

may have had a role in apical branching in the early vascular plants. Homologs of the angiosperm shoot apical meristem maintenance genes *CLV* and WOX-family genes were identified, although among the WOX genes none is a homolog of *WUS*. Homologs of auxin transfer and response genes, *PIN*, *TIR* and ARF genes are also present in *Selaginella*. Of the TCP genes identified in *Selaginella*, none is regulated by miR319 as they are in angiosperm; this indicates that miR319 regulation of these genes implicated in cell division in angiosperms has evolved only in the euphyllophytes or maybe even only in seed plants (Floyd and Bowman 2007). Numerous GRAS family genes are present in *Selaginella*, including homologs of the gibberellic acid signaling regulators DELLA, and of the *SCR* and *SHR* root radial tissue patterning genes. The presence of *SCR* and *SHR* in lycopsids and seed plants, two lineages that diverged prior to the evolution of roots, suggests that these two genes may have been responsible for the radial patterning of tissues in the undifferentiated dichotomous axes of the earliest vascular plants. However, the functional homology of *SCR* and *SHR* across extant vascular plants, and even across extant euphyllophytes, has yet to be proven (Tomescu 2008). Rychel et al. (2010) identified in *Selaginella moellendorffii* genes of the EPFL family, putative ligands that influence stomatal density in angiosperms. Finally, YABBY genes are the only family of major developmental genes that seems to be exclusively present in seed plants (Floyd and Bowman 2007); they were not identified in any seed-free plant to date.

6.8.7 MicroRNA Regulation of Genes

Floyd and Bowman (2004) have shown that the miR165/166 binding site of HD-ZIP III genes is conserved across all embryophytes. This gene regulation mechanism is absent in *Chara*, which suggests that this mechanism may have evolved in the common ancestor of embryophytes. An ancestral function of HD-ZIP III genes seems to be the control of apical growth, therefore the advent of this mode of HD-ZIP III gene regulation shared by all embryophytes may have been important for the evolution of three-dimensional growth in land plant sporophytes (Floyd et al. 2006). However, an alternative interpretation is that HD-ZIP III regulation by miR165/166 was plesiomorphic in the common ancestor of embryophytes and it was lost and possibly replaced by another mechanism, in *Chara* (Floyd et al. 2006).

Axtell and Bartel (2005) found several microRNAs shared by *Selaginella uncinata* and *Ceratopteris thalictroides* with all seed plants; some of these are also shared with the bryophytes (also reviewed by Axtell and Bowman 2008). These document as many microRNA-target interactions that have been constant throughout plant evolution. The fact that all of these deeply conserved microRNAs are primarily involved in developmental programs in *Arabidopsis* suggests that they affected the morphology of plants throughout their evolution (Axtell and Bartel 2005). In some cases, the pattern of shared and derived microRNA-target interactions gives clues to the phylogenetic position of evolutionary events in the control

of development. Such is the case of AP2 control by miR172, shared only among euphyllophytes, and that of miR319 regulation of TCP genes found only in the seed plants (Floyd and Bowman 2007).

6.9 Conclusion

Seed-free vascular plants encompass most of the morphological diversity known among land plants. Six distinct body plans can be defined in seed-free vascular plants based on major vegetative features of the sporophyte (growth polarity, organography, root and shoot branching), and they are characteristic of the lycopodiales, selaginellales, isoetaleans, psilotopsids, sphenopsids, and pteropsids. However, the evolutionary history of all this morphological diversity is not well understood. In fact, except for the major phylogenetic divide between lycopsids and euphyllophytes, the relationships among the major vascular plant lineages remain unresolved to date (Rothwell and Nixon 2006). Furthermore, the developmental anatomy features that have yet to be documented in detail and sampled broadly, from a taxonomic standpoint, are not few. All of these will provide as many improved criteria for comparing morphology and will add resolution to phylogenies of vascular plants.

In this endeavor, an additional layer of information is added by the molecular genetic pathways that control developmental programs and which can be used in addressing questions of morphological evolution. A number of developmental genes characterized in seed plants, and especially in angiosperms, have been isolated in the seed-free vascular plants. An extensive survey by Floyd and Bowman (2007) revealed that most of the known plant developmental gene families are present in mosses, lycopsids, and seed plants, indicating that the developmental toolkit of seed-free vascular plants probably includes members of many developmental gene families present in angiosperms. However, to date, the taxonomic sampling of studies providing information on developmental genes remains sparse – significant numbers of these genes have been isolated only in *Selaginella* among the lycopsids and *Ceratopteris* among the pteropsids. Of the genes identified, few have been characterized in terms of tissue-level expression patterns, and even fewer have undergone functional studies.

The developmental genetic pathways that have received attention are the interactions between class 1 KNOX genes and ARP-type genes, implicated in leaf development, and the MADS-box genes. The little that is known on the functions of these genes suggests that MADS-box genes have much more general functions in seed-free plants and are potentially involved in meristem activity and growth, whereas KNOX1-ARP interactions reveal a complex mosaic of shared and divergent pathways that mirrors the different independent evolutionary origins of leaves. Aside from directly available information on genes in different lineages, inferences can be based sometimes on collateral data. Such is the case of the shared genetic mechanism that controls the development of cells with a rooting function in mosses and seed plants (Menand et al. 2007). The broad taxonomic range of this mechanism brackets, phylogenetically, all seed-free plant lineages which are, hence,

hypothesized to share the same pathway. However, overall, we are far from a complete understanding of the genetic controls of development in the seed-free plant sporophyte, which would enable comparisons among the different lineages and between those and the seed plants.

Functional genetic analyses in seed-free vascular plants are in their infancy. The establishment of transformation techniques for representatives of each of the different lineages is still a remote beacon, but as it gains momentum, it will produce a wealth of exciting new data. The next decade should see more than a few significant advances and new hypotheses in the fields of plant evo-devo and phylogeny and maybe even some paradigm shifts.

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Part II
Propagation, Conservation and Control of Genetic Variability in Ferns

Chapter 7
From Spore to Sporophyte: How to Proceed *In Vitro*

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7.1 Introduction

Ferns existing today represent a genetic inheritance of great value as they include species of ancient vascular plants, which have direct connection with the crucial steps taken in the past for settling life on earth. This group of plants conserves in common a primitive organisation, linked to the water. The life cycle consists of two main generations: the gametophyte (haploid) and the sporophyte (diploid), which are chronologically and spatially separated. Both gametophyte and sporophyte can be cultured for different purposes. The culture of gametophyte, as the generation responsible of reproduction either by sexual or asexual means, offers us the opportunity to gain insight into the biological processes involved in sexual expression in plants, with important economical repercussions. From a practical point of view, a bigger knowledge about the *in vitro* culture of the sporophyte could contribute to exploiting in a more rational way those species demanded for its ornamental potential, which are taken nowadays directly from their habitat, and also to conserve those species living under risk of disappearing.

Traditionally, ferns are propagated by two methods: the sexual and the vegetative. The sexual method of propagation involves raising plants from spores. The vegetative method of propagation involves the use of rhizomes or other vegetative organs such as planting material. New plants are generally produced from pieces of rhizome isolated from the mother plant. Other parts of the plants such as bulbils, e.g. *Asplenium bulbiferum*; proliferating frond tips, e.g. *Adiantum caudatum*; stolons, e.g. *Nephrolepis*; offsets, e.g. tree ferns; stipules, e.g. *Angiopteris*; layering, e.g. *Lygodium*; tubers, e.g. *Nephrolepis*; root buds, e.g. *Ophioglossum* are also used as starting material for vegetative propagation (Fernández and Revilla 2003).

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Plant tissue culture is a practice used to propagate plants under sterile conditions, often to produce clones of a plant. Different techniques in plant tissue culture may offer certain advantages over traditional methods of propagation, including: the production of exact copies of plants that have desirable traits; to quickly produce mature plants; the production of plants in the absence of seeds or necessary pollinators to produce seeds; the regeneration of plants from genetically modified cells; the production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens; the production of plants from seeds that otherwise have very low chances of germinating and growing, i.e., orchids; to clean a particular plant of viral and other infections; and to quickly multiply these plants as "cleaned stock" for horticulture and agriculture.

Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency). Single cells, plant cells without cell walls (protoplasts), pieces of leaves, or (less commonly) roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones.

Nowadays, there is a large production of ferns in the world by micropropagation, the fern *Nephrolepis* being considered as one of the largest among *in vitro* propagated plants (Pierik 1991). In fact, we can find information about several companies specialising in the propagation and production of ferns, which are exported all over the world.

7.2 Spore Culture

Since tissue culture is widely employed as a technique for fern propagation or scientific studies, spores are widely used as a starting material. All the steps involved in the whole process for raising ferns from spores are the following: collection of spores at the right stage of maturity, sowing of spores, germination, growth of the prothallia and maturity of sexual organs, fertilisation and formation of sporophyte.

The mature fertile fronds are collected, wrapped in paper sheets, and dried at room temperature to release spores. At maturity, the sori on the undersurface of the leaf becomes brown in colour and, with practice, one is sure to pick up at the right stage. Within 1–2 days, the fronds get dried up and mature spores can be taken in the form of a power by tapping the fertile fronds, which are then ready for sowing.

In vitro culture of spores is carried out under fully axenic conditions on agar-plated petridishes. The use of spores to initiate culture avoids contamination of the propagation medium with bacteria, fungi, algae, and mosses, concomitant with the culture of explants taken from sporophytes grown in the field (Deberg 1994). Surface sterilisation is the first step for aseptic culture of ferns from spores (Dyer, 1979). An adequate protocol to ensure the cleaning of spores may be as follows.

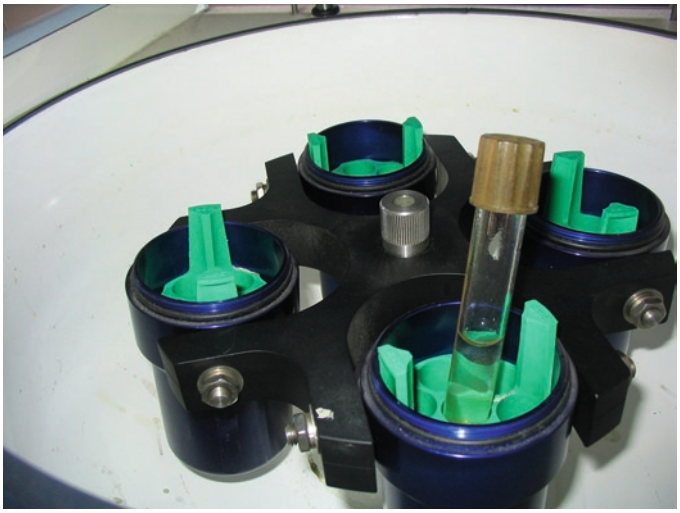

Fig. 7.1 Centrifugation step for recovering spores from cleaning solution

Isolated spores are placed in glass tubes, soaked in water for 2 h and then washed for 10 min with a solution of hypochlorite (0.5% w/v) containing a surfactant such as Tween 20 (0.1% w/v). Subsequently, they are rinsed three times with sterile distilled water. The suspended spores are centrifuged at 2,000 rpm for 3 min between rinses (Fig. 7.1) to change solutions and finally harvested in sterile distilled water (Fernández et al. 1993). Other sterilants that have been used include calcium hypochlorite, mercuric chloride, hydrogen peroxide, and even antibiotics such as streptomycin (Fay 1994; Cox et al. 2003). Other efficient methods for surface sterilisation of fern spores have been reported (Hua et al. 2009).

Spores are analogous to seeds as they contain all the required nutrients for early growth. Thus, the use of low-nutrient medium during initial stages of germination such as Knop (1865), Knudson (1946), or dilutions of the Murashige and Skoog (1962) medium (=MS) are typically employed and can be justified.

The spores of most ferns forming green gametophytes require exposure to light to germinate and grow up (Dyer 1979); however, some species, including *Lycopodium clavatum*, give rise to subterranean, nonphotosynthetic, mycorrhizal gametophytes and their spores germinate in the dark (Whittier 2008).

The germination time for fern spores has been found to vary from a few days to several months (Fernández and Revilla 2003; Chou et al. 2007). Recently, Fernández et al. (2007) have reported the use of confocal microscopy for helping us in the characterisation of fern spores based upon the fact that fern spores show autofluorescence as they have endogenous fluorescent compounds. Besides, they reveal specific fluorescent emission spectrum when they are excited with different wavelength lights (Fig. 7.2). The morphology and fluorescence spectrum could contribute to identification of fern species, which is interesting from a taxonomical point of view.

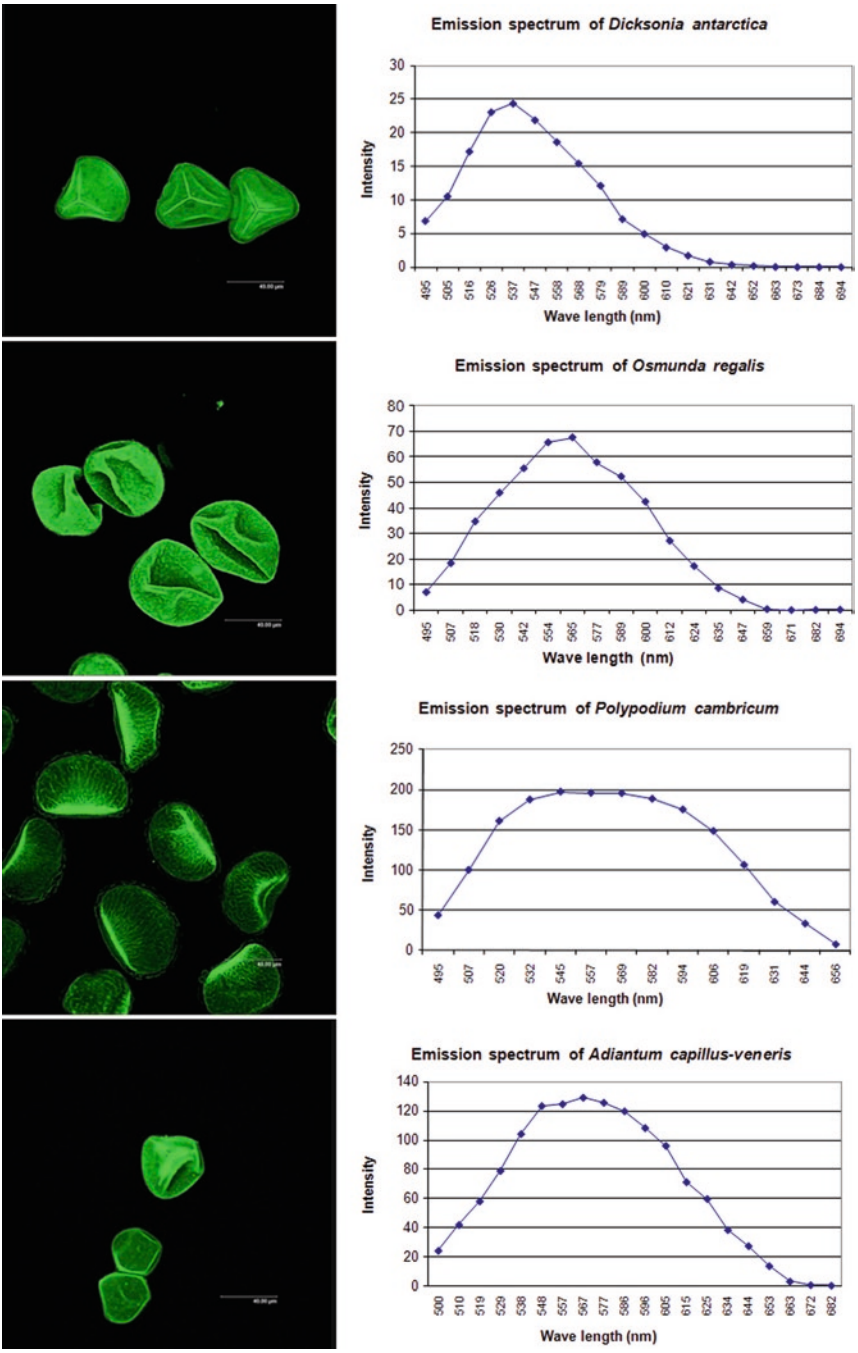

Fig. 7.2 Confocal images and emission spectrum of spores of several species of ferns

7.3 Gametophyte Culture: Nutritional and Environmental Conditions

Under natural conditions, the survival of gametophytes is strongly influenced by the environment. Insufficient moisture or desiccation is an important impediment (Peck et al. Farrar 1990). However, under controlled *in vitro* conditions, gametophytes may be successfully grown up on a variety media.

One striking feature when culturing gametophytes *in vitro* is the big growth rate shown during this phase of the fern's life cycle. Under appropriate conditions, gametophytes grow actively, forming spherical clusters. Nutrients as well as other physical and chemical factors such as light, pH, physical state of medium, and phytohormones affect all the processes involved in the growth and development of prothallium (Camloh et al. 1996; Fernández and Revilla 2003; Menéndez et al. 2006).

Nutritional requirements may differ depending on the species of ferns. In our laboratory, we have successfully cultured and germinated spores of a wide spectrum of leptosporangiate ferns such as *Adiantum capillus-veneris*, *Asplenium adiantum nigrum*, *Asplenium nidus*, *Blechnum spicant*, *Cyathea degrei*, *Culcita macrocarpa*, *Davallia canariensis*, *Dyksonia antarctica*, *Dryopteris affinis*, *Dryopteris corleyii*, *Polypodium cambricum*, *Pteris ensiformis*, *Woodwardia radicans*, *Woodwardia virginica*, … on liquid MS medium supplemented with sucrose 2% (Fernández et al. 1996a, b; Fernández et al. 1997a, b; Fernández et al. 1999; Somer et al. 2010). In contrast, gametophytes of *Osmunda regalis* (a protoleptosporangiate fern) grow better in Knop medium whereas growth was inhibited at high osmotica levels such as half- and full-strength MS (Fernández et al. 1997a). It has been observed a stimulatory effect of ammonium on germination and initial development of the nonphotosynthetic gametophytes cultured *in vitro* (Whittier 1990). According to our experience with photosynthetic gametophytes, under its reduced form, ammonium improves gametophyte development in species belonging to the gender *Asplenium*, *Blechnum*, *Dryopteris*, *Pteris,* and *Woodwardia* but not in *Osmunda* (Fernández et al. 1997a). For sporophyte production by sexual means, Kuriyama et al. (2004) reveal that reduction of total nitrogen to 25% of the original MS medium effectively promotes sporophyte yield in *A. capillus-veneris*.

Sucrose supports gametophyte growth when added to the nutrient media and the optimum concentration is afforded by its promoter effect as a nutrient and its inhibitory effect as an osmotic agent. In general terms, gametophyte dry weight increases when sucrose concentration of the culture medium increases but it has been reported that gametophytes of *O. regalis* are able to grow independently of the presence of sucrose in the culture medium (Fernández et al. 1997a). In relation to maturation of the sexual organs, it was clear that archegonia require the presence of carbohydrates in the medium to develop, but that antheridia can develop in less complex culture media. Addition of sucrose 2–3% to the mineral basal medium would be adequate.

Gametophytes of several species of ferns showed a great tolerance to pH changes from acid to alkaline conditions (Fernández et al. 1997b, c; Chang et al. 2007), *O. regalis* showed an optimum growth at pH 5.7 (Fernández et al. 1997a) and *D. affinis* growth was inhibited at pH 8.2 units (Fernández et al. 1996a, b).

The physical state of the culture medium – solid or liquid – affects gametophytic growth. The dry weight of *O. regalis* gametophytes cultured in a medium with 0.35% agar increased with respect to solid cultures (0.7% agar) (Fernández et al. 1997a), and in *B. spicant*, liquid medium favoured gametophytic growth but the gametophytes showed hyperhydricity, i.e. the culture appears as glassy, transparent (Fernández et al. 1997c).

In the most leptosporangiate ferns, addition to its effect on spore germination, light is considered necessary for two-dimensional growth of gametophytes, as in darkness or low light intensity, the gametophyte elongates but does not develop to the heart shape (Mohr 1962). The gametophyte of *O. regalis* again shows a particular behaviour, and developed a heart shape even in darkness.

Gametophyte density influences growth and sexual expression. In low-density populations gametophytes are usually large, and females or hermaphrodites, and small, spathulate and asexual in high-density populations (Huang et al. 2004; DeSoto et al. 2008). Possible explanations given could be related to competition for nutrients, presence of allelochemicals, etc. In the same report, it is proposed the sowing of spores sparsely, as it will lead to subsequent development of gametophytes of both sexes and promote successful sporophyte production.

7.4 Gametophyte Multiplication

7.4.1 By Natural Means

Gametophyte of fern exhibits two "natural means" of vegetative reproduction: gemmation and branching. In the first type, the basic pattern of gemma development consists of spherical units with several inner cells, surrounded by a layer of cells that divide anticlinaly. *In vitro* culture revealed, for the first time, this phenomenon in gametophytes of *O. regalis* (Fernández et al. 1997a).

In the second type of multiplication or branching, a new gametophyte begins as a one-dimensional filament that soon becomes two-dimensional, and it may occur in the filamentous gametophyte, and in the apical or basal area of two-dimensional gametophyte.

7.4.2 Homogenised Cultures

The gametophyte of ferns exhibits a great multiplication capacity when homogenised by mechanical means (Fernández, Bertrand and Sánchez-Tamés 1993). Gametophytes are mechanically fragmented using a waring blender for 15 s under

aseptic conditions. The homogenised tissue is cultured in 250 mL Erlenmeyer flasks containing 50 mL of liquid MS medium. The liquid cultures are placed on a gyratory shaker (75 rpm). Initially, explants become brown, apparently dead, but gradually, some cells start to divide and regenerate new gametophytes. Homogenisation of gametophytes is a recommended propagation system for species having a short life cycle, producing numerous sporophytes in a short period of time as occurs, for instance, with *Woodwardia virginica*, which completes its life cycle in 1 month (Fernández et al. 1999).

7.5 Sporophyte Formation

The formation of sporophytes in ferns is believed to be controlled in two possible directions: (a) from gametophyte to sporophyte, linked to the reproductive biology of the gametophytic phase; or (b) from preceding sporophyte to another sporophyte, linked to the morphogenetic potential of the sporophyte itself. In the first instance, sporophyte embryo development could occur through archegonia fertilisation, following a sexual mechanism or, in some cases, fertilisation is bypassed (apogamy).

7.5.1 Sexual Reproduction

Observations of fresh gametophytes under an optical microscope allow us to check for the presence of sexual organs, antheridia and archegonia, and the reproductive status of gametophyte. In species with sexual reproduction, the formation and maturation of antheridia and archegonia occur usually in the basal and apical parts of gametophyte, respectively. The presence of water is necessary for the displacement of the male gametes; adding water periodically is useful to the culture, favouring sexual reproduction.

Differences in the sequence of the ontogeny of gametangia and the proportion of sexual phenotypes in the gametophyte cultures have been reported in previous works (Tyron and Vitale 1977; Miller 1968; Klekowski 1969, Klekowski 1970; Haufler and Ranker 1985; Schneller et al. 1990; Hamilton and Lloyd 1991; Somer et al. 2010). In the last one, the proportions of sexual phenotypes in gametophytes of *A. capillus-veneris*, *A. adiantum-nigrum*, *D. canariensis*, *C. officinarum*, *D. dilatata*, *D. filix-mas*, *D. antarctica*, and *P. cambricum*, cultured on MS medium, were studied. Two main sexual tendencies were observed: maleness in *A. capillus-veneris*, *A. adiantum-nigrum*, *D. antarctica*, *D. dilatata*, and *D. filix-mas*, and femaleness in *D. canariensis*, *C. officinarum*, and *P. cambricum*. It does not seem that using a rich medium promoted femaleness in cultured gametophytes. On the other hand, regardless of the capacity of the gametophytes to form sexual organs in the same individual, only three species, *A. adiantum-nigrum*, *D. dilatata*, and

D. filix-mas, showed a moderate tendency to hermaphroditism when they are cultured under *in vitro* culture conditions. No correlation was found between hermaphroditism and sporophyte formation.

On the other hand, it has been reported that weak vegetative growth favours maleness and rapid growth favours femaleness or hermaphroditism (Korpelainen 1994). Somer et al. (2010) observed that high growth rate and maleness were observed in *A. capillus-veneris* under these culture conditions. The formation of sporophytes *in vitro* by sexual means varies among species. There are species showing very high rates of sporophyte formation such as *A. nidus* and *W. virginica* (Fernández et al. 1999), *P. cambricum* (Somer et al. 2010), and species showing difficulties for reproduction. Among these species, it is *B. spicant*, which acts an antheridiogen system controlling male sexual organ formation. The gametophytes of this species are mostly females when cultured on MS medium, and when mature, secrete to if you agree the medium antheridiogens, which induce maleness in young gametophytes. The periodical transfer of gametophtyes to a fresh medium might avoid accumulation of antheridiogens in the culture, and furthermore the formation of antheridia on young gametophytes, and as a result, the formation of sporophytes. A method for improving sporophyte formation in this species is to culture mature female gametophytes surrounded by young gametophytes (unpublished).

7.5.2 Apogamy

The formation of sporophytes from somatic cells, i.e., without the intervention of sexual organs is named apogamy. The term "obligate apogamy" describes a cycle in which a sporophyte is regularly produced in this manner. Approximately 10% of ferns and unknown proportion of other Monilophytes have life cycles of this type. In those species showing apogamy, a brown meristematic area develops near the apical notch and produce one sporophyte per gametophyte. The formation of apogamus sporophytes in *D. affinis* are visible 3–4 months after initiation of spore culture (Fernández et al. 1996a).

Apogamy can be induced *in vitro* altering the culture conditions. For instance, Whittier and Steeves (1960), Whittier and Steeves (1962), described the formation of apogamous plants in response to suitable concentrations of sugar in the medium. Later, the importance of osmotic potential is highlighted, suggesting that obligate apogamy may be an adaptation to xerophytic habit (Whittier 1975; Wagner and Smith 1993).

7.6 Sporophyte Multiplication

Once sporophyte appears in the culture, the following step will be to get its multiplication. In the formation of a sporophyte from preceding sporophytes, the morphogenic potential is involved. The process of sporophyte regeneration from sporophytic

tissue implies either direct regeneration, or indirect regeneration through callus or an aposporous gametophytic stage (Mehra and Sulklyan 1969; Kshirsagar and Mehta 1978; Bordonneau and Tourte 1987; Raghavan 1989; Kwa et al. 1995).

Different parts of the plant have been used for starting a clonal chain. At this end, the natural means of vegetative multiplication have been considered in the first instance. One of the classical successes was achieved culturing runner shoots of *Nephrolepis in vitro*. By this way, several leaf primordial were produced, which were isolated and grown into entire plantlets (Padhya and Mehta 1982). Prior to this finding, a great plasticity has been observed showed by excised leaf primordial when cultured *in vitro* (Steeves 1962). The rhizome of sporophyte had also showed a grand morphogenetic capacity *in vitro*, being used either for driving theoretical studies (Mehra and Sulklyan 1969; Kshirsagar and Mehta 1978) or to obtain an effective propagation system (Higuchi and Amaki 1989).

One prime consideration while deciding to set up a multiplication protocol in ferns, is the fact that the differentiation pattern towards gametophyte or sporophyte followed by sporophytic cells is labile. Furthermore, we must control the culture conditions acting in the developmental pathway which we are interested in.

In general terms, the differentiation pattern followed by gametophyte and sporophyte cells in ferns may be affected by many factors, such as the physiological isolation of cells, the age of plant material, nutrients (sucrose levels), exogenous phytohormones, and the physical state of the culture medium (von Aderkas 1986; Kwa et al. 1988, 1990; Materi and Cumming 1991; Bhambie and Gupta 1994; Camloh et al. 1994; Kwa et al. 1995; Teng and Teng, 1997; Fernández and Revilla, 2003; Martin et al. 2006).

Usually, the cultural conditions favouring differentiation of sporophytes do not favour the induction of aposporous gametophytes. For example, the ratio of sporophytes to gametophytes produced from leaves of *Microgramma vaciniifolia* increased when the sucrose concentration was increased from 0% to 4% (Hirch 1975). In *A. capillus-veneris*, 1–2% sucrose induced sporophytes whereas 7% induced aposporous gametophytes (Bhambie and Gupta 1994). The effect of the size of explant was recently investigated in *A. nidus* (H. Fernández, unpublished). The degree of disruption of rhizomes treated with 6-benzylaminopurine (BA), is associated with the posterior development of the explants. A large disruption of the rhizome tissue induced apospory and rhizome cells lost their capacity to differentiate sporophytes, but in larger size explants, sporophyte regeneration occurred. Teng and Teng (1997) investigating the regeneration pattern of *Platycerium bifurcatum* leaf cell suspension culture suggested that a threshold size of explant (in that case cellular aggregates) separated the two regeneration patterns. Moreover, the factors influencing the phase changes often interacted with each other. At 2% sucrose, 10 mm rhizome segments of *Ampelopteris prolifera* regenerated sporophytes, while 3–4 mm segments only regenerated gametophytes. At 0.2%, both 2–4 mm and 10 mm rhizome segments regenerated gametophytes (Mehra and Sulklyan 1969).

Concerning the role of phytohormones on multiplication of ferns, the effect of cytokinins, specially BA, on bud formation, inducing a high number of sporophytes, has been reported (Harper 1976; Loescher and Albrecht 1979; Beck and

Caponetti 1983; Hicks and von Aderkas 1986; Higuchi et al. 1987; Higuchi and Amaki 1989; Amaki and Higuchi 1991; Fernández et al. 1996b, Fernández et al. 1997b). Addition of BA to the culture of rhizome promoted a characteristic swelling of this organ as a result of proliferation centres located both on epidermal and subepidermal layers, as well as on the inner parenchyma, due to cell division in all planes. The organisation pattern they follow depends on the posterior supply of phytohormones and the contact period between explants and phytohormones (Fernández et al. 1997b). Subculture of rhizomes for 1 month on MS medium without added phytohormones induced frond development. Explants exposed to BA for 2 months induced apospory and gametophytes are formed from surface-located proliferation centres. The subculture of BA-pretreated rhizomes in media containing auxin-induced morphogenic callus, which regenerate sporophytes, and roots.

Morphogenesis in ferns might be induced by homogenisation or mechanical disruption of sporophyte, and subsequent culture in synthetic medium (Fernández et al. 1997b; Somer et al. 2010). The procedure is similar to that commented previously for gametophyte culture (Fig. 7.3). Sporophytes are homogenised with a waring blender for a few seconds (Fig. 7.3a) and then the plant material is cultured in liquid medium, and put under shaking in a growth chamber (Fig. 7.3b).

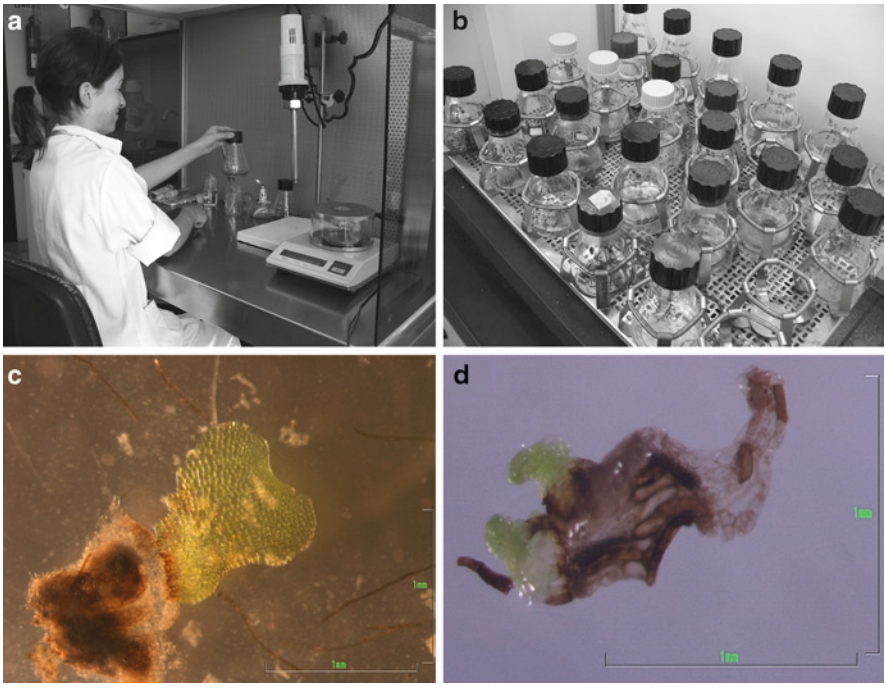

Fig. 7.3 Homogenised gametophytes from sporophytic tissue. (**a)** Chopping sporophytes with a waring blender. (**b)** Placement of cultures on a giratory shaker. (**c)** Aposporous gametophytes. (**d)** Sporophyte regeneration

Explants become initially brown and then some cells survive and regenerate either gametophytes (Fig. 7.3c) or sporophytes (Fig. 7.3d).

The role of phytohormones on the regeneration pattern in homogenised sporophytes has been studied, and varies both between and within species. For example, in *A. capillus-veneris*, *D. canariensis,* and *P. cambricum*, addition of BA to the medium induced the formation of aposporous gametophytes and/or dedifferentiated cellular aggregates, whereas the lack of BA favoured sporophyte regeneration. Conversely, in *A. adiantum-nigrum*, BA promoted the regeneration of both aposporous gametophytes and sporophytes (Somer et al. 2010). Kwa et al. (1988) reported the production of aposporous gametophytes from *Drymoglossum piloselloides* (L.) in juvenile fronds and in older fronds cultured in a modified MS medium supplemented with kinetin. Kwa et al. (1995) reported that low levels of auxins and cytokinins promoted apospory and high levels calli in *Pteris vittata* L. pinnae strips. Moreover, Kwa et al. (1997) described different morphogenetic capacities in two types of calluses cultured in a hormone-free medium, despite their common origin, in the fern *Platycerium coronarium*. Recently, Martin et al. (2006) found a synergistic effect from a combination of BA and kinetin that induced apospory.

In brief, we can assume that a wide range of culture conditions can affect morphogenesis in sporophytic tissues of ferns, and therefore, each species or part of the plant demands a singular study of its behaviour when cultured *in vitro*.

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Chapter 8
In Vitro Regeneration Systems of *Platycerium*

Marjana Camloh and Jana Ambrožič-Dolinšek

8.1 Introduction

The staghorn fern genus, *Platycerium* Desv., belongs to the family Polypodiaceae. It is an epiphytic genus of pantropical distribution, consisting of 15–18 species, cultivated worldwide because of their unique appearance (Hoshizaki 1972; Tryon and Tryon 1982; Hoshizaki and Price 1990; Hoshizaki and Moran 2001, Poremski and Biedinger 2001; Darnaedi and Praptosuwiryo 2003; Fernández and Vail 2003; Pemberton 2003). It has been stated that staghorns are the aristocrats of the cultivated ferns (Hoshizaki and Price 1990). Some *Platycerium* species, e.g., *P. grande* (Amoroso and Amoroso 2003) and *P. ridleyi* (Wee et al. 1992; Rodpradit 2003), are considered as endangered. These plants are distinguished from other ferns, among other characteristics, by the differentiation of the leaves into base fronds or mantle leaves and forked fertile leaves. Because of their great economic value, and their special place among ferns, these plants frequently have been used in cytological, morphological, developmental, physiological, and phylogenetic studies (e.g., Hoshizaki 1970; Nagmani and Raghavan 1983; Kwa et al. 1995a, b, Kwa et al. 1997a, 1997b; Camloh, et al. 1996, 1999; Teng and Teng 1997; Ambrožič-Dolinšek et al. 2002; Kreier and Schneider 2006; Espinosa-Matías et al. 2007; Janssen et al. 2007; Rut et al. 2008; Aspiras 2010).

Platycerium species are conventionally propagated by the sexual and vegetative methods. The first method involves raising plants from spores. The vegetative method of propagation, typical of at least some species of the genus *Platycerium*, is the development of new plants through root bud initiation (Hoshizaki 1970; Richards et al. 1983; Hoshizaki and Moran 2001). For some staghorn ferns (e.g., *P. grande*), it has been established that their spores are difficult to germinate in natural conditions (Amoroso and Amoroso 2003). As shown in studies of *in vitro* culture of spores, the duration of the process from spore to sporophyte can vary

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from 1 to 8 months in different ferns (Fernández et al. 1999). In *Platycerium* species, the length of this process, determined by using *in vitro* culture of spores, varies from 3 months in *P. bifurcatum* (Camloh et al. 2001), and 5 months in *P. ridleyi* (Rodpradit 2003) to 7 months in *P. grande* (Amoroso and Amoroso 2003). However, in a recent report on *P. coronarium* and *P. grande*, sporophyte development occurred only when gametophytes from the *in vitro* culture were transferred to sterilized potting medium (Aspiras 2010). Similarly, it was shown for *P. andinum* and *P. wandae* that sporophytes were not observed even 100 days after spore sowing in the medium (Espinosa-Matías et al. 2007).

It is known that the conventional propagation of ferns from spores is slow, while vegetative propagation is also hampered by low multiplication rates, and both observations are valid for staghorns also. Therefore, plant propagation methods using *in vitro* culture techniques have been intensively studied in ferns in recent decades (reviewed by Hegde and D'Souza 2000, Fernández and Revilla 2003, Somer et al. 2010). *Platycerium* species were also used for *in vitro* studies, and the great morphogenetic potential of these plants was revealed using different experimental systems (Wee et al. 1992, Camloh 2006 and references therein).

Besides mass propagation, *in vitro* cultures also have a role in the *ex situ* management of endangered species (Pence 2004; Soare 2008), a category to which some staghorns belong. Additionally, cryopreservation was studied in detail for *P. ridleyi* (Rodpradit 2003).

In this review, we provide an overview of research on *Platycerium* species using *in vitro* techniques, with special emphasis on the regeneration patterns obtained on sporophytic tissue. Regeneration systems in *Platycerium* are compared to those of other fern species and discussed with respect to their applicability to mass propagation. Furthermore, some similarities in regeneration patterns between *Platycerium* and seed plants are emphasized.

Firstly, the process of organogenesis regarding the initial explants, media, plant growth regulators, etc., is summarized. It has been established that various culture treatments can be used to induce and optimize organogenesis in seed plants and ferns; however, these factors are often manipulated in different ways (Sugiyama 2000; Philips 2004; George and Debergh 2008; Somer et al. 2010).

In ferns, regeneration on sporophytic tissue is usually initiated by growth regulators, although their usage is minimal (reviewed by Hegde and D'Souza 2000; Fernández and Revilla 2003). For some *Platycerium* species, it was reported that organogenesis of sporophytic tissue using different experimental systems was achieved without any growth regulators in the media (i.e., Wee et al. 1992; Camloh et al. 1994; Ambrožič-Dolinšek and Camloh 1997; Teng 1997; Rodpradit 2003). Furthermore, a high regenerative capacity was reported for some *Platycerium* species (Wee et al. 1992; Camloh 2006 and references therein). Regarding high regenerative capacity and simple culture requirements, a simple regeneration system applicable to mass propagation will be presented for *P. bifurcatum*.

In addition, the reports on apospory and apogamy, which were both studied in many ferns (reviewed by Fernández and Revilla 2003; Camloh 2006 and references therein, Martin et al. 2006; Somer et al. 2010 and references therein), including

staghorns (Kwa et al. 1995a; Teng and Teng 1997; Ambrožič-Dolinšek et al. 2002), are summarized for *Platycerium* species.

Secondly, we will point out the applicability of *in vitro* systems of *Platycerium* species, to obtain new insight into the effects of various culture treatments on regeneration patterns. For some *Platycerium* species, in addition to high regenerative capacity, high morphogenetic plasticity was claimed (Kwa et al. 1997a; Camloh 2006). For different *in vitro* systems of *P. bifurcatum*, e.g., the excised-leaf culture system, bud scale culture, and leaf cell suspension culture, it has been demonstrated that they can be easily manipulated for different morphogenetic responses: bud organogenesis, aposporous gametophyte development, rhizoid development, etc., by the practice of either wounding or cutting the explants, the concentration of sucrose, and jasmonic acid (JA) in the medium or cell aggregate size (Ambrožič-Dolinšek and Camloh 1997; Teng and Teng 1997; Camloh et al. 1999; Ambrožič-Dolinšek et al. 2002; Wee et al. 1992). Furthermore, in the case of the *P. coronarium* cell suspension culture, initiated from a gametophyte-derived callus, it was reported that morphogenesis into either gametophytes or sporophytes occurred without growth regulators, depending on the type of callus used (Kwa et al. 1997a). Thus, such systems are excellent tools for studying factors affecting morphogenesis.

8.2 *In Vitro* Regeneration Systems of Sporophytic Tissue

In seed plants, *in vitro* propagation is achieved either from pre-existing meristems (axillary buds), or from somatic cells through organogenesis (direct or indirect) or by somatic embryogenesis. A well-balanced mineral nutrient composition, an adequate supply of carbohydrates, and appropriate levels of growth regulators in the medium are major factors in morphogenetic expression *in vitro* (Ziv and Chen 2008 and references therein).

In ferns, when using sporophytic tissue, regeneration through axillary buds and direct or indirect organogenesis was reported (reviewed by Hegde and D'Souza 2000; Fernández and Revilla 2003). In ferns, homogenized cultures of gametophytic or sporophytic tissue are often used as propagation method (Bertrand et al. 1999, Fernández and Revilla 2003, Somer et al. 2010) and recommended for species with a short life cycle (Fernández et al. 1999). In several ferns, micropropagation was reported also through the proliferation of green globular bodies (GGBs) on different sporophytic explants, usually on media with cytokinin; these GGBs later develop into sporophytes (Higuchi et al. 1987; Amaki and Higuchi 1991; Fernández et al. 1996; Bertrand et al. 1999). The term GGB was first used by Higuchi et al. (1987) for the rapidly proliferating tissue developed on rhizome segments of *Nephrolepis cordifolia* cultured on a 6-benzylaminopurine (BA) containing medium. Somatic embryogenesis in Pteridophyta has rarely been described; it was reported for *Lycopodiella inundata* (Atmane et al. 2000) and *Huperzia selago* (Szypuła et al. 2005).

Two other morphogenetic processes have often been studied in ferns using *in vitro* techniques: apospory, the development of gametophytes on sporophytic tissue and apogamy, the development of sporophytes directly from the gametophytes without sexual fusion (reviewed by Fernández and Revilla 2003; Camloh 2006 and references therein, Martin et al. 2006 and references therein).

In *Platycerium* species, organogenesis has mainly been achieved directly. The regeneration of plantlets from homogenized leaf tissue (Cooke 1979; Teng and Teng 1997, 2000) and through the initiation of GGBs has also been described. Apospory and apogamy developments have also been studied. The reports carried out to date on *in vitro* regeneration of *Platycerium* from sporophytic tissue are summarized in Table 8.1; those already summarized by Hegde and D'Souza (2000) are not included.

8.2.1 Direct Shoot Organogenesis

In seed plants, direct shoot initiation on explants is rarely observed and is even unknown in many plant genera (George and Debergh 2008), while in ferns, it has been reported for several species (reviewed by Hegde and D'Souza 2000). In fern micropropagation through direct shoot organogenesis, rhizome tips or pieces, runner tips and section of fronds were mainly used as initial explants, and these explants were most commonly cultured on Murashige and Skoog (1962) and B5 media (Gamborg et al. 1968) or their modifications (reviewed by Hegde and D'Souza 2000).

In *Platycerium* species, direct shoot development was reported on the following explants: shoot tips (Hennen and Sheehan 1978; Thentz and Moncousin 1984); rhizome pieces (Wee et al. 1992); fragments of young leaves (Thentz and Moncousin 1984; Wee et al. 1992; Rodpradit 2003); entire juvenile leaf (Camloh and Gogala 1991; Camloh et al. 1994); shoots (Pevalek-Kozlina 1996); bud scales (Ambrožič-Dolinšek and Camloh 1997; Ambrožič-Dolinšek et al. 1999), and through GGB proliferation directly on pieces of juvenile leaves (Jámbor-Benczúr et al. 1995).

The following sections on direct shoot organogenesis in *Platycerium* are based on the initial explants used.

8.2.1.1 Shoot and Rhizome Culture

Micropropagation using shoot tips from greenhouse-grown plants as initial explants was first described by Hennen and Sheehan (1978) for *P. stemaria.* They also used this technique successfully for *P. wandae*, *P. veitchii*, *and P. wallichii*. Shoot tips, 2–3 mm^3 in size, were placed on modified MS with adenine sulfate (80 mg/L) and indole-3-acetic acid (IAA) (85.6 μM). Adventitious bud development occurred at the base of the explants, on the roots, and on leaves that were in contact with the medium. Thentz and Moncousin (1984) used shoot tips cultured on Linsmaier and

Table 8.1 The reports on in vitro regeneration of *Platycerium* from sporophytic tissue carried out to date (those reports already summarized by Hegde and D'Souza (2000) are not included)

Platycerium species	Initial explant	Media	Type of regeneration	References
P.bifurcatum	shoot tip, leaf fragments	MS, LS, different conc. of sucrose, agar, pH, 0.5-2.5 μM IBA and 4.4 μM BA (shoot tips), 8.9-13.3 μM BA (leaf fragments)	Sporophytes,	Thentz and Moncousin 1984
P.coronarium P.ridleyi	Rhizome pieces, juvenile leaf fragments	MS, 5.4 μM NAA (*P.coronarium*), no growth regulators (*P.ridleyi*)	Sporophytes	Wee, Kwa and Loh 1992
P.bifurcatum	Shoots	MS, Different conc. of sucrose, agar, pH, MS, 0.54 μM NAA and 9.3 μM KIN	Shoot multiplication	Pevalek-Kozlina 1996
P.bifurcatum	Juvenile leaf fragments	MS, with variations in strength, 0.54 μM NAA and 0.46 μM KIN	Sporophytes through GGBs	Jámbor-Benczúr et al.1995
P.bifurcatum	Leaf cell suspension culture	1/2 MS, 5.4 μM NAA and 4.4 μM BA	Sporophytes	Teng 1997
P.bifurcatum	Leaf cell suspension culture,	MS,	Sporophytes (from cell aggregates >500 cells) or aposporous gametophytes(from cell aggregates <100 cells)	Teng and Teng 1997
P.bifurcatum	Scales	MS, no growth regulators	Sporophytes, apospory	Ambrožič-Dolinšek and Camloh 1997
P.bifurcatum	Scales	MS, no growth regulators	Sporophytes	Ambrožič-Dolinšek, Camloh and Žel 1999
P.bifurcatum	Leaves, shoot base, homogenized sporophytes	MS, no growth regulators or 0.54 μM NAA	Sporophytes	Teng and Teng 2000
P.bifurcatum	Juvenile leaves	MS, low sucrose, wounding	Sporophytes, apospory	Ambrožič-Dolinšek et. al. 2002
P.ridleyi	Pieces of juvenile leaves	MS, no growth regulators	Sporophyte	Rodpradit 2003

MS, Murashige Skoog medium (1962), LS, Linsmaier Skoog medium (1965), GGBs, green globular bodies, *NAA* 1-naphtaleneacetic acid, *BA* 6-benzylamino purine, *KIN* kinetin, *IBA* 3-indolebutyric acid

Skoog medium (1965) with BA and 3-indolebutyric acid (IBA) to initiate culture. The adventitious buds and rhizoids developed at the base of the explants and on the roots. Wee et al. (1992) placed rhizome pieces, 2 mm^2 in size, on a growth regulator-free MS medium to initiate *in vitro* culture of *P. coronarium* and *P. ridleyi*. All rhizome explants produced sporophytes after 2 months in the culture. In some cultures, callus developed, but differentiation into sporophytes was not observed. Their report describes the acclimatization experiments in detail. Pevalek-Kozlina (1996) used shoots obtained *in vitro* from spores to initiate *P. bifurcatum* culture. The shoots were placed on MS medium with 1-naphtaleneacetic acid (NAA) and kinetin (KIN). The study focused on the effects of sucrose and agar concentration, and the pH medium on shoot multiplication. The highest multiplication rate (12.75–14.29 shoots per explant) was observed on a medium containing 3.0% sucrose and 0.9% agar, with a pH of between 5.7 and 7.0.

8.2.1.2 Leaf Culture

To initiate *Platycerium* leaf culture, fragments or entire leaves were used. Thentz and Moncousin (1984) obtained buds on leaf fragments using the same medium as for shoot tip culture, except that the concentration of BA was higher. After 6 weeks in the culture, the whole explant was covered with buds and rhizoids. After 10 weeks, the explants were subdivided. On 1 cm^2 of the explants, up to 30 sporophytes formed. Wee et al. (1992) also used fragments of young leaves for regeneration of *P. coronarium* and *P. ridleyi.* They cultured juvenile frond strips on an MS medium with 2,4-dichlorophenoxiacetic acid (2,4-D), NAA or BA. The best sporophyte production was obtained on *P. coronarium* with NAA, while for *P. ridleyi*, NAA and BA had no effect. For both species 2,4-D was inhibitory. Direct adventitious bud development was achieved in *P. ridleyi* on pieces of juvenile fronds (5 mm^2) cultured on an MS medium without growth regulators (Rodpradit 2003). After 2 weeks, several tiny, green, round sporophytes developed on the frond strips. The regeneration started at the margins of the explants and eventually covered the rest of the explants. From one initial explant, 30–50 sporophytes approximately 9 mm long with 2–4 fronds were obtained after 8 weeks in the culture. Jámbor-Benczúr et al. (1995) described *in vitro* regeneration of *P. bifurcatum* through the development of GGBs on fragments of juvenile leaf (0.5 mm^2 in size) cultured on a modified MS medium with NAA and KIN. In 12 weeks, along the cut surface of the explants, groups of GGB did develop even on the growth regulator-free medium. On the surface of the GGBs, first meristems and then shoot primordia appeared, but no development of callus was reported. For elongation, 2–3 mm colonies of GGBs were separated and placed on the same medium, without growth regulators. Rooting was achieved on a modified medium (Jámbor-Benczúr and Márta-Riffer 1990) with added NAA. Regeneration and rooting proved better when active carbon was added to the medium. From GGBs, plantlets fit for rooting developed in 6 months.

In our studies of *P. bifurcatum* regeneration, entire juvenile leaves, 0.8–1.2 cm in size, obtained from *in vitro* grown plants, were placed on the MS medium modified by Hennen and Sheehan (1978), but without adenine sulfate, so that the basal end

and the abaxial surface were in contact with the medium (Camloh and Gogala 1991; Camloh et al. 1994). Although the effects of some growth regulators on regeneration were tested, efficient regeneration was achieved on growth regulator-free medium. Buds appeared mainly on the abaxial surface of the leaf. The development of multicellular scales and rhizoids coincides with bud induction. Scale development was also observed during in vivo root bud development (Richards et al. 1983).

After 40 days of culture, 10–35 buds developed. Eight weeks after culture initiation, the buds were excised and elongated for an additional 4 weeks. To obtain more plants, explants could first be subdivided and cultured for 4 weeks or more before bud excision and elongation. Interestingly, at the base of shoots and sometimes on their leaves, the development of new buds occurred, and as in vivo, buds were occasionally also observed on the roots. Up to 150 shoots were obtained from a single leaf after 3 months in the culture. After elongation, roots already developed on several shoots. Shoots without roots were successfully rooted on ¼-strength modified MS, and after 6 weeks the plants were planted in soil. The whole regeneration process from initial explants to plants appropriate for planting in soil took 18 weeks (Fig. 8.1). Our further studies focusing on induced apospory revealed that wounding of the initial explants does affect bud development. On wounded leaves, a higher number of buds was obtained, and their development on the adaxial surface of the leaf was promoted (Ambrožič-Dolinšek 1998).

High regenerative capacity, especially when wounded explants are used to initiate the culture, good rooting of shoots, relatively simple culture requirements, and appropriate duration of the whole process from initial explants to plants make this system suitable for mass propagation of *P. bifurcatum*.

8.2.1.3 Culture of Bud Scales

In mature *P. bifurcatum* plants, scales cover the buds and the entire rhizome surfaces; they protect the buds from desiccation, animals, and excess water (Hoshizaki 1970).

Scales also cover the buds of *in vitro* cultured shoots, and their formation can be observed in the early stages of organogenesis (Camloh et al. 1994). During our studies on *in vitro* culture of *P. bifurcatum*, we observed different outgrowths on scales while they were still attached to the buds. Therefore, we made a detailed study of the regeneration patterns and morphogenetic potential of scales (Ambrožič-Dolinšek and Camloh 1997; Ambrožič-Dolinšek et al. 1999). To initiate scale culture, scales composed of a single layer of cells were detached from the *in vitro* grown shoots and placed flat on the same medium as used for leaf culture (Fig. 8.2). In the majority of scales, regeneration began during the first 30 days of culture as the proliferation of several cells, rarely one, at different sites on the scale. Newly proliferated zones formed three-, or occasionally two-dimensional groups of cells, which developed into buds, aposporous gametophytes, or remained undifferentiated. On the aposporous gametophytes, gametangia developed. The formation of rhizoids on the outgrowths was also observed (Fig. 8.2). Interestingly, after 90 days of culture, roots were already formed on nearly half of all developed buds. At the base of shoots, secondary shoots commonly developed, which most likely originated from scales of

Fig. 8.1 The regeneration of *P. bifurcatum* from juvenile leaf. (**a**) Initial explants, wounded (above) and intact (below) juvenile leaves. Bar = 1 cm. (**b**, **c**) Adaxial and abaxial side of the same intact leaf after 30 days of culture on growth regulator-free medium. Note the development of buds on the abaxial side of the leaf. (**d**), (**e**) Adaxial and abaxial side of the same wounded leaf after 30 days of culture on growth regulator-free medium. Note the development of buds also on the adaxial side of the leaf. (**f**) Buds elongated to shoots after 3 months of culture. Bar = 1 cm. (**g**) Shoots after elongation, several shoots already developed roots. Bar = 1 cm. (**h**, **i**) Rooted plants are transferred to the soil. (**h**), Plants after 6–7 months. Bar = 7 cm. (**i**), Plants grown in greenhouse, 10 months after culture initiation

the shoots. New shoots of such origin were simply detached from primary shoots and cultivated further. The described system of regeneration obtained on scales with no growth regulators in the medium additionally confirmed the high regenerative capacity of some *Platycerium* species.

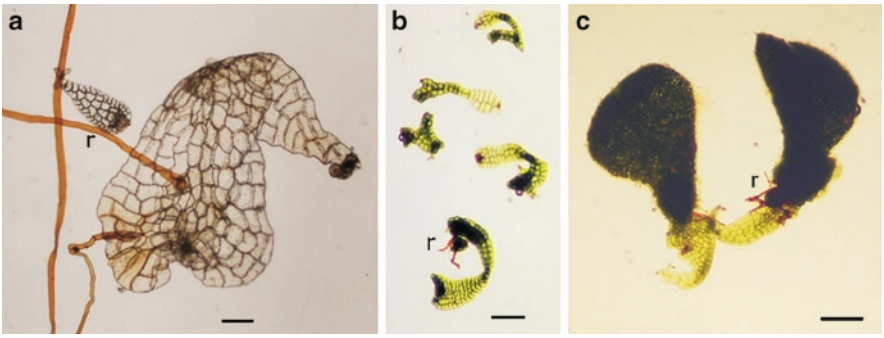

Fig. 8.2 The regeneration of *P. bifurcatum* from bud scales. (**a)** Scale, composed of a single layer of cells, after detachment from the in vitro grown shoot. Note the rhizoids on scale. Bar = 0.1 mm. (**b**, **c)** Scales placed flat on the growth regulator-free medium. (**b)** Scales after 30 days of culture, newly proliferated zones are clearly visible. (**c**), Shoots developed from scales after 60 days of culture. Bar = 0.5 mm

8.2.1.4 Homogenization of Sporophytes

George and Debergh (2008) define indirect shoot organogenesis as the formation of shoots on a previously unorganized callus, or in cell cultures. In homogenization studies of *Platycerium*, sporophytes were homogenized, and used directly to obtain regeneration. Since, no callus was observed during the regeneration, we placed these studies in the direct shoot organogenesis category. Cooke (1979) was the first to use homogenized sporophytes obtained *in vitro* for propagation of *Platycerium* species. Homogenized tissue was placed on a medium consisting of MS salts, sucrose, agar, inositol, and thiamine-HCl. Growth was evident in 2–3 weeks, and an excellent multiplication rate was reported. Teng and Teng (2000) used homogenized sporophytes, 1–3 cm long, to study the effects of antibiotic pulse treatment (ATP) on the regenerative ability of *P. bifurcatum.* As a control, they used nontreated homogenized sporophytes and obtained very high numbers of regenerants. Two milliliters of suspension (cell density was adjusted to 40–50 mg dry wt/mL) inoculated on a modified MS medium with or without NAA generally regenerated 800–900 and 100–200 sporophytes larger than 0.5 cm and 0.1–0.5 cm, respectively within 8–10 weeks.

8.2.2 Indirect Shoot Organogenesis

Propagation of ferns through indirect shoot organogenesis, the development of shoots from callus tissue, was rarely reported in ferns (reviewed by Hegde and D'Souza 2000). However, Hegde et al. (2006) obtained callus on rhizome tips of *Drynaria quercifolia* – a medicinal fern, when cultured on a Knop's salt solution and different concentrations of auxins. Sporophytic regeneration occurred on the medium with 6-(γ,γ-dimethylallylamino) purine (2iP). Suspension cultures were also induced from the friable morphogenetic callus.

In *Platycerium*, Wee et al. (1992) observed callus development on some cultures of *P. coronarium* and *P. ridleyi* initiated from rhizome pieces, but differentiation of shoots was not obtained. In their further studies, successful initiation of callus cultures from gametophytes was reported (Kwa et al. 1995b, Kwa et al. 1997a, b). However, these studies were not focused on *in vitro* propagation, but on some physiological aspects of callus cultures e.g., in the report of Kwa et al. (1995b) the establishment of a photoautotrophic callus culture system and its physiological analysis were described for *P. coronarium*. They also used this system to study the activity of Rubisco and phosphoenolpyruvate carboxylase (Kwa et al. 1997b).

8.2.3 Apospory and Apogamy

Apospory and apogamy will be described in detail in other chapters of this book; therefore, we will focus only on studies of these morphogenetic processes performed in *Platycerium* species. Kwa et al. (1995a) described IAA-induced apogamy in *P. coronarium* gametophytes cultured *in vitro*. The gametophytes were macerated into fine pieces using a scalpel, and clumps of about 70–75 mg were cultured individually on MS medium with IAA at different concentrations. The percentage of apogamy and the total number of apogamous sporophytes produced per gametophytic clump were the highest in the presence of 40 μM IAA. Teng and Teng (1997) obtained aposporous gametophytes from leaf cell suspension cultures of *P. bifurcatum*, but only from single cells or aggregates of up to 100 cells. This aposporous gametophyte later gave rise to sporophytes through apogamy. For suspensions, they used MS salts supplemented by thiamine-HCl, pyridoxine-HCl, nicotinic acid, glycine, myoinositol, and NAA.

In our experiments on *P. bifurcatum*, apospory was obtained during regeneration of bud scales (Ambrožič-Dolinšek and Camloh 1997), but a detailed study was performed on leaf culture (Ambrožič-Dolinšek et al. 2002) (Fig. 8.3). On the basis of

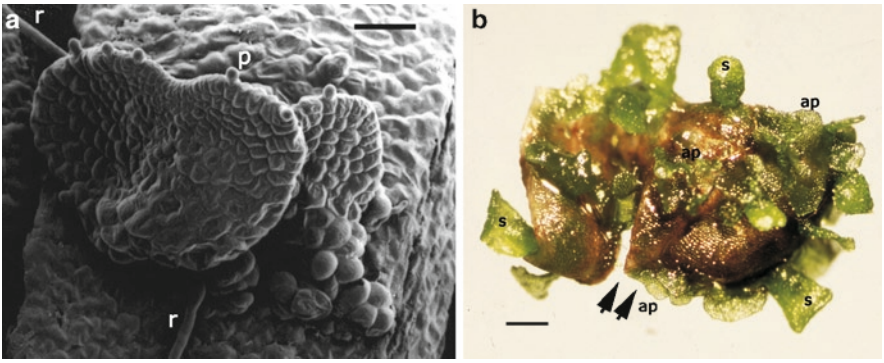

Fig. 8.3 The development of apospory on wounded leaves of *P. bifurcatum*, on modified MS medium with 0.01% sucrose. (**a**) aposporous gametophytes after 30 days of culture. Note rhizoids (r) and papillae (p). Bar = 0.1 mm. (**b**) the development of aposporous gametophytes (ap) and shoots (s) after 45 days of culture, arrows indicate wounds. Bar = 2mm

this study, we propose a set of conditions, which regularly and reproducibly induce apospory on most of the leaf explants of *P. bifurcatum*. Apart from the juvenility of explants, the most important factors for apospory induction are a combination of low sucrose concentration in the medium (0.01%) and wounding of the leaf.

8.3 *In Vitro* Cultures of *Platycerium* in Developmental and Physiological Studies

In vitro cultures of *Platycerium* species have often been used for different studies. In a recent review, the applicability of different experimental systems of *P. bifurcatum* to study the developmental and physiological processes was described in detail (Camloh 2006). Therefore, in this chapter, we will focus mostly on other staghorns and those studies using *in vitro* cultures of sporophytic tissue not described in the review, while studies on *P. bifurcatum* will be dealt with only briefly.

Kwa et al. (1995c) studied the role of ethylene on *in vitro* regeneration from frond and rhizome explants of *P. coronarium* sporophytes. They found that the addition of the ethylene action inhibitor silver thiosulfate at 25 and 15 μM resulted in an increase in the percentage of regeneration from frond and rhizome explants, respectively. Since in further experiments inhibitors of ethylene biosynthesis (which are effective in higher plants) were ineffective, they suggested that ferns do not produce ethylene via the same pathway as angiosperms. Kwa et al. (1997a) studied the morphogenetic plasticity of callus reinitiated from cell suspension cultures of *P. coronarium*. From callus initiated from gametophytes cultured on MS medium with 2,4-D, they established cell suspension cultures. When cells from the suspension cultures were plated on semisolid MS medium containing 10 μM KIN, two distinct types of callus masses, distinguished by their coloration, were obtained. Morphogenesis into either gametophytes or sporophytes occurred when these callus masses were cultured on growth regulator-free medium. They proposed this system as useful for comparative studies of developmental plasticity. Teng and Teng (1997) used leaf cell suspension cultures to study regeneration patterns of *P. bifurcatum in vitro*. They showed that morphogenetic events can be manipulated not only by environmental factors but also by cell status, i.e., aggregate size. From single cells and aggregates of up to 100 cells aposporous gametophytes developed, while from aggregates of more than 500 cells, direct regeneration of sporophytes was observed. In another report on *P. bifurcatum* (Teng and Teng 2000), they used leaf and shoot base explants and homogenized sporophytes to study the effects of ATP on the regenerative ability of *P. bifurcatum*. The response to ATP was dependent on the duration of ATP and the cell status at which the cells were treated. They considered the reaction to ATP as measured by changes in sporophyte regeneration and morphology as stress response. The cells' susceptibility to ATP was graded as tissue homogenized prior to the treatment > tissue homogenized after the treatment > leaf and shoot base explants.

In our experiments using the excised-leaf culture system, we found that JA affects organogenesis in *P. bifurcatum* (Camloh et al. 1999). JA stimulated development

of rhizoids and the development of adventitious shoots. Furthermore, it affects the site of shoot development. Therefore, we suggested that JA might be involved in regulating morphogenesis in this fern. We also studied the effects of wounding on organogenesis, and found that wounding has an effect on organogenesis similar to that with the addition of JA to the medium (Ambrožič-Dolinšek, 1998). In scale culture, we studied the effect of sucrose on organogenesis and found that increasing the sucrose concentration from 0 to 3% strongly increased bud development on scales, while the opposite effect of sucrose was observed on gametophyte development on scales (Ambrožič-Dolinšek and Camloh 1997; Ambrožič-Dolinšek et al. 1999).

In *P. ridleyi*, a detailed study on cryopreservation was performed using young sporophytes obtained *in vitro* from spores (approximately 2 mm in diameter) (Rodpradit 2003). Encapsulation/dehydration and encapsulation/vitrification techniques were tested. The encapsulation/dehydration technique gave a higher regrowth rate and faster regeneration. Each encapsulated young sporophyte usually develops into several new sporophytes, around 30–80 from the treated control and encapsulation/dehydration techniques, while 5–50 were yielded by the encapsulation/vitrification technique. The effect of cryopreservation on mass reproduction of *in vitro*-grown *P. ridleyi* was also studied using pieces of juvenile fronds (5 mm^2) cultured on an MS medium without growth regulators. No abnormality in regrowth or morphology was observed when comparing frond cultures initiated from noncryopreserved and cryopreserved frond strips; both could produce up to 50 new sporophytes on each strip after 8 weeks in the culture.

8.4 Conclusion

In vitro techniques have been intensively used in different *Platycerium* species in recent decades, and several regeneration systems applicable to mass propagation have been described. Successful cryopreservation has also been performed. For some staghorns, the great morphogenetic potential and plasticity of *in vitro* cultures is evident. Organogenesis was successfully achieved on different sporophytic explants (pieces or whole juvenile leaves, bud scales, rhizome pieces) with no growth regulators in the media. Furthermore, a simple, reproducible system for apospory induction was defined. Such a system represents an excellent tool for studying the factors affecting morphogenesis, and by using them, new information on the mechanisms involved in organogenesis has been obtained. Undoubtedly, such systems also represent a promising tool for studying developmental and physiological processes in ferns.

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Chapter 9
Stipule Propagation in Five Marattioid Species Native to Taiwan (Marattiaceae; Pteridophyte)

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9.1 Introduction

The eusporangiate family Marattiaceae includes six genera and ca. 260 extant species (Tryon and Tryon 1982). This family has an early origin and can be tracked back to the Carboniferous period (Collinson 1996). Many extant species are well adapted for growth on the shaded floor of wet, tropical forests (Tryon and Tryon 1982). Due to restricted sites and small populations, many of them are regarded as being endangered (e.g., Kuo 1997; Moore 2001; Chiou et al. 2006).

Five marattioid species of three genera, *Angiopteris lygodiifolia* Rosenst., *Angiopteris palmiformis* (Cav.) C. Chr., *Archangiopteris somai* Hayata, *Archangiopteris itoi* Shieh, and *Marattia pellucida* Presl, were documented in Taiwan (DeVol and Shieh 1994). Except for *Angiopteris lygodiifolia*, the other four species are restricted to a few sites and are considered to be endangered or critically endangered in Taiwan (Kuo 1997; Moore 2001).

Ex situ conservation, particularly of rare and endangered species, is an important supplement to the maintenance of ferns in the wild. Sexual propagation through spore culture is commonly used for the ex situ conservation of most ferns. However, gametophytes of marattioid species usually grow slowly, and/or their spores are hard to germinate (Chou et al. 2007). Because plantlets can be generated from stipules in marattioid species in the wild (Tryon and Tryon 1982; Sharpe and Jernstedt 1991), propagation from stipules was applied to this family for conservation and horticultural purposes (Uffelen 1994; Chiou et al. 2006).

In this study, sprouting rates and positions of five marattioid species native to Taiwan were determined. The effect of stipule sizes on sprouting rates and plantlet morphology were analyzed as well. The results showed that stipules of marattioid species are useful tissues for propagation and conservation.

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9.2 Materials and Methods

Five marattioid species native to Taiwan were used in this study. The source locations are given in Table 9.1 and Fig. 9.1. Depending on the rarity of a species in the wild, 9–117 stipules were removed from each of 2–29 sporophytes (Table 9.1). To avoid injury to the mother plants, only underground stipules, which were not firmly attached to the stems, were removed and cultured. As soon as they were harvested, stipules were sealed in plastic bags to prevent dehydration. In the laboratory, the

Table 9.1 Information on material sources

Species	Locations[a]	No. of plants	No. of stipules
Angiopteris			
Ang. lygodiifolia	WL, LHC, LG	25	71
Ang. palmiformis	LY	9	9
Archangiopteris			
Arc. somai	WL, LHC	24	117
Arc. itoi	WL, LHC	29	93
Marattia			
M. pellucida	LY	2	16

[a]Abbreviations are depicted in Fig. 9.1

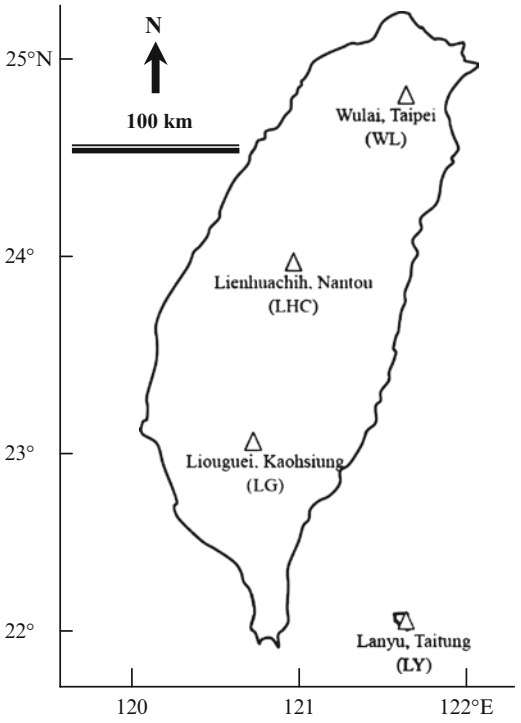

Fig. 9.1 Locations of the material sources in Taiwan and an offshore island (Lanyu)

stipules were rinsed for 3 min with clean water and after being half-covered by moist sphagnum moss, placed in plastic boxes. All cultures were maintained under white fluorescent light illumination (24 μmol m^{-2} s^{-1}, 12 h day^{-1}) at 20–28°C. After 1 year of culture, when no more sprouting was found, the positions from which the sprouts originated, sprouting rates, and the number of plantlets from each stipule were documented. The correlations of frond sizes (frond length × frond width) and stipule widths were analyzed.

9.3 Results

Plantlets were found at four specific sites on a stipule, namely the proximal (near the rhizome) right, proximal left, distal (toward the frond tip) right, and distal left margins (Fig. 9.2). One or more of the four points produced one plantlet in each stipule of *Angiopteris lygodiifolia*, *Angiopteris palmiformis*, *Archangiopteris itoi*, and *M. pellucida.* More plantlets were found at the proximal margins (70–97%) than at the distal margins (3–30%). Differences in sprouting rates between the right and left margins of the proximal site were larger (2–12%) than what they were for the distal sites (0–1%) among *Angiopteris lygodiifolia*, *Angiopteris palmiformis*, and *Archangiopteris itoi*. In *M. pellucida*, on the contrary, the sprouting rates between the right and left margins of the distal site (8%) was larger than it was for the proximal site, which showed no difference between the two margins. In *Archangiopteris somai*, however, plantlets were found only on the proximal margins (Table 9.2).

Stipules began to sprout after 1 month of culture in all the five species. The sprouting rate and the number of plantlets per stipule somewhat differed among species. After 1 year of culture, every stipule of *Angiopteris lygodiifolia*, *Angiopteris palmiformis*, and *M. pellucida* had sprouted plantlets, and the average numbers of plantlets per stipule

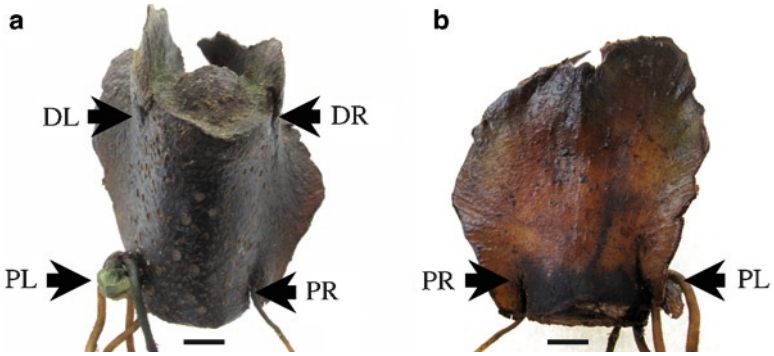

Fig. 9.2 Positions of sprouting on stipules, an example of *Angiopteris lygodiifolia*. (**a)** View of the abaxial side. (**b)** View of the adaxial side; positions of the distal right (*DR*) and distal left (*DL*) are hidden. The DR, DL, proximal right (*PR*), and proximal left (*PL*) positions at the stipule margins are indicated by *arrows*. Scale bars = 1 cm

Table 9.2 Sprouting rate (%) by stipule position of five marattioid species

Species	Distal right	Distal left	Proximal right	Proximal left
Angiopteris				
Ang. lygodiifolia	15	15	36	34
Ang. palmiformis	6	6	50	38
Archangiopteris				
Arc. somai	0	0	52	48
Arc. itoi	1	2	47	50
Marattia				
M. pellucida	18	10	36	36

Table 9.3 Sprouting rates and plantlet number for stipules taken from five marattioid species

Species	Sprouting rate (%)	No. of plantlet per stipule bearing plantlets (minimum–maximum)
Angiopteris		
Ang. lygodiifolia	100	2.68 (1–4)
Ang. palmiformis	100	2.00 (1–3)
Archangiopteris		
Arc. somai	85	1.66 (1–2)
Arc. itoi	98	1.92 (1–2)
Marattia		
M. pellucida	100	2.44 (1–4)

Table 9.4 Widths of sprouting and nonsprouting stipules in *Archangiopteris somai* and *Archangiopteris itoi*

	Stipule width (mm)		
Species	Sprouting stipules	No-sprouting stipules	*t*-test
Arc. somai	13.46 ± 4.04 ($n=100$)	10.27 ± 3.38 ($n=17$)	$t=3.0786$, $p<0.01$
Arc. itoi	23.32 ± 7.81 ($n=93$)	16.33 ($n=2$)	$t=1.2585$, $p=0.21$

were 2.68, 2.00, and 2.44, respectively. *Archangiopteris somai* and *Archangiopteris itoi* had relatively lower sprouting rates of stipules, at 85 and 98%, respectively, and lower average numbers of plantlets per stipule, at 1.66 and 1.92, respectively (Table 9.3). It was found that the size (width) of nonsprouting stipules was significantly smaller than those of sprouting ones in *Archangiopteris somai*, whereas the sprouting abilities of these two kinds of stipules did not significantly differ in *Archangiopteris itoi* (Table 9.4).

Stipule size had a positive correlation with sprouting rates among the five species. In *Angiopteris lygodiifolia*, stipule sizes (widths) had a significant modestly positive correlation with sprouting rates. When the widths of stipules were >40 mm, four plantlets could be produced per stipule. Small stipules had a tendency to produce fewer plantlets (Fig. 9.3a). In *Angiopteris palmiformis*, stipule sizes (widths) had a nonsignificant modestly positive correlation with sprouting rates. When the widths

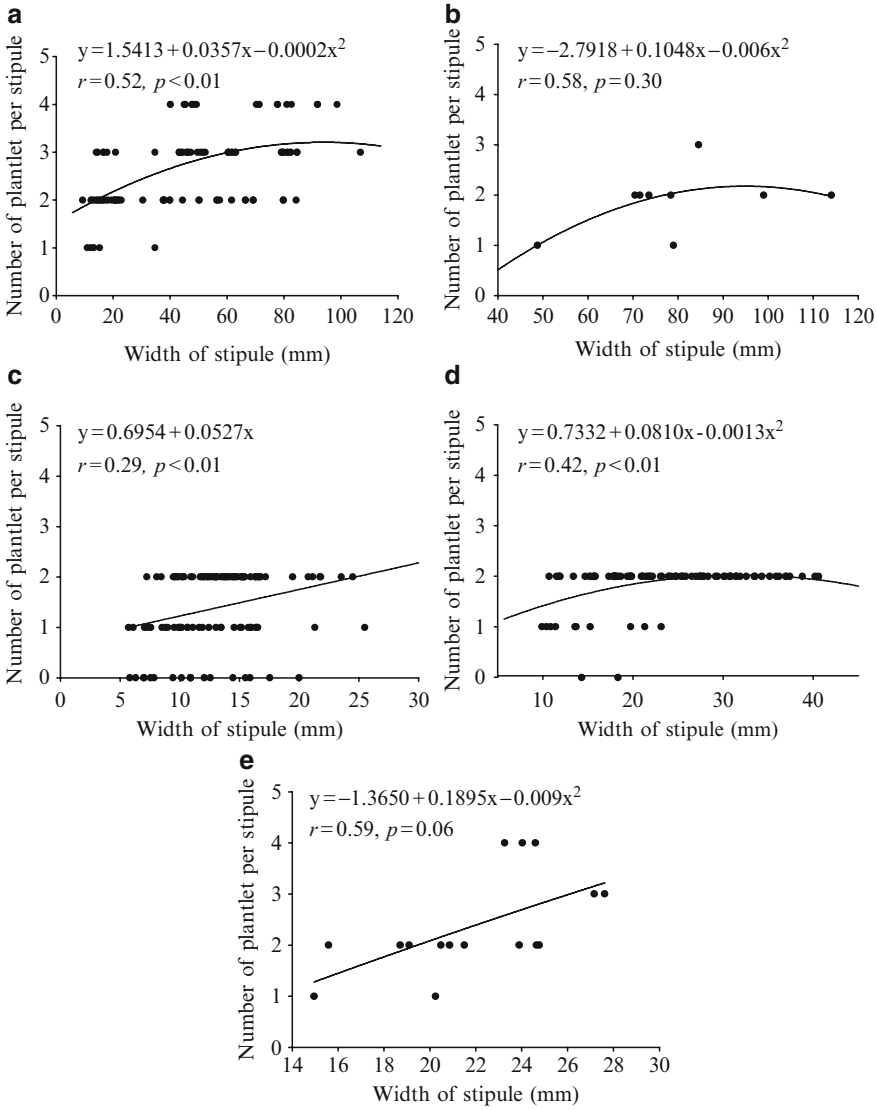

Fig. 9.3 Correlation between the width of stipules and the number of plantlets in five marattioid species. (**a**) *Angiopteris lygodiifolia*; (**b**) *Angiopteris palmiformis*; (**c**) *Archangiopteris somai*; (**d**) *Archangiopteris itoi*; and (**e**) *Marattia pellucida*

of the stipules were >70 mm, only two plantlets were produced per stipule. A small stipule (ca. 50 mm wide) only produced one plantlet (Fig. 9.3b). In *Archangiopteris somai*, stipule sizes (widths) had a significant weakly positive correlation with sprouting rates. Their stipules produced two plantlets at the most (Fig. 9.3c). In *Archangiopteris itoi*, stipule sizes had a significant modestly positive correlation with sprouting rates. When the widths of stipules were >25 mm, two plantlets were

produced per stipule. Smaller stipules (with widths of <10 mm) produced no (zero) to two plantlets (Fig. 9.3d). In *M. pellucida*, stipule sizes (widths) had a nonsignificant modestly positive correlation with sprouting rates. Plantlet numbers per stipule almost linearly increased with stipule size (width). Larger stipules (with widths of >22 mm) produced two to four plantlets. However, smaller stipules (with widths of <22 mm) only produced one to two plantlets (buds) (Fig. 9.3e).

Correlations between the sizes (widths) of stipules and the first fronds (width × length) varied among these five species. They were significantly correlated in *Angiopteris lygodiifolia*, *Archangiopteris somai*, and *Archangiopteris itoi*, whereas they were not significantly correlated in *Angiopteris palmiformis* and *M. pellucida* (Fig. 9.4). The first fronds reproduced from bigger stipules tended to have more-complicated structures, i.e., pinnate to bipinnate, whereas those from smaller stipules tended to be simpler (Fig. 9.4).

9.4 Discussion

In our field investigation, no stipular bud development was observed if the stipules attached to the mother plants among these five Taiwan marattioid species. This suggests that mother plants release some chemical signals to stipules that inhibit the development of plantlets (White 1979; Hillman 1984). Old stipules of *Angiopteris palmiformis* can spontaneously detach from the mother plants and might produce plantlets in the wild. On the other hand, stipules of the four other marattioid species do not detach from their mother plants, and no plantlet is naturally produced from stipules.

In the family Marattiaceae, buds of stipules were found at four specific positions. Gwynne-Vaughan (1905) noted that meristematic tissues were only found at two proximal points of the stipule margins in *Archangiopteris henryi* and *Kaulfussia aesculifolia* (=*Christensenia aesculifolia*). This is identical to our observations in *Archangiopteris somai.* In contrast, buds of *Angiopteris lygodiifolia*, *Angiopteris palmiformis*, *Archangiopteris itoi,* and *M. pellucida* also occur at two distal points, similar to *Danaea wendlandii* as reported by Sharpe and Jernstedt (1991).

A previous study showed that 40% of sprouts in *Archangiopteris somai* and 90% in *Archangiopteris itoi* were produced after 1 year of stipule culture (Chiou et al. 2006). In this study, the sprouting rates of stipules were up to 85 and 98% in *Archangiopteris somai* and *Archangiopteris itoi*, respectively. This indicates that sphagnum moss, which was used in this study, is more suitable as a culture medium than what was previously used (4:1, soil: peat moss), probably due to the moisture stability and ventilation of sphagnum moss.

Generally, a bigger stipule reproduced more plantlets with relatively larger fronds that had more-complicated structures. This suggests that larger stipules can provide more nutrients for plantlet growth, both in terms of number and size. The situations, however, in *Angiopteris palmiformis* and *M. pellucida* are not consistent with this generality. This contradiction is probably caused by too-small sample sizes being used.

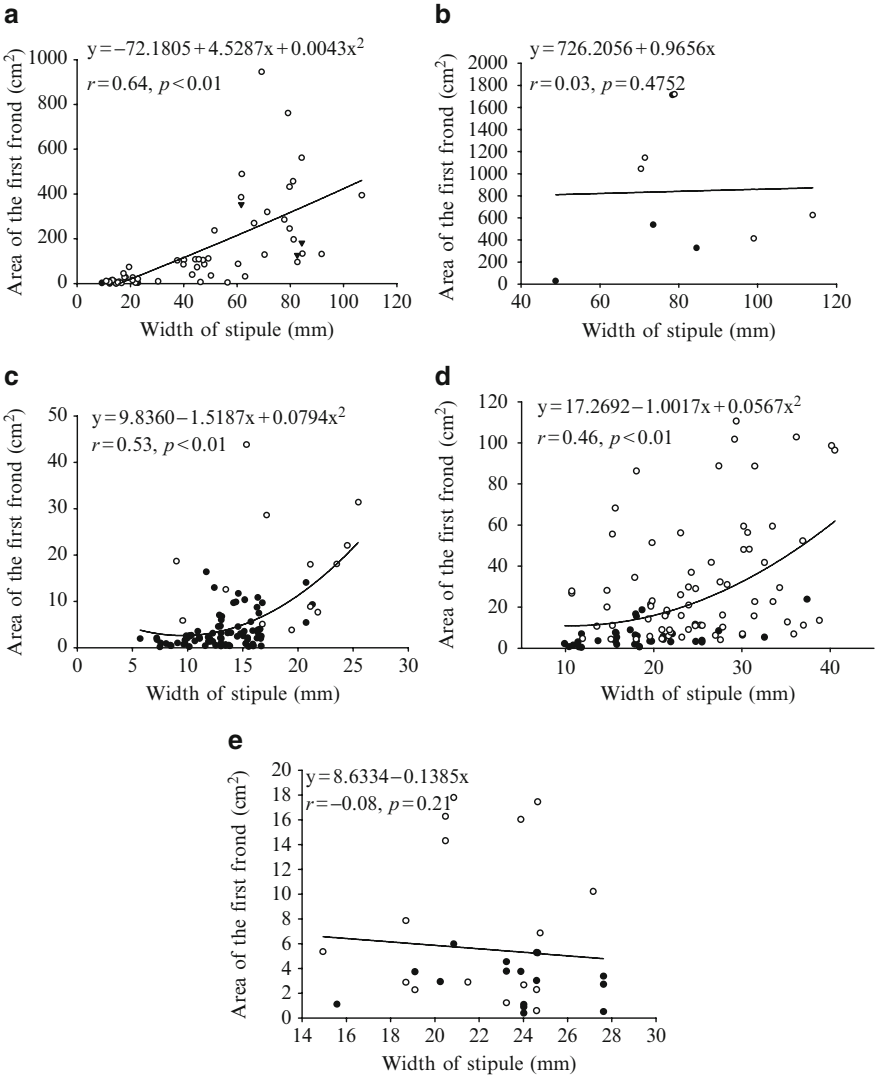

Fig. 9.4 Correlation between the width of stipules and the area and morphology of the first frond of five marattioid species. (**a**) *Angiopteris lygodiifolia*; (**b**) *Angiopteris palmiformis*; (**c**) *Archangiopteris somai*; (**d**) *Archangiopteris itoi*; (**e**) *Marattia pellucida*. ●: simple frond; ○: pinnate; ▼: bipinnate

This study demonstrates that the five marattioid species are capable of stipular budding. Plants can be reproduced in a shorter period compared to sexual propagation, e.g., the gametophytes of *Archangiopteris somai* did not produce sporophytes until 13 months of culture (Chou et al. 2007). Thus, it can be efficiently applied to horticulture and ex situ conservation. However, a limitation of stipule culture is that each sporophyte of *Archangiopteris somai*, *Archangiopteris itoi*, and *M. pellucida*

only produced a few fronds per year (unpublished data). Therefore, the number of stipules per plant is strictly limited, and materials for asexual propagation must be seriously considered.

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Chapter 10
Tree Ferns Biotechnology: From Spores to Sporophytes

Jan J. Rybczyński and Anna Mikuła

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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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

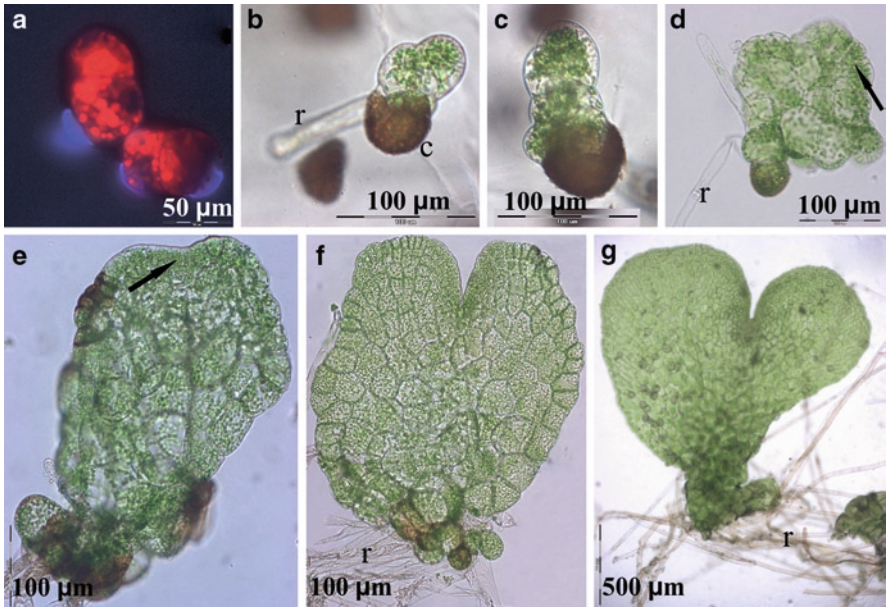

Fig. 10.1 *Cyathea australis* spore germination and prothallium formation on ½MS medium supplemented with 2% sucrose: (**a**) 9-day-old culture of two spores after the first division indicated by red autofluorescence of chlorophyll and blue autofluorescence of spore coat induced by blue-violet light (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).

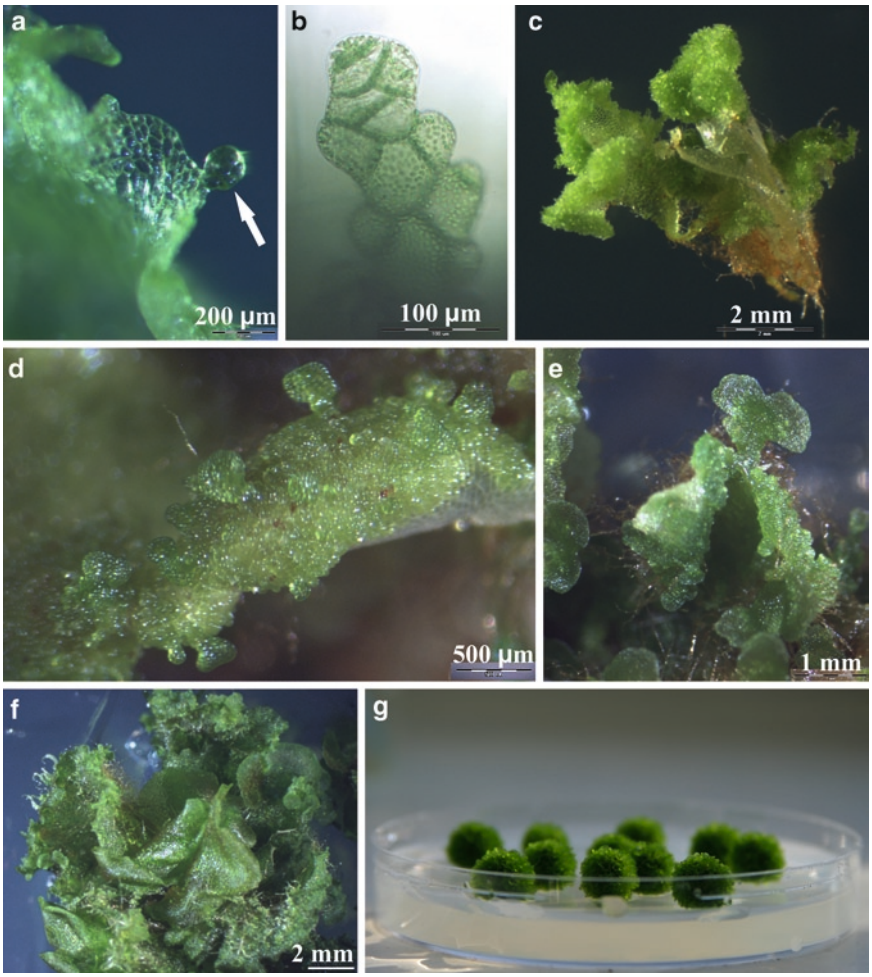

Fig. 10.2 Secondary gametophyte production on primary explants of various tree fern species: (**a**) the first stage of secondary prothallium proliferation on the *Cibotium glaucum* gametophyte (*arrow*); (**b**) specific cell divisions leading to young gametophyte formation; (**c**) multiplication of 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

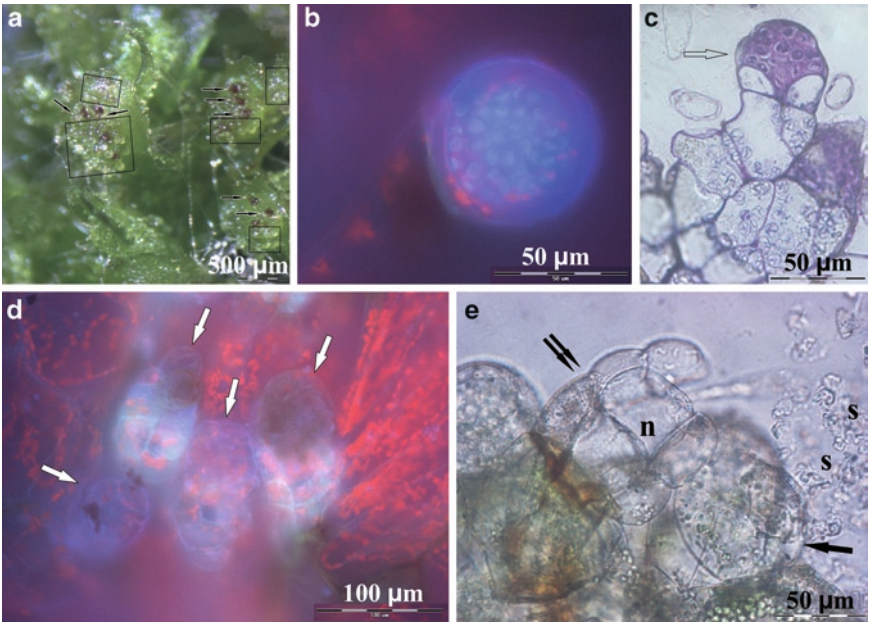

Fig. 10.3 Sexual organs of tree fern gametophytes cultured in vitro: (**a**) a few archegonia (*arrows*) and numerous antheridia (*in frames*) on the bottom side of gametophytes; (**b**) mature antheridium with male gametes in blue-violet light; (**c**) long section of mature antheridium (*arrow*) dyed 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

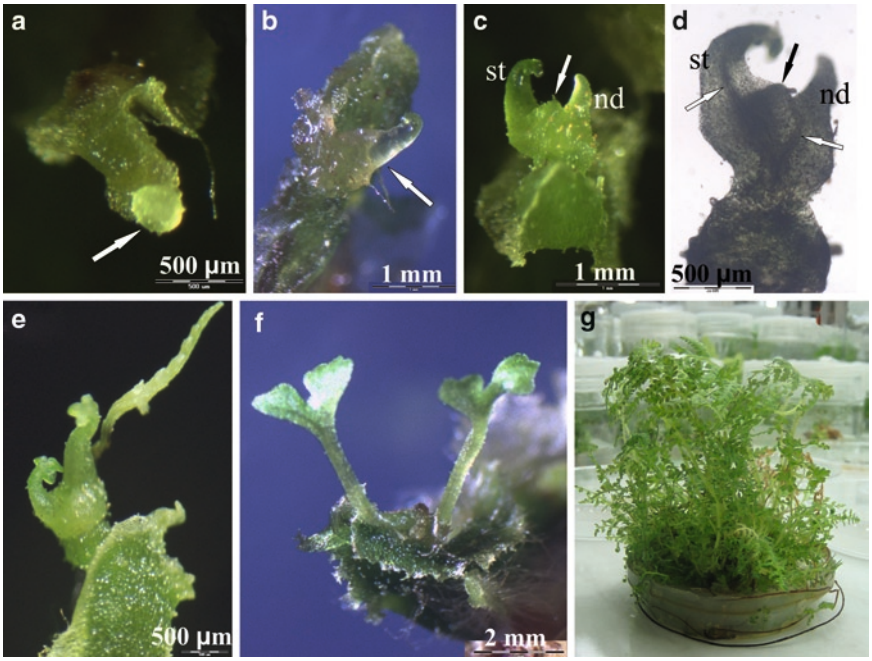

Fig. 10.4 Tree fern sporophytogenesis in vitro: (**a**) archegonium after fertilization with early embryo inside (*arrow*); (**b**) the first leaf primordium (*arrow*) of *Cyathea delgadii* embryo; (**c**) two leaf stage embryo of *Cyathea delgadii* (st – first and nd – second leaf primordiium; *arrow* 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

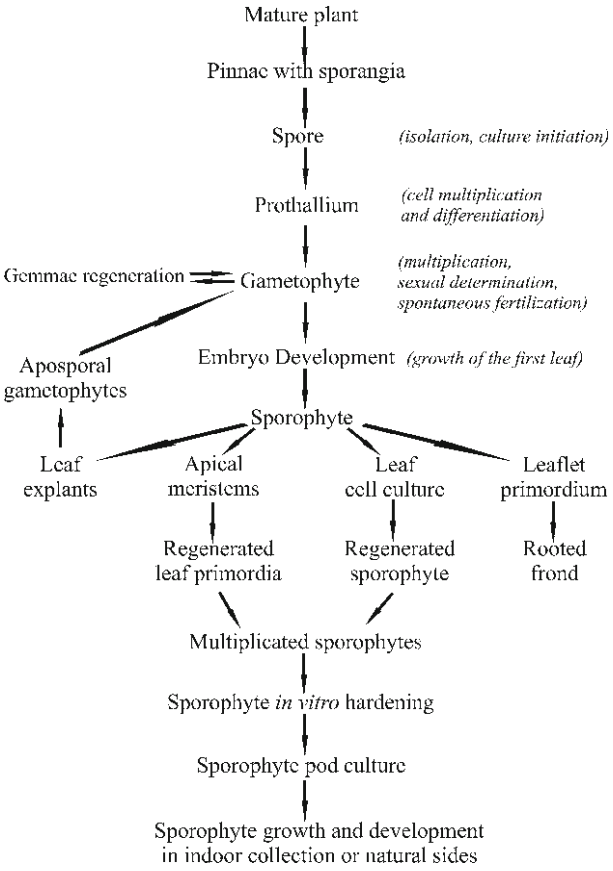

Fig. 10.5 Diagram summarizes the alternation of generation and developmental modification due to in vitro culture methods application in tree fern life cycle

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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Chapter 11
In Vitro Propagation of Rare and Endangered Serpentine Fern Species

Jowita Marszał-Jagacka and Krystyna Kromer

11.1 Introduction

Fern species of the *Asplenium* genus – *Asplenium adulterinum* Milde, *Asplenium adiantum-nigrum* L., and *Asplenium cuneifolium* Viv. – are often called serpentine ferns, because their occurrence is almost strictly related to serpentine areas. These ferns were considered to be an endemic European species and glacial relics (Holderegger 1994), before one of them, *A. adulterinum*, was found on Vancouver Island, B.C., Canada (Klinkenberg 2008).

The largest serpentine massifs in Europe occur in the Alps, the Balkan Peninsula, southeast Portugal, and Great Britain (Roberts and Proctor 1992); in Poland, they are found in the region of Lower Silesia only (Karpowicz 1969; Szczęśniak 2006). Serpentine rocks are deep rocks formed as a result of hydrothermal transformation of ultra alkaline magmatic rocks from the Devonian period. They are characterized by a gray-green color, with a specific chemical composition in which the relation of calcium to magnesium is exceptionally low, as well as having a low content of N, P, and K; a high content of Ni, Co, and Cr; and a comparatively high value of pH amounting to 7.2 (Proctor and Woodell 1975).

The habitat on serpentine rocks is xeric, which is caused by the physical proprieties of soil. That soil contains many fragmented serpentine rocks and is water-penetrable (Sarosiek and Sadowska 1961; Proctor and Woodell 1971). Dispersion of vegetation growing on serpentine rocks also induced the xeric character of serpentine communities; it facilitates a warming of the bedrock by the large quantities of light reaching its surface. As a result of irradiation, high temperature, and soil evaporation, the microclimate of plants' habitat on serpentine rocks is dry (Whittaker 1954; Proctor and Woodell 1971).

All factors mentioned thus far determine the infertility, and even the toxicity, of serpentine soil for plants. These factors are called "the serpentine complex"

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(Sarosiek and Sadowska 1961). Therefore, plants growing on serpentine rocks show features of adaptability, permitting them to live in conditions normally unfavorable to their development.

Serpentine ferns are extremely rare and have been declining at an alarming rate. *A. adulterinum* was included in the list of species as an extension to Appendix II of the Habitat Directive (92/43/EEC Directive) as a Species of Community Interest, which protection requires the designation of a Special Area of Conservation. It was also listed together with *A. cuneifolium* in Annex IV of The Convention of European Wildlife and Natural Habitats (Bern Convention) concerning plant species demanding strict protection in all European Union countries. *A. adulterinum* and *A. cuneifolium* are regarded as seriously threatened and have an IUCN category from EN to CR in Poland (Zarzycki and Kaźmierczakowa 2001), the Czech Republic, Slovakia, Great Britain, and many other countries. The increase of ecological threats and the dying out of serpentine ferns forces a rethinking of these decisions; this is most urgent as so much is being assumed and expected about the maintenance of the biodiversity of our ecological systems. Therefore, it is necessary to undertake procedures for protection and conservation of fern populations creating complex examples of *A. serpentine*; of which the *A. adulterinum* and *A. cuneifolium* are characteristic species (Żołnierz et al. 2008).

The aim of the study is to elaborate the method of *in vitro* cultures of these valuable ferns, by initially using material gathered from natural habitat spores. This is essential in order to gain knowledge on nutritional requirements and reproductive cycle, as well as the whole life cycle of the plants. The qualification of critical factors threatening these ferns, in their natural environment, is also within the aim of this work.

11.2 Materials and Methods

11.2.1 Initiation of Culture

Spores of two serpentine fern species: *A. adulterinum* Milde, *A. cuneifolium* Viv., as well ferns such as *A. septentrionale* (L.) Hoffm. and *Polypodium vulgare* L. were collected from plants growing in their natural habitats. A two-step method of disinfection was used: (1) a first-grade disinfection in 70% ethanol for 3 min, and (2) a second-grade disinfection in 0.1% NaClO for 10 min. Single leaves from fern fronds, with developed sporangia, were collected in paper envelopes and left to dry at room temperature for 7–10 days. After this period, the spores resulting from the sporangium were packed in blotting paper filter bags – called packets. The spores in the packets were soaked in disinfecting solutions, and then washed three times with sterile distilled water. Then the aseptic spores were sown in 100 mL flasks, each containing 35 mL of solid medium. The culture medium for spore germination was 1/4 MS macroelements, supplemented with 30 g L^{-1} of sucrose, and solidified with 8 g L^{-1} of agar, finally the pH value of the media was adjusted to 6.2 before

sterilization (18 min at 121°C). Then, 2 mL of sterile distilled water was added to each flask of sown spore cultures to increase germination. All the cultures were then incubated at 18–20°C under cool white fluorescent lamps of 14.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to provide a 16 h photoperiod.

11.2.2 Growth of Gametophytes

The growth and development of the gametophytes of the four fern species were compared in experiments. The prothallial colonies were cultured in the following media: full-strength, 1/2, 1/4, and 1/8 dilutions of MS macronutrients, and modified MS with 6.8 g L^{-1} of agar and 20 g L^{-1} of sucrose, with pH values of 6.8–7.0, and changed proportions of macroelements (NH_4NO_3 – 825 mg L^{-1}; KNO_3 – 950 mg L^{-1}; $CaCl_2 \times 2\ H_2O$ – 110 mg L^{-1}; $MgSO_4 \times 2\ H_2O$ – 370 mg L^{-1}; KH_2PO_4 – 42.5 mg L^{-1}), and 1 mg L^{-1} of glycine (called MSZ medium).

The influence of incremental doses of sucrose (0, 5, 10, 20, 30, 40 g L^{-1}) on growth and development of the gametophytes was analyzed in *A. adulterinum*, *A. septentrionale*, and *P. vulgare.* The percentage of decaying gametophytes was determined after 5 weeks of culture. After a subsequent 5 weeks, the fresh weight and number of prothalli were calculated.

The influence of several sugars, such as glucose, fructose, mannose, sucrose, maltose, and lactose (in the same concentration of 0.087 M) on the growth and development of gametophytes was studied. The 0.087 M concentration of sugar corresponds to 30 g L^{-1} of sucrose, so the osmotic pressure of the medium was maintained at the same level. Forty specimens for each experimental treatment were used – each flask containing ten heart-shaped gametophytes.

During the culture period, a portion of sterile water (2–3 mL per flask) were added to increase the moisture of the gametophytes so as to increase fertilization and trigger the developmental program of the diploid phase of the organism.

11.2.3 Gene Bank of Prothalli

In order to create a "gene bank" of prothalli from *A. adulterinum* and *A. cuneifolium*, specimens were stored for a year at low temperature. The single heart-shaped gametophytes of the fern species were then transferred to 50 mL flasks, each containing 20 mL of medium. They were cultured on 1/2 MS mineral medium with different doses of agar and sucrose. The 1/2 MS medium was also supplemented with the following compounds: Kinetin (KIN), indol-3-acetic acid (IAA), and gibberellic acid (GA_3). The *in vitro* cultures were stored for a year at low temperature: +2°C, continuous darkness; and +8°C under a cool white fluorescent lamp of 0.15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in a 16 h photoperiod. After that, the prothalli were subcultured on fresh 1/2 MS medium and the survival rate and size of the gametophytes of *A. adulterinum* and *A. cuneifolium* were calculated.

11.2.4 Culture of Sporophytes

Culturally formed sporophytes were carefully separated from the gametophytes and subcultured on 1/2 MS medium with various doses of KIN and 0.1 mg/L^{-1} NAA. Several types of cultures and substances were applied to decrease the growth of the gametophytes, among others – growth inhibitors and polyphenols.

11.2.5 Regeneration of Shoot Buds

Young fronds of growing *in vitro* sporophytes were removed from the rhizome and inoculated horizontally on 1/2 MS medium with KIN and IAA. One part of the isolated fronds remained intact, while the other was cut off with a razor blade along its main nervation.

11.2.6 Acclimation of Plants Ex Vitro

Before planting, the sporophytes were transferred from the agar medium to a liquid medium with perlite, where the sporophytes developed roots, and then for the last 7 days of culture, they were placed under light with an irradiance of 34.6 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Subsequently the flasks with the fern plants were placed in a greenhouse, and after 5 days, the aluminum foil covers were removed; 3 days later, the plants were potted in a variety of gardening soils, including a commercial mix for ferns at pH 6.5 and pH 7.0. The pots were covered with polyethylene foil, which was gradually removed over time. After 4 weeks the number of plants continuing to grow was counted, and then the plants were transferred to a hotbed 5 weeks later.

11.2.7 Measurements of Stomata Cells and Spores Size

In the observation, five plants grown from a controlled *in vitro* environment, and five plants from a natural habitat were used. Leaf blades from the middle part of fully developed plants were collected in June. The epidermis from the abaxial side of the leaf blades was removed and placed on microscope slides with a drop of water and covered with a covering glass and the length of the stomata was then analyzed. In the same manner, a length of spores of diverse origin, collected in September, was measured under a microscope.

11.3 Results and Discussion

The spores of *P. vulgare* (20 days) and *A. adulterinum* (30 days) germinated first, then spores of *A. septentrionale* (40 days) and *A. cuneifolium* (40 days). Heart-shaped gametophytes, with sex organs, were formed after 3 months of culture for *P. vulgare* and *A. adulterinum*, and after 4 months for *A. septentrionale* and *A. cuneifolium*. Six months later, sporophytes could be observed on *P. vulgare* and *A. adulterinum*, and 8 months on *A. septentrionale* and *A. cuneifolium.*

The influence of full-strength, and 1/2, 1/4, and 1/8 dilutions of MS macroelements and MSZ medium for serpentine ferns was tested in these experiments (Fig. 11.1). The actual experiments and written observations permit us to affirm that nutritional conditions, just like different physical and chemical factors, influence the course of the multiplication of gametophytes, and as a consequence, the formation of sporophytes. During the investigations it was shown that ferns grown on a modified MSZ medium with a macronutrient quantity similar to the composition of serpentine soil did just as well as on diluted to 1/2 MS medium (Fig. 11.2). The weaker multiplying of gametophytes on a full concentration of a macronutrients shows that in natural conditions, substratum rich in nutritional components may limit their development and consequently their expansion.

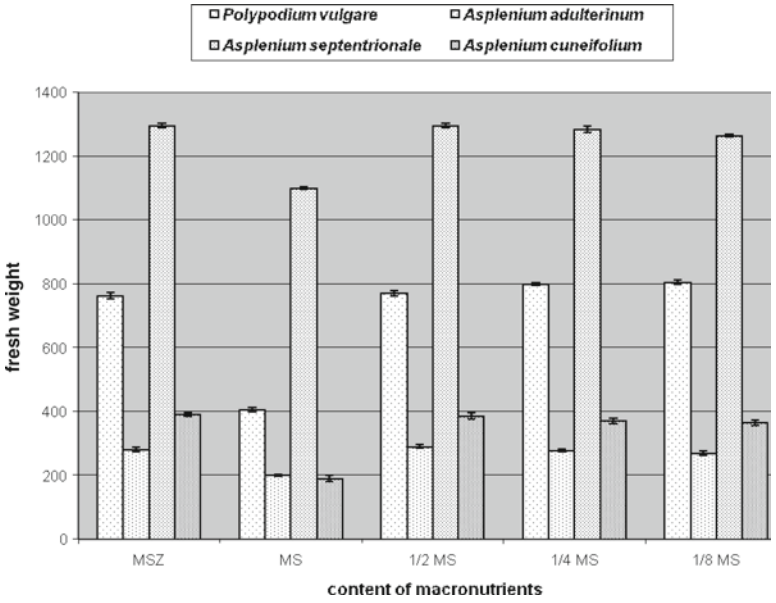

Fig. 11.1 Influence of MS medium and its modification on the growth and development of gametophytes of *Polypodium vulgare, Asplenium adulterinum, Asplenium septentrionale, and Asplenium cuneifolium* after 2 months of culture (From Kromer et al. 2006)

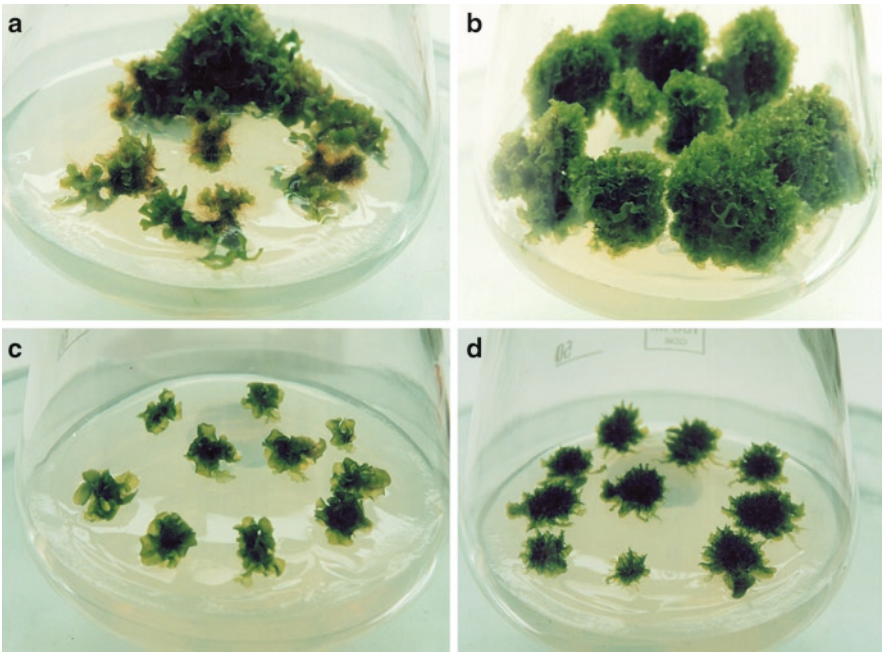

Fig. 11.2 Multiplication of gametophytes on 1/2 MS media after 6 months of culture: (**a**) *Polypodium vulgare*; (**b**) *Asplenium septenrionale*; (**c**) *Asplenium adulterinum*; (**d**) *Asplenium cuneifolium*

Full composition of macronutrient of MS medium was useful for the propagation of gametophytes and sporophytes of *A. nidus* (Fernández et al. 1993), as well as *Blechnum spicant* and *Pteris ensiformis* (Fernández et al. 1996a). The gametophytes most sensitive to salinity turned out to be those of *A. cuneifolium* (a decay of 50%), but the least sensitive were gametophytes of *A. septentrionale* (a decay of 10%). Studying the influence of Knop, Knudson, Klekowski and Murashige and Skoog media in full composition of macroelements, as well as its dilutions to 1/2 and 1/4, on increment of fresh and dry weight of *B. spicant* gametophytes, Fernández et al. (1997a) discovered that the use of full-strength MS medium increased gametophyte growth, but delayed antheridia and archegonia formation. Media poorer in nutrients (Knop, Knudson and Klekowski) decreased gametophyte growth, but allowed sex organ formation over a short period of time. On the contrary, working with *Osmunda regalis*, Fernández et al. (1997b) observed that gametophytes grew best in poor Knop culture medium.

Therefore, serpentine ferns probably grow on serpentine rocks, which are characterized by infertility and even toxicity, where other plant species would be unable to grow. It is possible that serpentine ferns possess adaptable features permitting them to grow in such unfavorable conditions on serpentine substratum. The comparison of nutritional requirements for serpentine fern gametophytes and published data related to other fern species, testifies to the fact that they may be different for various species.

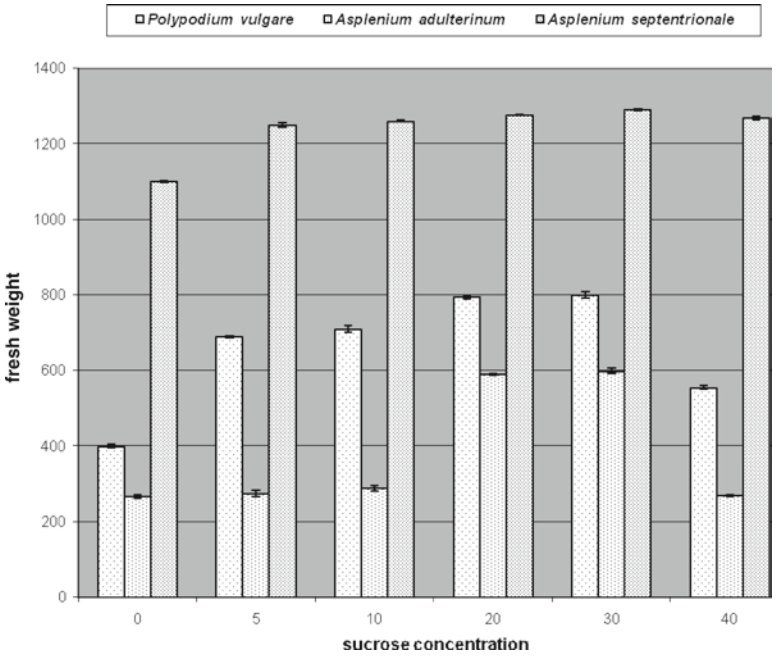

Fig. 11.3 Influence of sucrose concentration in medium on the growth and development of gametophytes of *Polypodium vulgare, Asplenium adulterinum*, *and Asplenium septentrionale* after 2 months of culture (From Kromer et al. 2006)

These investigations show that the doses of sucrose contained in the medium influenced the growth and development of the studied fern species (Fig. 11.3). The most suitable dose for gametophyte growth and development of *A. adulterinum* and *P. vulgare* was 20 and 30 g L^{-1}, respectively. In these concentrations, gametophytes of all the studied species obtained the highest biomass. *A. septentrionale* attained an equal increase of mass, as well as the number of the first row of gametophytes in the studied sucrose concentrations of the medium. This fern appeared to be unusually tolerant to both lack of sucrose and its high concentration. For the remaining species (*A. adulterinum* and *P. vulgare*), the addition of 40 g L^{-1} of sucrose to the medium was an over-optimal concentration.

Zenkteler (2000) also investigated the influence of a concentration of sucrose in the medium on the vitality of eight fern species, which similarly to our experiences, showed the best development using a medium with an addition of 20 g L^{-1} of sucrose.

Hirsch (1975) studied the influence of sugars in a medium on the regeneration of gametophytes and sporophytes from leaves of *Microgramma vacciniifolia*. She stated that the multiplying of gametophytes, as well as the number of formed sporophytes, depended on the concentration of sugar in the medium. Fernández et al. (1997a) affirmed that the dry weight of gametophytes increased as concentrations of sucrose in the medium increased, but increase of fresh weight was the highest near a concentration of 20 and 30 g L^{-1}. In the investigations on *A. adulterinum*, *A. septentrionale*, and *P. vulgare*, similar results were obtained.

Table 11.1 Influence of sugars applied in 1/2MS medium on the growth and development of gametophytes of *Asplenium adulterinum, Asplenium cuneifolium, Asplenium septentrionale Poypodium vulgare* after 2 months of culture (From Kromer et al. 2006)

	Fresh weight of gametophytes [mg]					
	Sugars 0.087 M					
Species	fructose	maltose	glucose	mannose	sucrose	lactose
Asplenium adulterinum	necroses of gametophytes	70	26.25	38.75	66.25	21.25
Asplenium cuneifolium	necroses of gametophytes	41.25	161.25	161.25	177.5	7.5
Asplenium septentrionale	necroses of gametophytes	193.75	1351.25	453.75	1705.0	166.25
Polypodium vulgare	necroses of gametophytes	123.75	293.75	81.25	253.75	necroses of gametophytes

The influence of fructose, maltose, glucose, mannose, sucrose, and lactose in the medium on growth and development of gametophytes of four fern species was tested (Table 11.1). The results of the investigations conducted showed that each of the fern species studied attained the best increase of fresh weight and gametophyte size on a medium with the addition of sucrose. The application of glucose provided similar results; with the exception of *A. adulterinum*, which preferred maltose in the medium, and *A. cuneifolium* which grew equally well on a medium of glucose and mannose. The remaining sugars, and particularly fructose, inhibited the increase of protallial fresh weight or caused its decay. Moreover, fructose turned out to be the most toxic, and gametophytes of all the studied fern species decayed after its use in the medium. Romano et al. (1995) observed a decrease in the number of roots in cork oak cultivated on a medium with an addition of fructose. This effect can probably be connected with the degradation of sugars during autoclaving into furfural and its derivatives, which may be toxic components (Bogunia and Przywara 1999).

The *in vitro* cultures are an effective method of preserving genetic supplies of *A. adulterinum* and *A. cuneifolium* ferns. Heart-shaped gametophytes of both plant species studied best tolerated storage in + 8°C, a light intensity of 0.15 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and grew on a 1/2 MS medium, without growth regulators (Kromer et al. 2006). The agar and sucrose doses applied in the experiments had no influence on the condition of the stored gametophytes. When transferred into fresh 1/2 MS medium, the gametophytes of both fern species developed correctly and produced sporophytes. In the experiments conducted with preserved genetic fern supplies from the genus of *Asplenium,* well-shaped gametophytes were applied, which similarly to mature gametophytes of the *Matteuccia struthiopteris* (Zenkteler, 1992), tolerated storage well. Zenkteler (1992) stated that a lack of light and the effect of low temperature had been unfavorable in the early developmental stage of gametophytes such as germ filament, at this stage of development a protonema inappropriately tolerated the process of adaptation and showed disturbances in gametogenesis.

11.3.1 Sporophyte Formation

The formation of only one to five sporophytes after 2 months of culture was a rare phenomenon, considering the thousands of gametophytes grown in the flasks. An analysis of the phenotypes revealed a high number of female gametophytes in contrast to only a few males, and the hermaphrodite heart-shaped prothalli were not numerous. A low number or even absence of gametophytes with antheridia is most likely a limiting factor in the fertilization and development of sporophytes (Kempinska et al. 2006; Kromer et al. 2008).

Those sporophytes that appeared were isolated from the gametophyte culture, yet removal of all filaments from the culture was almost impossible. The coexistence of two generations, i.e., sporophytes and gametophytes, in the culture is dangerous to the former. The extension of filaments, which entwine and cover specifically the growing sporophyte meristem, has a lethal effect on them. In considering all the applied methods of prothalli elimination, the best results came from a culture in liquid medium. In this medium, the regeneration of filamentous protonema was delayed and a part even died due to inappropriate external factors. It should then be considered that prothalli vitality goes beyond the sporophyte's ability to grow, this is probably due to an unlimited number of prothallus cells giving rise to new prothallia.

11.3.2 Sporophytes Multiplication

To induce propagation of sporophytes, phytohormones were added and their influence on growth and development of serpentine ferns was studied. Single, separated plant rhizomes were placed on agar media with one dose of NAA (0.1 mg L^{-1}), and increasing amounts of KIN (0.5–6.0 mg L^{-1}). It was observed that kinetin stimulates the growth of fronds and expansion of rhizomes in doses no higher than 1.0 mg L^{-1} (Fig. 11.4). Further increments of its concentration caused an inhibition of sporophyte development. A high concentration of kinetin provoked a decrease in plant size, but increased the number of meristematic points in rhizomes, and their multiplication as well. The largest number of plants, i.e., 14, was noted on the medium with a kinetin concentration of 1 mg L^{-1} (Fig. 11.5). Further increments of kinetin content were not beneficial to sporophyte growth and development, while a dose of 6.0 mg L^{-1} was toxic for plants.

In the experiments carried out on the ferns, it was noticed that there was a strong influence by the applied phytohormones on the number of fronds formed and on induction of new growth points on the rhizomes. The NAA positively increased the number of leaves and sporophytes in *A. septentrionale*, whereas in *A. cuneifolium* this auxin initiated a larger number of fronds.

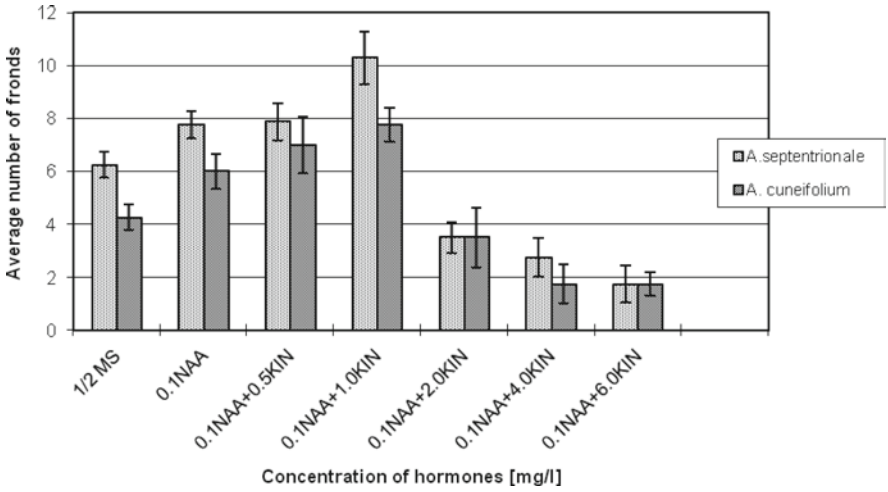

Fig. 11.4 Influence of phytohormones on number of developing fronds of *Asplenium septentrionale* and *Asplenium cuneifolium* on 1/2 MS after 2 months of culture

Fig. 11.5 Sporophytes of *Asplenium adulterinum* on 1/2 MS medium with kinetin (0.5 mg L^{-1}) and NAA (0.1 mg L^{-1}) after 2 months of culture (From Kromer et al. 2006)

11.3.3 Regeneration from Sporophytes

Growing sporophytes from spores is a long-term process. The development of a technique for regeneration of sporophytes from part of a plant, especially from detached leaves (the removal of which does not destroy the naturally growing endangered specimens of the species), can be very valuable. To establish a possible procedure, cultured *in vitro* fronds of ferns were studied. Juvenile leaves, detached from rhizomes of three fern species, were transferred and inoculated horizontally on 1/2 MS medium. These explants showed varying ability for regeneration which depended on the phytohormones present in the medium and the wounding of stipe and blade.

The inoculated leaves showed initial swelling and callus formation on the stipe base, later however, along the whole stipe length, a number of initial growth points were formed. Following this, shoot buds from these places regenerated forming rhizomes and roots, eventually developing into young sporophytes. The blades, in most cases, turned brown and decayed after a few weeks; only occasionally did they regenerate shoots. Additionally, parallel to the sporophytes, gametophytes were present. The unwounded explants of the species regenerated sporophytes on a basic medium and on media supplemented with IAA and KIN. A direct correlation between the hormones applied and the formation of sporophytes was not found (Table 11.2).

The wounding of fern stipes specifically stimulated the regeneration process. In this process, the auxin added to the medium decreased the formation of sporophytes, and the kinetin inhibited this ability completely in *A. adulterinum* and *A. cuneifolium*, while it strongly decreased in a dose-dependent manner in *A. septentrionale* (Table 11.3).

The unique morphogenic potential of sporophytes was also pointed out by Fernández et al. (1993, 1997a, 1997b, 1999); Somer et al. (2010). It has been reported that, in homogenate cultures, both gametophyte and sporophyte regeneration take place. However, the establishment of *in vitro* cultures from isolated fern

Table 11.2 Influence of growth regulators on regeneration per cent of isolated intact fronds of *Asplenium adulterinum*, *A.cuneifolium*, *A. septentrionale* and *Polypodium vulgare* after 4 months of culture

	Phytohormones [mg l^{-1}]				
Species	0	0.1IAA	0.1IAA+0.5KIN	0.1IAA+1.0KIN	0.1IAA+2.0KIN
Asplenium adulterinum	33.3	0	0	0	33.3
Asplenium cuneifolium	0	0	0	16.6	16.6
Asplenium septentrionale	20.0	33.3	13.3	13.3	13.3
Polypodium vulgare	0	0	0	33.3	33.3

Table 11.3 Influence of growth regulators on regeneration per cent of isolated and wounded fronds of *Asplenium adulterinum*, *A.cuneifolium*, *A. septentrionale* and *Polypodium vulgare* after 4 months of culture

	Phytohormones [mg l^{-1}]				
Species	0	0.1IAA	0.1IAA+0.5KIN	0.1IAA+1.0KIN	0.1IAA+2.0KIN
Asplenium adulterinum	100	66.7	0	0	0
Asplenium cuneifolium	100	66.7	0	0	0
Asplenium septentrionale	66.7	77.8	44.4	22.2	0
Polypodium vulgare	33.3	33.3	0	0	0

fragments is very difficult, because of its sensitivity to surface-sterilizing agents. Therefore, in the light of our experience, the only suitable explants in this respect are the fronds.

11.3.4 Plant Preparation Before Transfer to Greenhouse

The transfer of the plants from agar into a liquid medium with perlite caused better rooting of the propagated fern species. The rooted sporophytes, small plants with leaves 5–8 cm in length, were exposed for 7 days to increasing light intensity from 14.2 to 34.6 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and for the next 5 days the plants in open flasks were placed in a greenhouse (the end of March or beginning of April). The influence of various horticultural substrata on the survival rate of the transplanted sporophytes was then monitored. To lower the level of stress created by this radical change in conditions, the potted plants were maintained in a propagation chamber and the containers for 2 weeks of acclimation, were covered with polyethylene film, and watered regularly. After 2 weeks, the potted plants were placed in a cold frame, primarily covered with a glass window, and artificially shaded. All the tested species required a relative high humidity and strict control of the hygienic state of the medium and plants, thus a preventive phytostationary treatment was applied to avoid the development of *Botrytis cinerea* and insects (*Diptera*) from genus *Sciaride*. The sporophytes of *A. adulterinum*, and *A. cuneifolium* acclimated best in commercial fern substrates of pH 6.5 at 62.5% and pH 7.0 at 50.0%, respectively, whereas *A. septentrionales* acclimated best in substrates containing peat, compost, leaves, and sand (3:2:1). The results obtained in serpentine soil were 6–15% lower.

The best growth of plants was observed when they were positioned along the edge of the concrete frame (Fig. 11.6). It may be supposed that the soil dries out regularly here during the day. It was observed that *A. adulterinum* and *A. cuneifolium* formed sporophylls in the second year of cultivation whereas *A. septentrionale* formed sporophylls in the third year only.

Fig. 11.6 Plants of *Asplenium adulterinum* originated in vitro after 2 years of cultivation in cold frame (From Żołnierz et al. 2008)

11.3.5 Comparison of Plants from Natural Stands and Originated In Vitro

A. adulterinum is a hybrid, which develops from two diploids, *A. trichomanes* and *A. viride* (Lovis and Reichstein 1968; Reichstein 1984), and contains 144 chromosomes (2n). The developmental anomalies of gametophytes are often observed in *in vitro* cultures. Very characteristic of ferns is the occurrence of apogamy, i.e., the formation of sporophytes without gametes and apospory, which is defined as a direct production of gametophytic material from sporophytic tissues. Apogamy is a natural process of propagation for triploid and tetraploid fern species.

In a broader context, apospory and apogamy (and other asexual modifications of the life cycle) are referred to as forms of apomixis (Reiger et al. 1976). This situation has been documented in many different ferns (Raghavan 1989; Fernández et al. 1993; Fernández et al. 1996b; Somer et al. 2010), including *A. nidu*s and *A. adiantum-nigrum*. This phenomenon, however, is unprofitable from the perspective of species preservation, where the main goal is to retain unchanged – in regard to the natural environment – but genetically diverse individuals. Additionally, such sporophytes are incapable of sporogenesis. This is the reason why the level of the ploidy of sporophytes

derived *in vitro* should be compared with sporophytes growing in a natural habitat. The polyploidy species possess several characteristic features that can be used for such a comparison, such as cell volume, dimension of pollen, length of stomata, number of chloroplasts in guard cells, and size of plants. The length and number of stomata, as well as dimension of spores of *A. adulterinum* plants from natural environments, as well as of those derived from the *in vitro* culture were compared. The guard cells of plants collected from cold frames were slightly longer, and their average size was 29.57 μ, whereas the average length of *A. adulterinum* collected from a natural habitat in Książnica was 29.38 μ (Table 11.4). The number of stomata on 1 mm^2 of the abaxial epidermis of plants grown in a cold frame was insignificantly higher than of those originating from a natural habitat. Similarly, spore dimension in plants developing in an *in vitro* environment was slightly larger (Table 11.5). These minor observable changes may result from better mineral nutrition of the soil and better growth circumstances in a substituted habitat. Moreover, this confirms the diploid character of the *in vitro* plants derived from *A. adulterinum*, and proves the usefulness of the described techniques in the propagation of protected and endangered fern species.

The serpentine ferns studied from *in vitro* cultures reproduced an entire generative cycle and formed sporophytes, which were successfully acclimated in the greenhouse, and later in a garden cold frame and grew satisfactorily in a substitutive habitat. Thus, populations were created, which could be used to conserve fern cultivation in substitutive habitats. The sporophytes' growth and vegetative propagation was positively sup-

Table 11.4 Length of stomata cells on abaxial side of *Asplenium adulterinum* fronds from natural stands and originated *in vitro*

Plants	Avg. length of 100 stomata cells [μ]	Avg. length of stomata from 5 plants [μ]
Cold frame		
specimen 1	30.02	29.57
specimen 2	29.55	
specimen 3	29.50	
specimen 4	29.52	
specimen 5	29.26	
Natural stand		
specimen 1	29.36	29.38
specimen 2	29.39	
specimen 3	29.47	
specimen 4	29.55	
specimen 5	29.14	

Table 11.5 Average diameter of spores of *A. adulterinum* collected from natural stands and originated *in vitro*

Plants	Avg. length of spore [μ]
Natural stand	7.573 ± 0.588
Cold frame	7.782 ± 0.738

ported by small doses of auxin and kinetin. Once detached from plant fronds, especially stipes basis possess a regenerative ability and formed shoot buds in reaction to applied phytohormones or exclusively in response to wounding. This feature might be very useful in the propagation of a specific genotype of serpentine ferns from a single or specific locality without the destruction of the propagated plants, and should be helpful in the variability of genetic maintenance in ex situ collections. The studies proved that ferns require average resources of substratum, and that insufficient or excess nutrition decreases their growth rate. With reliance on measurements of stomata length and spore diameter, it was indirectly shown that derived *in vitro* sporophytes represent the same level of ploidy as plants growing in a natural habitat, so it might be thought that they possess double sets of chromosomes per cell. The undertaken studies, related to the propagation and long-term storage of genetic resources of serpentine ferns, should contribute to the preservation of these species in natural flora.

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Chapter 12
Conservation of Fern Spores

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12.1 Introduction

Ferns are cultured and propagated largely through germination and growth of spores. Viable spores can be germinated and gametophytes cultured through a variety of established methods (an example can be found in Chap. 7 of this volume); however, methods to maintain the viability of freshly harvested spores during storage are less understood. Spore banks for ferns (analogous to seed banks for angiosperms) are a promising *ex situ* conservation tool, because large quantities of germplasm with high genetic variation can be conserved in a small space with low economic and technical costs (Dyer 1994; Pence 2004; Ballesteros 2008). Can we store spores for long periods of time without affecting their viability and genetic integrity?

12.2 Background: Fern Spore Longevity and Storage

Fern spore longevity and aging was broadly described in the late 1960s and 1970s (e.g., Gullvag 1968, 1969; Hauke 1969; Lloyd and Klekowski 1970; Jones and Hook 1970; Smith and Robinson 1975; Kato 1976; Dyer 1979), coincident with a growing interest in pteridology. Large differences in longevity between species were found, and a correlation between spore type (green vs. nongreen) and longevity was observed (Lloyd and Klekowski 1970; Smith and Robinson 1975). The rapid deterioration of green spores was attributed to intolerance to desiccation, high metabolic rates, and lack of dormancy (Kato 1976), and these hypotheses launched the idea that green spores exhibit a recalcitrant behavior analogous to seeds (Roberts 1973). Nongreen spores, on the other hand, could be treated as orthodox seeds (*sensu lato* Roberts 1973), and dry conditions, either at ambient or low temperature, were the recommended storage conditions (Dyer 1979).

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The first spore banks were developed in a cooperative program between C. N. Page and D.G. Mann at the Royal Botanic Garden of Edinburgh (RBGE) and A. F. Dyer at Edinburgh University (Page et al. 1992), and research led by UK scientists supported fern spore conservation efforts (e.g., Rita 1990; Ide et al. 1992; Agrawal et al. 1993; Beri and Bir 1993; Whittier 1996; Lebkuecher 1997; Simabukuro et al. 1998b; Camloh 1999). Negative effects of sterilization and low temperature (<0°C) exposure on spore viability during storage were shown (Simabukuro et al. 1998b; Camloh 1999). Wet storage was presented as a new technique that was more effective than dry storage at maintaining viability (Lindsay et al. 1992). Moreover it was shown that spores could survive liquid nitrogen exposure (Agrawal et al. 1993). While preservation methods for fern spores rapidly improved, few advances were made to understand the mechanisms underlying the viability loss of spores during storage. Aging mechanisms were suggested by Beri and Bir (1993), who showed that the loss of soluble substances during storage of nongreen *Pteris vittata* spores led to lower water uptake upon imbibition. Lebkuecher (1997) showed that green spores of *Equisetum hyemale* did not survive after 2 weeks under extreme desiccating conditions (2% RH, 25°C), eventually losing the ability to recover photosynthetic capacity.

The first decade of the new century opened with two important international symposia concerned with the topic of fern conservation: *Fern Flora Worldwide: Threats and Responses* (University of Surrey, Guilford, UK, 23–26 July, 2001) and *Ferns for the 21st Century* (Royal Botanic Garden Edinburgh, Scotland, UK, 12–16 July, 2004). Fern conservation appeared as one of the new focal points in pteridology, attracting scientists from all over the world. The Global Strategy for Plant Conservation (GSPC) was also established, emphasizing *ex situ* conservation as a key feature under the 1992 Convention on Biological Diversity (CBD). In response to the increasing interest on fern spore conservation, some interesting works were published during this period (Constantino et al. 2000; Morini 2000; Pence 2000, 2004; Rogge et al. 2000; Quintanilla et al. 2002; Aragón and Pangua 2004; Ballesteros et al. 2006). These works continued to test hypotheses proposed the decade before on the basis for negative effects of freezing on spores, the beneficial effects of wet storage (Constantino et al. 2000; Quintanilla et al. 2002; Aragón and Pangua 2004), and the possibility of fern spore cryopreservation (Pence 2000, 2004; Rogge et al. 2000; Ballesteros et al. 2006).

Most recently, the use of physicochemical approaches adapted from seed storage science (Ballesteros and Walters 2007a, b) have lent new perspectives, offered new tools, and revealed interesting physiological questions about fern spore longevity, storage behavior, cryopreservation, and the glassy state in spores, reviving an interest in fern spore conservation for the next decade.

12.3 Water Content of Fern Spores and Storage Stability

The water content at which orthodox germplasm (i.e., tolerant to desiccation and low temperatures during storage) is stored has an enormous impact on longevity of the accessions and tolerance to storage at low temperatures (FAO/

IPGRI, 1994, Roberts and Ellis 1989; Gómez-Campo 2001; Walters 1998, 2004). Fern spore water content can be easily manipulated by equilibration at different relative humidity (Ballesteros and Walters 2007a). Conventional storage conditions for fern spore conservation recommended storage at 4°C, air-dry or over a desiccant (Dyer 1994). Optimum moisture conditions were proposed as 20% RH at the storage temperature which corresponds to about 0.025 gH_2O/g dry weight for *Pteris vittata* and 0.039 gH_2O/gdry weight for *Polystichum setiferum* (Ballesteros and Walters 2007a). However, fern spores are generally stored after dehiscence from sporangia at ambient conditions of the laboratory with little regard to moisture (Hoekstra 2002; Ballesteros 2008). Ambient conditions differ among laboratories preparing fern spores for storage, introducing potential uncontrolled variation in procedures and results. For example, the annual average relative humidity in Fort Collins, Colorado (USA) is ~35%, ~55% in Madrid (Spain), and ~68% in Valencia (Spain), while in Cincinnati, Ohio (USA) or Santander (Spain) it is ~75%, and ~80% in Edinburgh (UK). Water contents within this RH range vary from 0.036 to 0.053 gH_2O/gdry weight for *Pteris vittata* spores or from 0.055 to 0.085 gH_2O/gdry weight for *Polystichum setiferum* spores according to water sorption isotherms calculated for several species of nongreen spores at 25°C (Ballesteros and Walters 2007a). Thus, the spore water content reached under room conditions usually exceeds the recommended water content for the optimum storage of these species (Ballesteros and Walters 2007a).

Currently a very broad RH range, between 7% and 70% RH, is recommended for fern spore storage, suggesting the need for greater refinement of storage protocols to maximize spore longevity at different temperatures (Ballesteros and Walters 2007a, b). Studies that explore the effect of drying fern spores to relative humidity (RH) <20% had mixed results with nongreen spores of different tree fern species: longevity of *Lophosoria quadripinnata* spores increased whereas longevity of *Cyathea caracasana* decreased at storage RH<20% (Constantino et al. 2000). Longevity of *Woodwardia* sp. spores appeared to be maximum at about 20% RH (Walters et al. 2005). Moreover, it has been shown that green spores of *Equisetum hyemale* survive initial exposure to, and 1 week storage under, extreme desiccating conditions (2% RH, 25°C) (Lebkuecher 1997). Also, green spores of *Osmunda regalis* appear to survive exposure to low relative humidity (RH<20%) overnight (Pence 2000). These data suggest that green spores could be more tolerant to desiccation than was originally thought, and storage at different temperatures could be optimized by controlling water content before storage as with nongreen spores. More studies are needed to understand and improve dry, cold storage of fern spores. Cryopreservation is not only a feasible alternative, but may be necessary to maximize spore longevity in long-term storage (Pence 2004; Ballesteros 2008; Ballesteros et al. 2006).

"Wet" storage of fern spores has been shown to be an effective alternative technique for spore viability conservation (Lindsay et al. 1992; Simabukuro et al. 1998b; Quintanilla et al. 2002; Aragón and Pangua 2004), particularly for spores that were believed to have recalcitrant (*sensu lato* Roberts 1973) storage behavior such as green spores (Lindsay et al. 1992) or those produced by *Culcita macrocarpa*

and *Woodwardia radicans* (Quintanilla et al. 2002). Wet storage is intended to mimic natural soil spore banks by sowing spores over nutrient media and storing them in the dark at lower temperature (Lindsay et al. 1992). Germination of hydrated spores is prevented by continuous darkness and, analogous to seeds in secondary dormancy (Villiers 1974), maintenance of viability is believed to result from ongoing cellular repair. The continuous metabolism presumably exhausts the storage reserves within the spore, eventually, reducing the ability of the spore to germinate and develop into a gametophyte, though published data to confirm this are lacking. Wet storage also presents logistical problems for spores that germinate in the darkness, maintaining cultures free of bacterial or fungal contamination, and economizing on preparation time and storage space requirements (Quintanilla et al. 2002). As a solution to short and medium term storage needs, wet storage appears to be effective and inexpensive; but dry storage and cryopreservation methods are needed for longer term storage (Ballesteros 2008).

12.4 Fern Spores: Orthodox or Recalcitrant Storage Behavior?

Seeds have typically been classified as orthodox or recalcitrant depending on their storage behavior. Orthodox seeds tolerate both desiccation and low temperatures during storage without affecting their viability, while recalcitrant seeds do not tolerate desiccation and usually have high water contents thus do not tolerate freezing (Roberts 1973; Berjak and Pammenter 2008). This traditional definition of storage behavior has been modified over the last 30 years to include "intermediate" storage behavior, which includes those seeds that usually survive exposure to either low water contents or low temperatures, but do not survive when exposed to both stresses simultaneously (Ellis et al. 1990a, b; Berjak and Pammenter 2008). Seeds with intermediate storage physiology commonly have lipids with relatively high crystallization and melting temperatures (Crane et al. 2003).

Conventionally, nongreen and green spores have been considered orthodox or recalcitrant in the literature, respectively. However, both nongreen and green fern spores are tolerant to desiccation (as explained in above paragraphs), so they cannot be considered strictly recalcitrant, though negative effects are detected when some species are stored at RH < 20% (Constantino et al. 2000), and other species seem to die faster when they are stored at room conditions compared with wet conditions (Lindsay et al. 1992; Quintanilla et al. 2002). Also, fern spores are usually affected negatively by temperatures reached in a conventional freezer (−10 to −30°C), although not all the spores in the population are killed at these temperatures (Lindsay et al. 1992; Simabukuro et al. 1998b; Quintanilla et al. 2002; Aragón and Pangua 2004). Based on their unusual response to low temperature, it has been suggested that fern spores exhibit a storage physiology that can be described as intermediate between recalcitrant and orthodox, or that they exhibit a storage physiology that remains uncharacterized (Ballesteros and Walters 2007b; Ballesteros et al. unpublished).

Recognition that storage at −25°C may compromise germplasm longevity if triacylglycerols (TAG) within cells crystallize between 0 and −25°C has led to a reevaluation of the intermediate storage physiology characteristic. In nongreen fern spores, lipid transitions are detected using Differential Scanning Calorimetry (DSC) between −4.1°C and −33.6°C and suggest that 25–40% of the dry mass of spores is TAG based on the enthalpy of the melting signals (Ballesteros and Walters 2007b). This estimate is consistent with earlier reports that TAG comprises between 20% and 50% of the dry mass of nongreen spores from a variety of fern species (Robinson et al. 1973; Towill and Ikuma 1975; Gemmrich 1977; DeMaggio et al. 1980; Gemmrich 1980; Minamikawa et al. 1984; Randi and Felippe 1988; Simabukuro et al. 1998a). Moreover, lipid droplets have been observed in green spores of Osmunda sp. (Hiyama et al. 1992; Ballesteros et al. unpublished), and appear to enlarge during storage at −25°C (Ballesteros et al. unpublished). A similar observation was made in seeds (Volk et al. 2006). Rapidly cooling germplasm to −80°C or LN treatment can prevent damage because TAG crystallization is inhibited (Vertucci et al. 1991; Crane et al. 2006). A brief exposure to ≥35°C also ameliorates damage incurred by storage at TAG crystallization temperatures by fully melting TAG before imbibition (Crane et al. 2006; Volk et al. 2006). Studies using DSC to detect TAG phase transitions could help us further optimize storage conditions for both green and nongreen spores of ferns.

12.5 Conclusions

Compared to seed storage, fern spore storage for *ex situ* conservation has received little attention during the last 50 years. Fern spores, both green and nongreen, appear to be tolerant to desiccation, but there are substantial variation in longevity between the two types of spores and among different species within each category. Little is known about the causes of aging of spores during storage and whether green and nongreen spores succumb by the same mechanisms. Longevity of both spore types is reduced when they are stored at temperatures of a conventional freezer (−10 to −30°C), suggesting that fern spores may have analogous physiologies to seeds classified in the intermediate storage category or that they exhibit novel and previously uncharacterized storage behavior. Based on current knowledge about fern spore storage in conventional freezers, cryopreservation is not only feasible, but is necessary to maximize spore longevity for long-term preservation.

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Chapter 13
Exploration of Cryo-methods to Preserve Tree and Herbaceous Fern Gametophytes

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13.1 Introduction

13.1.1 Fern Gametophytes as Germplasm for Genebanks

Many ex situ genebanks are investigating the feasibility of storing spores to preserve biodiversity of ferns because of the stress tolerance and availability of this germplasm (Agrawal et al. 1993; Pence 2000b; Rogge et al. 2000; Ballesteros et al. 2004; De Brum and Randi 2006; Ballesteros and Walters 2007; Mikuła et al. 2009). However, some fern species produce spores that are short-lived and lose viability within weeks or months (e.g., *Osmunda regalis* and other species from the *Cyatheaceae* family) (Lloyd and Klekowski 1970; Large and Braggins 2004), and some interspecific hybrids produce non-functional spores that have incompatible chromosome pairing (Large and Braggins 2004). Spore banking is difficult in these instances and alternative methods to preserve genetic diversity in genebanks are needed.

The fern gametophyte provides a novel source of germplasm that can circumvent many of the problems associated with fern spores and so serve an important complementary role in fern conservation. In the natural habitat, the gametophyte is a relatively short-lived stage of fern development. However, this life form can be easily and quickly multiplied by in vitro culture, often without application of plant growth regulators (Fernández and Revilla 2003; Goller and Rybczyński 2007). Fern gametophytes possess the remarkable ability to regenerate whole organisms from tissue chopped into small bits; the chopping might even stimulate propagation (Fernández et al. 1999). In vitro cultured fern gametophytes can provide consistent and sufficient germplasm for model system studies of organogenesis and reproductive biology, reliable regeneration of desired genotypes, and materials to bolster population sizes of endangered species. Maximizing the potential of gametophytes in scientific research

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(Schulte and Reski 2004; Tanurdzic and Banks 2004) and cost-effective conservation (Li and Pritchard 2009) will require integration of in vitro techniques with cryopreservation practices (Engelmann 2004; Sarasan et al. 2006; Pence et al. 2007; Keller et al. 2008). Cryopreservation methods for gametophytes of mosses (Christianson 1998; Pence 1998; Burch and Wilkinson 2002; Burch 2003; Schulte and Reski 2004; Rowntree et al. 2007; Rowntree and Ramsay 2009) and liverworts (Pence 1998; Rowntree and Ramsay 2009) appear to be established, but successful cryopreservation of fern gametophytes is reported in only seven species: *Davallia fejeensis, Drynaria quercifolia, Cibotium glaucum, Adiantum trapeziforme, Adiantum tenerum, Polypodium aureum* (Pence 2000a), and *Cyathea australis* (Mikuła et al. 2009).

13.1.2 Current Status of Gametophyte Cryopreservation

Classical approaches to plant cryopreservation have well-established advantages and disadvantages (Gonzalez-Arnao et al. 2008) which also apply to cryoconservation of fern gametophytes. In the few published studies, fern and moss gametophytes were cryopreserved using encapsulation/dehydration or controlled rate cooling (i.e., two step cooling) techniques, though air drying to optimum moisture levels and cooling to liquid nitrogen at appropriate rates was successful for species that are innately tolerant of desiccation (e.g., the moss *Bryum rubens*). There are no known reports of cryoprotection of gametophyte explants of ferns and bryophytes using vitrification techniques.

Desiccation tolerance is a key for successful cryopreservation (Panis et al. 2005) and innate differences are found among gametophyte species (Pence 1998; Burch 2003; Pence et al. 2005). A culture pretreatment in which explants are grown on medium supplemented with sugars, osmotica, abscisic acid (ABA) and/or proline enhances survival of cryoprotected germplasm exposed to liquid nitrogen (COST Action 871, 2006). Preculture on ABA-enriched medium is considered essential for survival after cryoexposure of some fern, moss, and liverwort gametophytes and lycopod shoot tips (Christianson 1998; Pence 1998, 2000a, 2001), and most pretreatments involve a 3 day to 3 week incubation on medium supplemented with some combination of 10 μM ABA, 100 mM proline, and 5% sucrose (Pence 2008). Pretreatments with higher concentrations of sucrose also gave excellent survival of explants encapsulated into alginate beads and dried slowly (Pence 1998, 2000a; Burch and Wilkinson, 2002; Mikuła et al. 2009). Preculture on ABA and proline, followed by routine application of cryoprotectant solutions containing 5% DMSO and 10% glucose, was needed for survival of cryoexposed moss gametophytes using the controlled cooling rate approach (Christianson 1998; Schulte and Reski 2004). One week preculture on medium supplemented with the osmoticum mannitol resulted in 100% regrowth of more than 650 mutants of *Physcomitrella patens,* regardless of phenotype or growth properties, enabling high-throughput cryopreservation of moss mutants (Schulte and Reski 2004). Clearly, culture under water stress conditions, which presumably induces greater tolerance to desiccation,

is a critical step in effective cryopreservation protocols. Culture pretreatments that induce greater stress tolerance in explants will expand the types of germplasm that can be effectively cryopreserved.

13.1.3 Brief Summary of Habitat for Fern Species in This Study

In this paper, we study fern species that exhibit variable tolerance to dry conditions and a range of tolerances to cold in their dominant sporophyte stage. Here, we select seven tree fern species and two herbaceous ferns (deciduous and evergreen), originating from three ecoregions where temperatures stay above 0°C, decline to a minimum of about −5°C in winter, or frequently decline to less than −32°C in winter (Table 13.1).

The most frost sensitive fern species in our study are *Cibotium glaucum* (Sm.) Hook. & Arn., *Cibotium schiedei* Schlecht. & Cham., and *Cyathea delgadii* Sternb. These tree ferns are integral to the ecology of humid tropical rainforests; they survive full sun in wetter areas, but need protection when the climate is drier (Large and Braggins 2004). *Ci. glaucum* (3 m height) is endemic to the Hawaiian Islands and grows in windward forests, dry or damp, up to an elevation of 1,800 m where annual temperatures range between 26°C and 32°C. This species' leathery fronds are ideally adapted to repel the burning cinders of Hawaiian volcanoes. A congener, *Ci. schiedei* (5 m height), is native to Mexico and is broadly distributed among shady or open areas, usually with plentiful rainfall (from 600 up to 1,400 m) (Large and Braggins 2004). The species is moderately sized and easy to grow. *C. delgadii* (10 m height) grows within the deep shade of rain forests and mid- to high-elevation cloud forests of the South American Andes, Central America mountains, and Brazilian highlands (from 100 up to 2,730 m) (Oliveira-Filho and Ratter 1995). It is a very fast growing plant and abundant in undisturbed, degraded, or gallery forests (Arens 2001).

Tree fern species that survive occasional snow and temperatures of about −5°C originate from high latitude regions of South America, Tasmania, southern New Zealand, and the sub-Antarctic Auckland Islands. These species have limited cold tolerance; for example, *Cyathea dealbata* (G. Forster) (10 m height) survives to about −3°C if kept moist (Large and Braggins 2004), and other species can survive winters in maritime areas of Great Britain, Ireland, the Mediterranean, Japan, Southern California and the Southeast USA (up to −15°C) with special protection (Barclay 2002; Large and Braggins 2004). This study includes the species *Cyathea australis* (R. Brown) Domin (10 m height), which is endemic to Australia and Tasmania, and *Cyathea smithii* Hook. (8 m height) and *Dicksonia fibrosa* Col. (6 m height), which are endemic to New Zealand (Large and Braggins 2004).

The most cold-hardy species of fern in this study are represented by *Osmunda regalis* L. and *Phyllitis scolopendrium* (L.) Newman. *O. regalis* (1.5–2 m height) is a cosmopolitan, subatlantic species, found in areas of the Americas, Europe, North Africa, Asia, and New Zealand, mainly in western Poland. This deciduous herbaceous fern grows in wet habitats such as woodland bogs and both sporophyte and gametophyte are sensitive to water stress (Zarzycki et al. 2002). The species has

Table 13.1 Geographic distribution, growth habit, and climate of fern species used in this study

Species	Common name	Distribution	Altitude above sea level (m)	Winter temperature (°C)
Cibotium glaucum (Sm.) Hook. & Arn.	Hawaiian tree fern; Hapu'u	Hawaii (endemic)	≤1,800	[1]tropical
Cibotium schiedei Schlecht. & Cham.	Mexican tree fern	Mexico	600–1,400	[1]tropical
Cyathea delgadii Sternb.	-	Caribbean, Central America, South America	100–2,730	[2]tropical
Cyathea dealbata (G. Forster) Swartz	Silver tree fern; Ponga	New Zealand (endemic)	-	[1]≥−3
Cyathea australis (R. Br.) Domin	Rough tree fern	Southeast Australia, Tasmania, Norfolk Island (endemic)	0–1,200	[1]≥−5
Cyathea smithii Hook.	Soft tree fern; katote	New Zealand (Auckland Islands) (endemic)	-	[1]≥−5
Dicksonia fibrosa Col.	Wooly tree fern; Kuripaka	New Zealand (endemic)	≤800	[1]≥−5
Osmunda regalis L.	Royal fern; Flowering fern	Europe, North Africa, North and South America ([3]vulnerable in Poland)	65–580	[4]≥−32 (deciduous)
Phyllitis scolopendrium (L.) Newman	Hart's-tongue fern	Europe, Macaronesia, North Africa; Western Asia ([3]declining – critically endangered in Poland)	600	[5]≥−32 (evergreen)

[1] Large and Braggins (2004)
[2] Oliveira-Filho and Ratter (1995)
[3] Mirek et al. (2006)
[4] Data from Botanical Garden – CBDC PAS, Warsaw, Poland
[5] In Polish conditions, the temperature up to −32°C did not have negative effect on the species (Jan Bodziarczyk, Agricultural University, Kraków, Poland, personal communication, 2010)

distinct morphologies for fertile and sterile fronds (Landi and Angiolini 2008). *P. scolopendrium* is distributed in Macaronesia (except Cape Verde Island), North Africa from Morocco to Libya and Western, Central, and Southern Europe, eastward to Crimea, the Caucasus, Turkey, and north Iran (Ivanova and Piękoś-Mirkowa 2003). Populations of *P. scolopendrium* are found in southern Poland at about 600 m above sea level. A mountain fern, *P. scolopendrium* grows in moist, shaded deciduous forests along northern or northeast rocky slopes, producing evergreen, leathery whorled fronds.

Tree ferns are an important plant group which help to create a sheltered and moist forest floor, providing ideal habitat for many bryophytes and filmy ferns and a "nursery" for recruited seedlings of many species (Forest Practices Authority 2009). Some tree ferns, for example, members of the *Cyatheaceae* and *Dicksoniaceae* families, are commercialized ornamental plants, leading to overexploitation of natural populations (Forest Practices Authority 2009). Tree ferns also have a rich ethno-botanical history which includes using the roots of *Cyathea delgadii* to make crafts, the trunk pith of *Cibotium glaucum* as a source of starch, and the hairs of *C. glaucum* to dress wounds and stuff pillows and mattresses (Hensley et al. 2003). Once abundant, tree ferns are now at risk by deforestation and industrialization. They are difficult to preserve in reserves or ex situ plantations because of their large growth habit and cold-sensitivity. In case of Polish studied species we should stress that both of them are included into Red List (Mirek et al. 2006). Thus, methods to preserve their biodiversity through genebanking methods are urgently needed.

The aims of the study are to develop a generalized protocol to cryostore in vitro-derived fern gametophytes and to test the widely held assumption that germplasm from species originating from tropical areas is less amenable to cryopreservation.

13.2 Materials and Methods

13.2.1 Plant Material

Experiments were carried out on gametophytes of nine fern species. Spores of *Osmunda regalis* and *Phyllitis scolopendrium*, herbaceous species that are cold tolerant were collected from adult individuals growing in the field collection of Botanical Garden - CBDC, Polish Academy of Sciences in Warsaw. Seven tree fern species from three genera were studied and culture procedures have been previously reported (Goller and Rybczyński 2007). *Cyathea* species include *C. australis*, *C. dealbata*, *C. delgadii*, and *C. smithii*; *Cibotium* species include *Ci. glaucum* and *Ci. schiedei;* and *Dicksonia* species include *D. fibrosa.* Gametophyte tissues of all studied fern species were grown on agar medium (Murashige and Skoog 1962) consisting of half-strength micro- and macro-nutrients with full composition of vitamins (½MS), 2% (w/v) sucrose, 0.8% (w/v) Difco agar, pH 5.8. Cultures were incubated at 22 ± 1°C with 16 h light (3.5 μE m^{-2} s^{-1})/8 h dark cycles. Tissues were transferred to fresh medium every 6 months.

Four to six weeks before cryopreservation treatments, cultures were transferred to fresh medium and newly formed prothalia or gametophytes producing prothalia were selected for further experimentation (Mikuła et al. 2009). Preculture consisted of a 1–2 week culture treatment on agar or liquid ½MS medium with 0.25 M sucrose (w/v) and with or without supplementation with 10 μM ABA (ABA was used during the last week of preculture treatment). This preculture protocol was also given to gametophytes that had been previously encapsulated within alginate beads according to methods described by Fabre and Dereuddre (1990).

To encapsulate gametophytes, newly formed prothalia were suspended for 10 min in distilled water containing 3% (w/v) sodium alginate (Sigma) and 20 g l^{-1} sucrose (without pH adjustment). About 50 droplets of this mixture was pipetted into 50 ml of ½MS liquid medium containing 0.1 M $CaCl_2$ and 20 g l^{-1} sucrose (pH 5.8) over a 45 min period and alginate beads 4–5 mm wide formed around each gametophyte.

13.2.2 Cryoprotection Procedures

Vitrification. Pretreated gametophytes (2 weeks with ABA) were bathed in 2 M glycerol + 0.4 M sucrose loading solution at 22°C for 20 min and then exposed to either PVS2 or PVS3 vitrification solution for 5 min, 0.5 h, 1 h, 2 h, and 3 h. The PVS2 solution contained 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO, and 0.4 M sucrose (Sakai et al. 1990) and was delivered to gametophytes at 2–3°C (on ice). The PVS3 solution contained 40% (w/v) glycerol and 40% (w/v) sucrose in basal culture medium (Nishizawa et al. 1993) and was delivered to gametophytes at 22 ± 2°C (room temperature). Samples were washed twice in fresh PVS2 or PVS3 solutions, loaded into 2 ml cryovials and placed directly into liquid nitrogen (LN).

Encapsulation/vitrification. Gametophytes, encapsulated and precultured (2 weeks with ABA) using procedures described above, were exposed to the vitrification loading solution (2 M glycerol + 0.4 M sucrose) for 20 min, then to either PVS2 or PVS3 solutions for up to 3 h and finally to LN as described above.

Encapsulation/dehydration. Gametophytes, encapsulated and precultured (½MS + 0.25 M sucrose and 0 μM or 10 μM ABA) for 1 or 2 weeks using procedures described above, were dried over 3 days by daily increases in sucrose concentration from 0.5 to 0.75 to 1 M. Beads were then harvested and surface-dried in a laminar flow chamber at room temperature for 5 h and loaded into 2 ml cryovials (15 beads per 2 ml cryovial). Cryovials were immersed directly into LN where they remained for 1–3 days.

13.2.3 Thawing of Gametophytes

Cryotubes submerged in liquid nitrogen for 1–3 days were plunged into a +38°C water bath for 3 min. Gametophytes prepared using vitrification or encapsulation/vitrification procedures were then washed with 1.2 M sucrose solution for 0.5 h to

remove cryoprotectants. All gametophytes were then transferred to Petri dishes containing agar supplemented with 1/2 MS and 20 g l^{-1} sucrose. Recovering plant material was transferred onto fresh medium within 2 days.

13.2.4 Survival Assessments

Survival was assessed by the retained green color of gametophytes 3–7 days after thawing. The 3–7 day culture time is necessary to observe greening because all gametophyte cells are green directly after thawing, but necrosis within cells becomes obvious 3 days after thawing. Location of surviving cells was verified by chlorophyll autofluorescence 3 days after thawing using a light microscope (Vanox-Olympus) equipped with computer image analysis system (analysis program ver. 3.1). Fluorescence was induced by blue-violet light (BV filter: 400–440 nm). Rate of recovery was estimated by the time after thawing required for gametophytes growing in vitro to develop sexual structures.

Results were analyzed using a single-factor analysis of variance (ANOVA) and a Fisher's Least Significant Difference Test (LSD) using Statgraphics Plus software. Significance is stated at $P < 0.05$. Statistical analyses were performed on two independent experiments with at least 75 gametophytes in each experiment.

13.3 Results

13.3.1 Gametophyte Survival Following Various Cryo-methods

Survival was assessed in gametophytes from ferns that were cryoprotected using three common methods and then exposed to liquid nitrogen. Survival varied considerably among the various preculture and cryoprotection procedures.

Cells from gametophytes that were directly exposed to PVS2 or PVS3 (nonencapsulated) plasmolyzed within 5 min (Fig. 13.1a). After 1 h, only cells within the meristematic area called the apical notch were alive (Figs. 13.1b, c). Exposure of explants to LN was completely lethal (examples not shown).

Encapsulated gametophytes survived exposure to PVS2 or PVS3 solutions for up to 3 h (Table 13.2 gives data for *Dicksonia fibrosa*, which is representative of other species). These solutions provided some cryoprotection as indicated by increasing survival with increasing soaking times up to a maximum of 50% survival for gametophytes treated for 3 h in PVS2 delivered on ice or 2 h in PVS3 delivered at room temperature. Major portions of the gametophyte tissue were necrotic after cryoprotection by encapsulation/vitrification; however some cells survived and proliferated (Fig. 13.2), demonstrating the resilience and regenerative capacity of these cells.

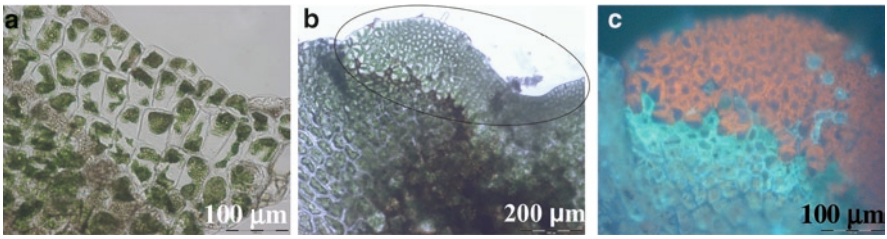

Fig. 13.1 Gametophytes exposed to vitrification solutions; (**a**) part of *Cyathea smithii* gametophyte after 1 h treatment with PVS2. All visible cells are irreversibly plasmolyzed; (**b**) Gametophyte of *Cyathea delgadii* 3 days after 1 h treatment with PVS2 solution showing survival of cells in the apical meristem (*boxed area*) and irreversibly plasmolyzed cells elsewhere; (**c**) red chlorophyll autofluorescence of living portion of *Cyathea delgadii* gametophyte

Table 13.2 Survival of *Dicksonia fibrosa* gametophytes after cryoprotection using the encapsulation/vitrification technique with or without exposure to liquid nitrogen

Time of PVS2 or PVS3 treatment (h)	PVS2 on ice		PVS3 at room temperature	
	−LN	+LN	−LN	+LN
0.5	100	6.3 ± 2.9 c	100	22.5 ± 0.4 c
1.0	100	22.6 ± 4.1 bc	100	30.0 ± 2.8 c
2.0	100	34.2 ± 4.0 ab	100	54.0 ± 7.6 a
3.0	100	50.6 ± 14.9 a	95.3 ± 1.7	41.8 ± 1.6 b

Values marked by the same letter are not significantly different at $P < 0.05$ (LSD test)

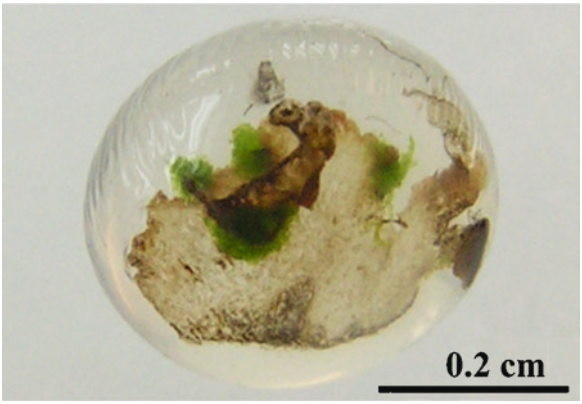

Fig. 13.2 *Dicksonia fibrosa* gametophyte protected by encapsulation/vitrification procedure, 2 weeks after thawing. A few cells on only a small portion of gametophyte surface survived (*green*) and the remaining portions became necrotic (*brown*)

Encapsulation/dehydration procedures resulted in the highest and most consistent survival of the three cryprotection methods studied (Figs. 13.3, 13.4, and 13.5). Even still, survival varied among species ranging from 67.5% for *C. dealbata* under optimized conditions to 100% for *P. scolopendrium* and *D. fibrosa* (Figs. 13.4 and 13.5).

The dehydration steps were critical to the survival of encapsulated explants to LN. Gametophytes dried within 1 day using 0.5, 0.75, and 1.0 M sucrose did not survive the cryoexposure (data not shown). The procedures reported here use a day-long incubation at each sucrose concentration. This 3-day-long osmotic desiccation was as effective as it extended up to 6 days (in each above mention sucrose concentrations for 72 h/48 h/24 h, respectively) (data not shown). The optimal period of air drying was previously assessed and it is as long as 5 h (Mikuła and Rybczyński 2006).

13.3.2 Preculture Effects on Detectable Damage

Preculture promoted from two- to more than sixfold greater increases in gametophyte viability after cryopreservation than without it (except *Phyllitis scolopendrium*) (Fig. 13.5). For *Cyathea dealbata* application of preculture was necessary for its survival after cryoexposure. Factors during preculture had variable effects on survival of fern gametophytes. Of the nine species of ferns studied, only *P. scolopendrium* gametophytes gave 100% survival following exposure to LN regardless of pretreatment factors (Fig. 13.4). Response of *O. regalis* explants was not significantly affected by the preculture treatment, and survival over ranged between 69% and 82%. *Dicksonia fibrosa* gametophytes were induced to high tolerance of LN conditions by 1 week pretreatment (with or without added ABA) giving 90% and 100% survival after 1 and 2 weeks pretreatment, respectively (Figs. 13.3 and 13.4). For the other species, ABA, 2 week preculture, and agar medium generally gave higher survival compared to no ABA, 1 week preculture, and liquid medium, and the combination of

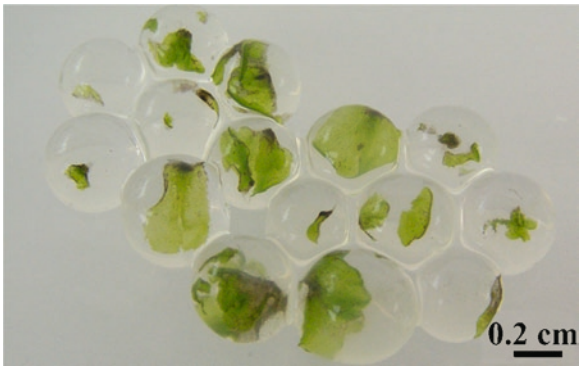

Fig. 13.3 *Dicksonia fibrosa* gametophyte protected by encapsulation/dehydration method, 3 days after thawing. Whole gametophytes survived the cryopreservation procedure

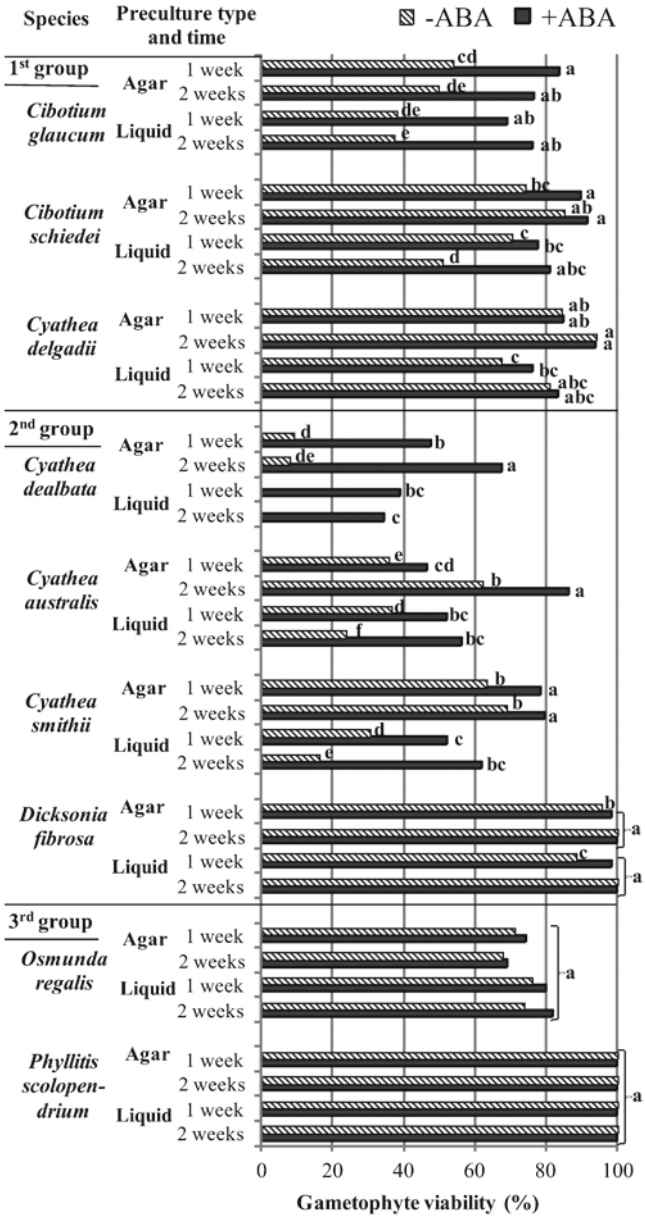

Fig. 13.4 Gametophyte survival (%) of nine fern species after preculture on liquid or agar medium in the presence and absence of ABA. Explants were cryoprotected using encapsulation/dehydration procedures and then exposed to LN. Species are categorized by ecogeographic region of origin: 1st group – tropical rain forest; 2nd group – mild, subfreezing winters; 3rd group – temperate species with winter temperatures as low as −32°C. Values marked by the same letter are not significantly different at $P < 0.05$ (LSD test)

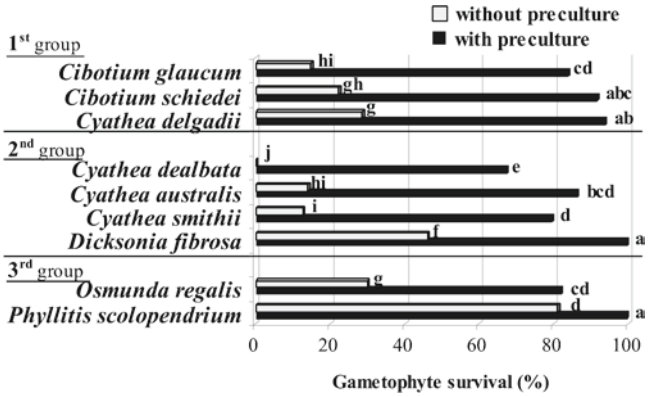

Fig. 13.5 Gametophyte survival (%) of ferns under optimal conditions of preculture treatment and without any preculture, depending on the ecogeographic region of the origin: 1st group – tropical rain forest; 2nd group – mild, subfreezing winters; 3rd group – temperate species with winter temperatures as low as −32°C. Values marked by the same letter are not significant different at $P<0.05$ (LSD test)

these factors often gave the highest survival after exposure to LN (Fig. 13.4). Congeners of *Cyathea* (except *C. delgadii*) and *Cibotium* were highly responsive to ABA and survival increased by 10–90% in ABA treated materials. Gametophytes of these species were also fairly sensitive to LN exposure, with non-ABA treated gametophytes often giving 0–40% survival (Fig. 13.4).

Between 70% and 100% survival was achieved for fern gametophytes exposed to LN after preculture and cryoprotection. Despite this relatively high level of survival, visible damage was apparent within explants as areas of necrotic tissue (Figs. 13.2 and 13.6). Preculture duration and species' sensitivity to cryopreservation stresses affected the extent to which necrosis developed in post-thawed gametophytes, and this was quantified for two species in Table 13.3. Necrosis in *P. scolopendrium* (the species with the highest tolerance to cryoexposure) was apparent in over 70% of explants that did not receive a preculture treatment (Table 13.3 and Figs. 13.6a, b). This level of damage was not lethal, as 81% of these explants survived LN treatment and regenerated. Extent of necrosis decreased to a minimum of 6% after 2 weeks of preculture (Table 13.3 and Fig. 13.6d). In contrast, all explants of *C. australis* displayed necrosis following LN exposure (Table 13.3 and Fig. 13.7). Longer preculture was essential for higher survival in this species (Fig. 13.4), but never gave full protection (i.e., no necrosis) of the whole gametophyte (Table 13.3).

13.3.3 Recovery of Gametophyte in Culture and Sporophyte Production

Time for gametophytes growing in vitro to develop sexual structures depended on the proportion of surviving and necrotic cells of explants (Figs. 13.6, 13.7, 13.8). Explants

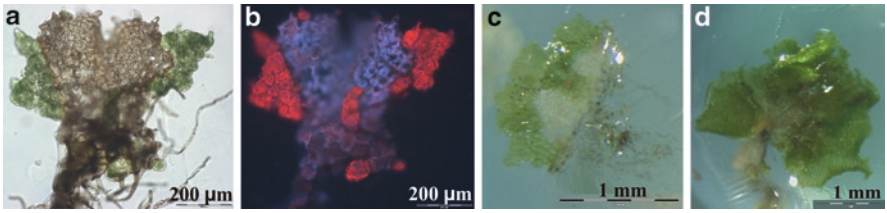

Fig. 13.6 Effect of preculture duration in 0.25 M sucrose and 10 µM ABA on the extent of necrosis in *Phyllitis scolopendrium* gametophytes after cryopreservation; (**a**) without preculture in white light; (**b**) the same explant in UV light; (**c**) after 1-week preculture; (**d**) after 2-week preculture. Three days after thawing

Table 13.3 Effect of preculture duration on survival and incidence of necrosis of *Cyathea australis* and *Phyllitis scolopendrium* gametophytes cryopreserved using the encapsulation/dehydration technique. Preculture using agar, 0.25 M sucrose, and 10 µM ABA

Time in preculture (week)	Gametophyte survival %		% explants with necrotic tissue	
	C. australis	*P. scolopendrium*	*C. australis*	*P. scolopendrium*
0	14.1 ± 1.1	81.3 ± 7.6	100.0	72.0 ± 6.1
1	46.8 ± 2.8	100.0	100.0	19.2 ± 6.9
2	88.4 ± 6.9	100.0	100.0	6.4 ± 2.7

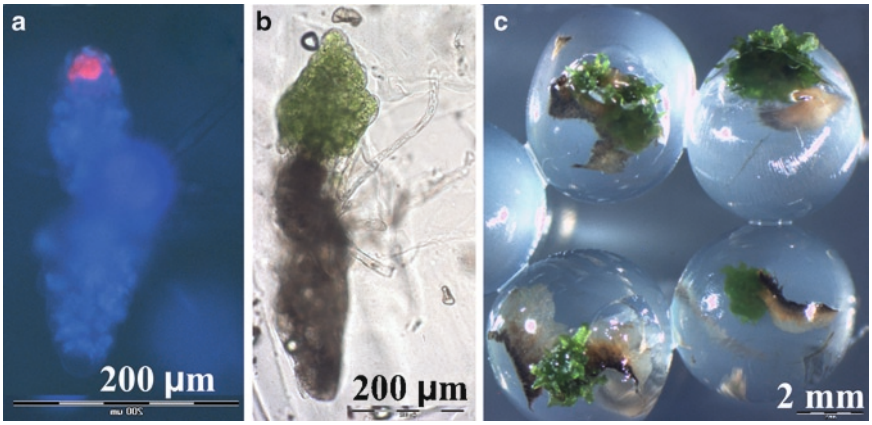

Fig. 13.7 Recovery of *Cyathea australis* gametophyte culture; (**a**) explants derived from cryopreservation without preculture with a single surviving cell in UV light (*red*), 3 days after thawing; (**b**) gametophyte recovered from single living cell, 2 weeks after thawing; (**c**) gametophytes derived from cryopreservation with 2-week preculture with extensive necrosis and numerous young prothalia, 2 weeks after thawing

showing low levels of necrosis showed rapid recovery and multiplication of prothalia (Figs. 13.8c and 13.8e); lush new cultures were obtained within 8 weeks (Fig. 13.8f). *D. fibrosa* and *C. delgadii* gametophytes, which appeared relatively tolerant of LN exposure, formed sporophytes within 8 and 10 weeks post thawing, respectively

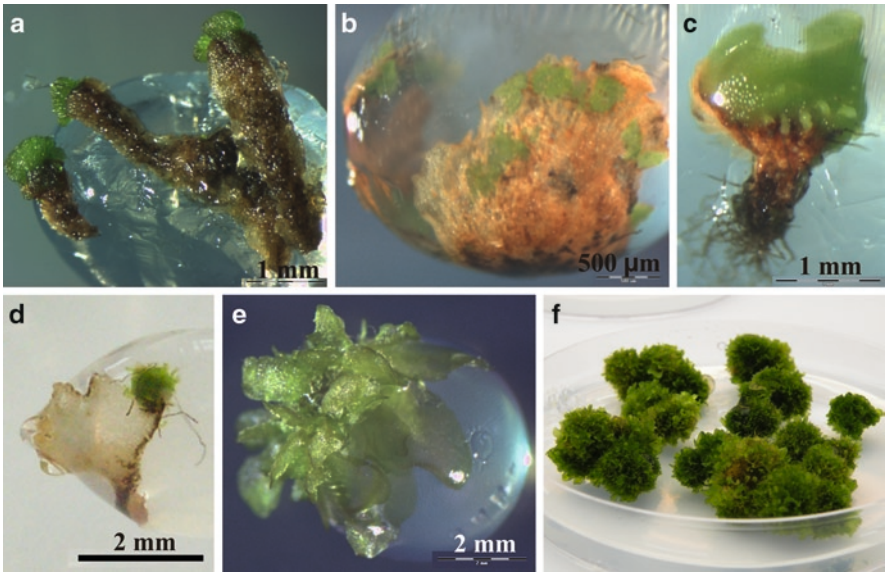

Fig. 13.8 Recovery of gametophyte in post-thawing culture: (**a**) living apical part of *Cyathea delgadii* elongated gametophytes used for study, 7 days after thawing; (**b**) a few living cells of *Cyathea dealbata* gametophyte, 3 days after thawing; (**c**) majority of cells of *Cibotium schiedei* gametophyte survived LN exposure, 3 days after thawing; (**d**) recovering gametophyte cultures of *Cyathea delgadii* from single living cells of heart-shape gametophytes cryopreserved without preculture, 2 weeks after thawing; (**e**) recovering gametophyte cultures of *C. delgadii* from whole living explants preserved by 2-week preculture with sucrose and ABA, 2 weeks after thawing, and later (**f**) lush gametophyte culture after eight weeks

when cultured on standard ½MS medium (Figs. 13.9a and 13.9b). Slow recovery was observed in gametophytes experiencing extensive tissue damage (i.e., *C. australis* (Figs. 13.7a–c), *C. dealbata* (Fig. 13.8b), *C. smithii,* and *Ci. glaucum* (not shown); cultures became lush after about 5 months and sporophytes were eventually formed after about 1 year. Gametophytes that were so damaged that only one or just a few cells survived, could recover (Figs. 13.7a, b), and eventually produced fully mature cultures. Sporophytes derived from cryopreserved gametophytes (Fig. 13.9c) grew quickly and easily acclimated to greenhouse conditions (Fig. 13.9d) and were not distinguishable from sporophytes derived from non-cryopreserved prothalia.

13.4 Discussion

This study investigates the response of fern gametophytes to cryoprotection procedures and LN exposure. We show large differences in gametophyte responses to vitrification, encapsulatation/vitrification, and encapsulation/dehydration procedures, with only the latter procedure showing high survival (Figs. 13.4 and 13.5).

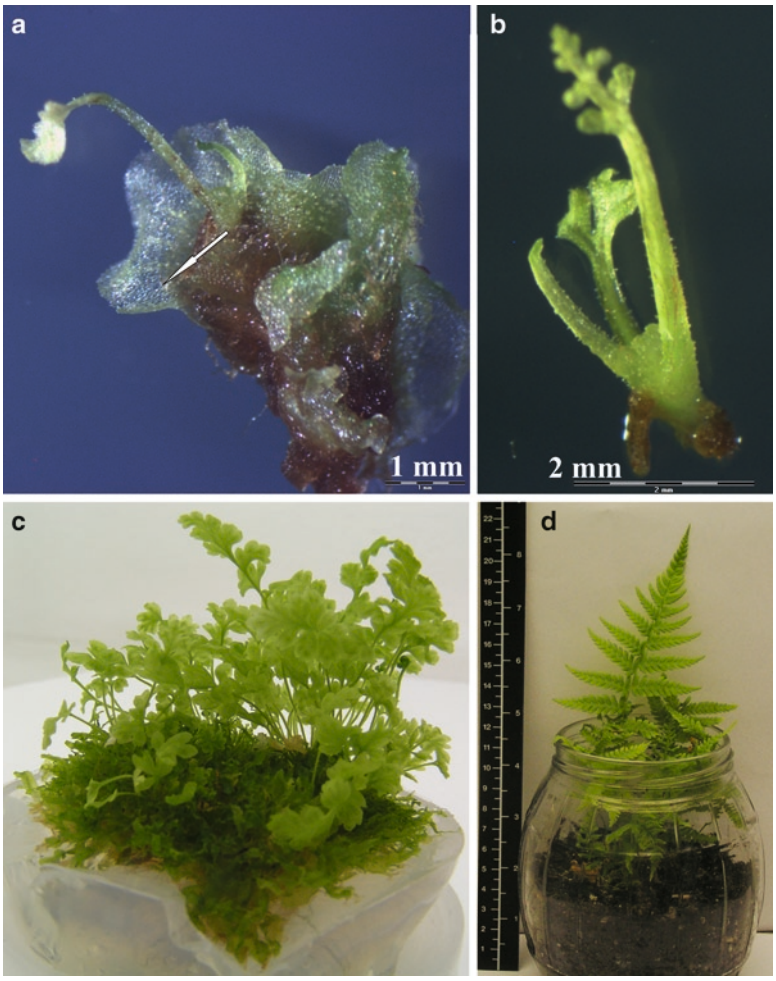

Fig. 13.9 Sporophytes production in recovering gametophyte culture; (**a**) sporophyte of *Dicksonia fibrosa* begins 10 weeks after thawing (arrow); (**b**) young sporophyte of *Cyathea delgadii*, three young frond and root are visible; (**c**) mass production of *Dicksonia fibrosa* sporophyte culture, 6 months after thawing gametophytes; (**d**) *Cyathea delgadii* sporophytes after acclimatization in open jar

Survival percentages using encapsulation/dehydration procedures varied among the nine species tested, and preculture conditions that exposed explants to ABA and agar (as opposed to liquid) medium enhanced survival of sensitive species. Contrary to expectations, gametophytes from tropical species exhibited comparable or greater tolerance of cryoexposure compared to gametophytes from species originating in regions with mild winters having occasional subfreezing temperatures or severe winters having minimum temperatures as low as −32°C (Figs. 13.4 and 13.5).

13.4.1 Cryoprotection and Gametophyte Survival

The toxicity of PVS2 and PVS3 solutions on nonencapsulated fern prothalia was distinctive, with only a few cells within the meristematic region surviving a 1-h exposure. In the presence of the ca. 8 M PVS2 and PVS3, cells irreversibly plasmolyzed, demonstrating the chemical toxicity and limited tolerance of these tissues to low water stress (Fig. 13.1a) (Volk and Caspersen 2007). The alginate bead coatings protected cells of the fern gametophyte from some of the damage induced by PVS2 and PVS3 and encapsulated gametophytes survived 1–3 h exposures to the vitrification solutions (Table 13.2). Protection is only moderate, and the encapsulation/vitrification procedure resulted in only about 50% survival of cryoexposed explants, which is consistent with a previous report using young *Platycerium ridleyi* sporophytes (Rodpradit et al. 2003). The high toxicity of PVS2 and PVS3 solutions necessitates alternative chemical dehydrants such as solutions developed for gentian axillary buds (Suzuki et al. 2008) or mint shoot tips (Volk et al. 2006) possessing, for example, $CaCl_2$ or polyethylene glycol 8000.

Encapsulation of gametophytes in alginate beads is believed to be effective because it slows dehydration rate and maintains final tissue water content above levels achieved by vitrification or air drying alone (Pence 2000a; Burch 2003). We report that nonencapsulated gametophytes of the nine fern species studied here did not survive open air drying (data not shown). Encapsulation increased survival of cryoexposed gametophytes of *Cibotium glaucum, Polypodium aureum, Adiantum trapeziforme*, *Davallia fejeensis, Adiantum tenerum,* and *Drynaria quercifolia* (Pence 2000a). However, the ≤50% survival of some of these species and complex drying procedures needed for alginate beads suggests that encapsulation alone does not confer tolerance to LN. Survival of encapsulated explants in LN required precise drying procedures, usually involving stepwise desiccation using concentrated sucrose for a prescribed period and then a brief period of air drying (Mikuła and Rybczyński 2006; Mikuła et al. unpublished). Treatment at 0.75 M sucrose appeared to give mixed results, with an 18 h exposure substituting for ABA pretreatment in some studies (Pence 1998, 2000a), but a 6-day-long exposure following stepwise drying at 0.3, 0.5, and 0.75 M (Mikuła and Rybczyński 2006) or daily, stepwise desiccation until 1.0 M sucrose giving poor survival in our fern gametophytes.

Preculture in sucrose and ABA, as compared to without any preculture treatment, increased survival of all species of fern gametophytes studied (Fig. 13.5). Maximum survival was observed after a 1 week preculture treatment for most species except *C. australis* and *C. dealbata*, for which maximum survival was observed after 2 weeks preculture. Preculture in ABA plus proline also increased chloronema cell survival of three moss species and two species of *Sphagnum* (Christianson 1998). Our data are consistent with the observation of significant increases in survival following ABA preculture for explants with low tolerance to cryoexposure, and limited benefit of ABA preculture for explants already exhibiting high tolerance (Pence 1998, 2001; Pence et al. 2005). For example, *P. scolopendrium, D. fibrosa,* and *C. delgadii* gave >90% survival with or without ABA preculture; while ABA preculture increased survival from near 10% to above 60% for *C. dealbata* (Fig. 13.4).

13.4.2 Autofluorescence as a Simple Methods Assessment of Gametophyte Viability

Regrowth of cells in post-thawing culture is the most sensitive test of viability, but the method is time-consuming. Other viability tests based on cellular absorption of dyes (2,3,5-triphenyltetrazolium chloride – TTC test or fluorescein diacetate – FDA test) (Mikuła et al. 2006) can be used for various plant materials, for example: cell suspension culture, callus, shoot meristems, or buds, but it is not appropriate to green gametophyte explants. Gametophyte survival could be assessed by retaining their green color, which in dead prothalia disappeared the 3rd day after thawing. Green autofluorescence of chlorophyll, induced by blue-violet light, could help in location of surviving cells (Figs. 13.1c, b), determining their number (Fig. 13.7a) (Mikuła et al. 2009) and estimation range of gametophyte necrosis. This simple and quick method may also help assess spore viability after cryoexposure (Mikuła et al. 2009).

13.4.3 Cellular Damage to Gametophytes and Totipotency

Damage to cryoexposed gametophytes was usually evident by the development of necrotic regions on the explant (Figs. 13.2, 13.6–13.8, and 13.8d). The number of surviving cells and cell types reflect the severity of the stress and the effectiveness of the cryoprotection, and this, in turn, influenced the rate at which a surviving gametophyte recovered, developed, and reached sexual maturity (Fig. 13.8d–f). Our results showed faster growth and shorter time to maturity (Fig. 13.8d–f) in surviving gametophytes with less necrosis which was achieved by longer preculture treatment with sucrose and ABA (Table 13.3). A similar trend was reported for protonemata cells of the moss *Ditrichum cornubicum* and *Brium rubens* in which thorough cryoprotection by preculture in sucrose and ABA or encapsulation, reduced overall necrosis and increased recovery rate (Burch and Wilkinson 2002; Burch 2003). Therefore, we believe the additional time in preculture is critical for effective cryoprotection of tree and herbaceous fern gametophytes even if survival percentages do not indicate a benefit.

The resiliency of gametophytic tissue following the severe stress of cryoexposure is also noteworthy and introduces fern gametophytes as a potentially excellent model system to study totipotency and cell differentiation in plants. Significant portions of young gametophytes became necrotic after cryoexposure; for example, about 75% of *C. australis* gametophyte cells were killed (Mikuła et al. 2009). In this study, we observed survival of the gametophyte even if only one cell survived (e.g., Figs. 13.7a and 13.8d). Survival of a small population of cells suggests different sensitivities and different levels of protection among cell types. Studies have shown that tissues within a plant organ respond differently to cryopreservation stresses and that differentiated cells are likely to succumb while meristematic cells may survive (Volk and Caspersen 2007; Wang et al. 2009).

13.4.4 Desiccation and Cold Tolerance and Cryopreservability

We compared cryopreservability among fern gametophytes in species originating from different ecoregions to address the hypothesis that cryopreservability would be correlated with tolerance to the minimum temperature experienced in that region. This hypothesis was not supported. Rather, gametophytes from areas with mild frosts but low risk of water loss appeared to be less tolerant of cryopreservation stress compared to gametophytes from tropical environments that seldom experience subfreezing temperatures but may experience wetting and drying cycles. Moreover, *Osmunda regalis* explants were relatively sensitive to cryoexposure despite plant tolerance to severe winters (Fig. 13.4). Both sporophyte and gametophyte stages of *O. regalis* require wet habitats, so this plant may not have effective mechanisms to cope with water loss. In moss and liverwort gametophytes, survival following cryoexposure is correlated with their tolerance to desiccation. Extremely desiccation tolerant moss species are able to survive fast drying and LN exposure without cryoprotection (Burch 2003), and survival of less desiccation tolerant cells can be enhanced to some degree by cryoprotection solutions (Pence 1998; Burch and Wilkinson 2002; Burch 2003). This suggests that ability to survive cryoexposure is more related to desiccation than cold tolerance.

The range of desiccation tolerance among bryophyte gametophytes is well established (Oliver 2007). There are some reports that fern gametophytes, like bryophytes, exhibit a broad range of longevities and tolerances depending on their habitat (Watkins et al. 2007b). Gametophytes of terrestrial fern species from temperate areas typically survive for just 6 months (Watkins et al. 2007a); however, stress tolerance in the gametophyte may exceed that exhibited in the sporophyte (Sato 1982). In contrast, gametophytes of tropical epiphytes may live for years (Watkins et al. 2007a). During the gametophytic stage, tropical epiphytes routinely experience desiccation and rehydration cycles, sometimes daily. The relatively high survival of fern gametophytes measured in this study and the variation in cryopreservability among species may be related to desiccation tolerance in these cells. Growth on ABA and osmotica appear to enhance this tolerance, suggesting that it is an inducible response.

13.5 Conclusions

We report a range of tolerances to cryopreservation stress among genera and congeners of fern gametophytes. Despite the variation, the encapsulation/dehydration technique consistently protected gametophytes during cryoexposure, giving >70% survival of explants and lowering the incidence of necrosis in recovering explants. A preculture treatment that exposes explants to sucrose and ABA is necessary to confer full protection. Reduced necrosis speeds up recovery time and gametophytes reach sexual maturity sooner. The ability to effectively cryopreserve gametophytic tissues makes them useful germplasm for scientific research and ex situ conservation.

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Chapter 14
Pteridophyte Spores Viability

Jose María Gabriel y Galán and Carmen Prada

14.1 Concept of Spore Viability – Biological Importance

Pteridophyte spores are unicellular structures of variable size depending on the species. They are produced in the sporangia via meiosis when sporophytes reach maturity after a period of growth. The spore is the first cell of the haploid phase in the life cycle of a pteridophyte. Once released from sporangia, and under suitable conditions, it has the capacity to create a new haploid individual, the gametophyte, through a series of developmental processes. In essence, and despite a certain variation in the developmental pattern, these processes comprise the germination of the spore, the growth of a filamentous prothallus -first uniseriate, then biseriate and planar-, the organization of a meristem and, finally, the emergence of a pre-sexual gametophyte. The spore germination is the first sign of the entire gametophytic developmental course. Basically, it consists of a molecular and physiological preparation phase, the division of the cell into two daughter cells, the rupture of the spore wall and the appearance and growth of those cells (one rhizoidal cell and other prothallial cell). Spore viability is defined as the time that spores retain its capacity to germinate.

Hybridization and apogamy are biological phenomena with a very high incidence in ferns. Both of them imply the occurrence of abnormal meiosis that yields variable percentages of aborted or atypical spores. Aborted spores are misshapen and unviable. Atypical spores are so due to alterations on size, content, laesurae or any other character. Most of these spores are either non-viable but sometimes certain percentages of atypical spores can maintain their viability and germinate. In *Psilotum nudum*, for example, atypical spores with coagulated oil droplets germinated, though rarely (Whittier 1990). Pentaploid cytotype of *Pteris vittata* produced 70% of unbalanced nuclei that derived in misshapen sterile spores, and a 30% of tetrahedral typical spores and atypical monad and bilateral spores that

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germinated (Khare and Kaur 1983). Similar observations have been done in other *Pteris* species (O. Martínez, personal communication).

It is frequent to see that pteridophyte spores, faced up to an optimal artificial environment (humidity, atmosphere, nutrients and temperature), can rapidly germinate. This phenomenon has been called quiescence. Dormancy could be defined as the set of developmental characters that derived in the failure of viable spores to germinate under suitable conditions that would promote germination of quiescent spores (Raghavan 1989). This set of characters is supposed to be some kind of physical and chemical changes in the spore, established during its maturation. Only when some conditions are met, the chemical state is reversed and the spore is able to germinate. Light, temperature, water and hormonal conditions are some of the presumable aspects affecting the dormancy, thus controlling the establishment and cease of the dormant state (Weinberg and Voeller 1969; Nauyalis 1989; Whittier 1990; Perez-Garcia et al. 2007). Spores of some ferns (Hymenophyllaceae, Grammitidaceae) exhibit no or limited dormancy, since they are known to germinate within the sporangium. On the other hand, spores of some Pteridaceae, Schizaeaceae and Marsileaceae remain dormant for a very long period (Courbet 1963).

The successful establishment of species individuals and populations in a new habitat after spore dispersal depends, among other factors, on how long the spores retain their ability to germinate (Sheffield 1996; Shorina 2001). Spore dormancy is the ecological mechanism that provides pteridophyte species with a correct dispersal over the time and through the establishment of spore banks of different ages (Lindsay and Dyer 1990; Dyer and Lindsay 1992; Dyer 1994; Penrod and McCormick 1996). There is unequivocal information to consider that dormancy has evolved as a response to environmental pressures. This is particularly explicit in groups with subterraneous, non-chlorophyllous gametophytes, as Lycopodiaceae and Ophioglossaceae. In these plants, a mycorrhizal relationship is needed to be made during the first germination stages with an endophytic fungus in order to develop the gametophyte. So, a longer dormancy period of the spores derives in more opportunities to contact the appropriate mycelium (Raghavan 1989; Whittier and Moyroud 1993; Whittier 1998). However, in general, the adaptative significance of more or less extended spore viability remains obscure.

When spore viability decreases, various significant aspects of individual development and population dynamics are modified. With regard to the development, it has been proved that the gametophytes could grow abnormally and even the production of sporophytes from germinated spores is affected (Smith and Robinson 1975; Beri and Bir 1993). With respect to the populations, the reduction of spore viability has a direct effect in the germination percentage, which could be highly reduced. In consequence, so would be reduced the number of successful gametophytes in the subsequent population. In this same sense, the rate at which germination occurs is slowed down and the time from spore release to germination is increased (Pence 2008), factors that could derive in important consequences on natural population dynamics.

14.2 Viability Variation Among Species

Pteridophytes as a whole can typically maintain spore viability for a long period, measured in the rank of years, but it is largely known that there is a great disparity among species (Okada 1929). One of the principal features contributing to the variation in spore viability is the presence of chrolophyll pigments in the cell. In this sense, two kinds of fern spores could be defined based on their colour: chlorophyllous (named also as green spores) and non-chlorophyllous (non-green spores, generally brown, yellow or similar). Germination rate and viability of both kinds of spores are extremely different.

Green spores are those that contain chloroplasts. Among their features, two are specially relevant (Lloyd and Klekowski 1970). First, they exhibit a clear tendency to a quick germination, which is in mean of 1.5 days. Some species are especially notable in this feature, since the spores could germinate within the sporangia, as happens with the Hymenophyllaceae and Grammitidaceae (Stokey 1940; Stokey and Atkinson 1958). Second, their germination ability is very rapidly lost, with a mean viability of around 48 days. *Equisetum hyemale* is particularly sensitive, as all the spores are deceased in 2 weeks, at room temperature (Lebkuecher 1997). *Osmunda regalis* showed a bit longer viability of around a month (Stokey 1951), while *Onoclea* and *Matteuccia* species continue viable after 1 year (Lloyd and Klekowski 1970).

Most of the ferns have non-chlorophyllous spores whose germination takes between 4 and 210 days, with a mean germination time of 9.5 days, but the principal difference between green and non-green spores is that the latter show a clearly higher viability, with a mean of 1,045 days (Pence 2008). Within this non-green spores group, some homosporous species produce spores that become completely inviable in a few months, for example, *Culcita macrocarpa* (Quintanilla et al. 2002), while others, like *Pellaea truncata*, are able to germinate after more than 50 years from harvest (Windham et al. 1986). The absolute record to date of spore viability is reported within the heterosporous genus *Marsilea*: megaspores obtained from 100-year-old sporocarps of *M. oligospora* germinated forming rhizoids and archegonia (Johnson 1985). Irrespective of these extreme cases, the normal rule for most of the ferns is to present spore viability that ranges between 1 and 3 years.

In addition to the facts informing about a great interspecific variation in spore viability, data have been reported on the existence of an also noticeable intraspecific variation between individuals and populations in the spore functionalities (Kiss and Kiss 1998; Schneller 1998; Schmitz et al. 2006).

14.3 Factors Affecting Viability

Beyond the yet remarked importance of the presence of chlorophyll in the cell, there are some other known factors that directly affect the viability of spores. These factors are either intrinsic, such as genotype and age, or extrinsic, such as the conditions of physical environment or presence of competitors.

14.3.1 Genotype

The correlation between genetic dosage and spore viability is still under debate, since results of research point out in divergent directions.

There is some evidence suggesting that there is a positive direct relationship between the genetic dosage and, among other aspects of pteridophyte development, the spore viability. In some investigated taxa, polyploids seem to have a better behaviour in this sense, producing higher percentages of viable spores which hence yield higher germination percentages than their diploid ancestors. Pioneer observations were made on *Dryopteris* species (Whittier 1970), *Polypodium virginianum* (Kott and Peterson 1974) and, more recently, on *Polystichum* (Pangua et al. 2003). For some species of the heterosporous *Isoetes*, a similar correlation between megaspore viability and ploidy levels has been demonstrated (Kott and Britton 1982).

However, some other research point out in other direction. A comparison between diploid and allotetraploid species of *Dryopteris* concluded that, irrespective of the ploidy level, percentages of aborted spores, germination times and final germination percentages where similar (Quintanilla et al. 2002; Quintanilla and Escudero 2006). In conclusion, the authors stated that the allopolyploidization process did not imply a change in the success of spore germination. Analogous results were achieved in some complexes of *Asplenium* involving taxa with different ploidy level, for example, *Asplenium adiantum-nigrum* (Prada et al. 1995) and *Asplenium foreziense* groups (Herrero et al. 2002). In some cases, noticeable differences have been observed: in tetraploid cytotypes of *Pteris vittata* a 100% of germination was achieved but only 30% in pentaploid cytotypes (Khare and Kaur 1983).

One general interpretation of all this information is that the relationship between spore viability and ploidy level is taxa-specific, i.e., is variable among taxa, and no generalization can be done. In addition, as it has been pointed out, viability is reduced at first in recently formed polyploids until they "learn" how to overcome the evolutionary barrier of an irregular meiosis, thus enhancing the percentage of abnormal spores and, subsequently, the low germination and viability rates. So, to get a more general perspective, data from spore fitness and ploidy levels should be addressed along with the time of polyploids origins.

14.3.2 Age

Spore viability is definitely correlated to its age, defined as the time elapsed from the meiosis. This fact is known for a long time, and has been mostly studied under artificial cultures and frequently in relation to the search of a suitable storage method for conserving fern spores.

The perception of this relationship in the green spore species is unambiguous, as there is a drastic reduction of the viability in just a few days or weeks from the meiosis. Among the non-green spore species, the studies of decay in viability related

to spore age are less easy, since the viability can be maintained for a very long period. In consequence, experiments that start from spores collected at the same time should be extended for even years. The reduction in spore viability is measured by the reduction of the germination percentage of spores cultured at different times from the collection. A short way is to use spores from preserved fertile plants of different ages, for example, from herbaria (Windham et al. 1986). This permits a less prolonged experiment by simultaneously culturing spores of different ages. The problem is that results could be somewhat affected by endogenous individual uncontrolled variables, since multiple sporophytes are used. In any case, the preservation method of the spores is critical because it is known that different storage methods affect viability and other spore functional features (Aragon and Pangua 2004; Ballesteros et al. 2006; Pence 2008).

Avoiding the analysis of the storage conditions, which exceed the objectives of this work, two examples can be used to illustrate the effect of age over spore viability. *Pteridium aquilinum* spores viability have been calculated to exceed 10 years, but germination percentages result seriously decreased in a 30% by the 4th month after harvest and by a 50% after around a year (Conway 1949). In *Polypodium vulgare* the reduction of spore viability is less dramatic, varying from ca. 90% of germination percentage in spores recently collected to ca. 55% in 4-year-old spores (Smith and Robinson 1975).

Another phenomenon related with spore age is the delay in the germination times, i.e., the time between release of spore and germination (Fig. 14.1).

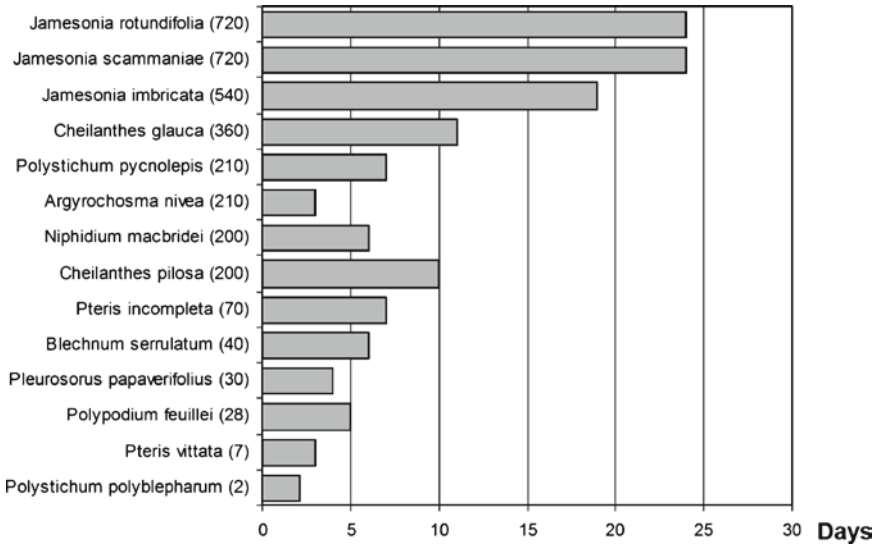

Fig. 14.1 Relationship between age (in parentheses for each species measured in days from collection to sowing) and germination time (days from sowing to first germination) for a diverse number of species. In general, there is a direct positive relation between age and days to germination

14.3.3 Temperature

Temperature is one of the better known external factors to influence spore functionality. There is a quite amount of data stating an unequivocal correlation between environmental temperature and spore germination, because temperature is known to be one of the germination triggering factors that breaks spore dormancy (Bhardwaja and Sen 1966; Raghavan 1989). Some recent studies indicate that germination is optimum within a tolerance range of temperatures, more or less ample depending on the species. In other words, there is evidence in some species for the existence of maximum and minimum temperature limits out of which the spores are unable to germinate or they do it at abnormal rates, including delay in the germination timing (e.g., Pangua et al. 1994; Quintanilla and Escudero 2006; unpublished data) (Fig. 14.2). However, since a delay in the germination timing is not the same matter as a decrease in the viability, difficulties arise to demonstrate a direct relation between temperature and spore viability. Even more, some experiments result in an almost complete recovery of normal germination numbers in spore cultures moved from an environmental temperature of 10°C to a one of 20°C, the latter assumed as the optimal temperature for growing a majority of fern spores. The conclusion is that germination is (partially) controlled by temperature, but not the spore viability.

Nevertheless, a clear relationship between temperature and spore viability could be encountered in assays to prevent the deceased of the spore over the time, i.e., to extend its viability. Much of these experiments are made in a conservational context. Among other techniques, storing spores at very low or freezing temperatures allow to obtain moderate or high germination percentages after a time that exceeds in

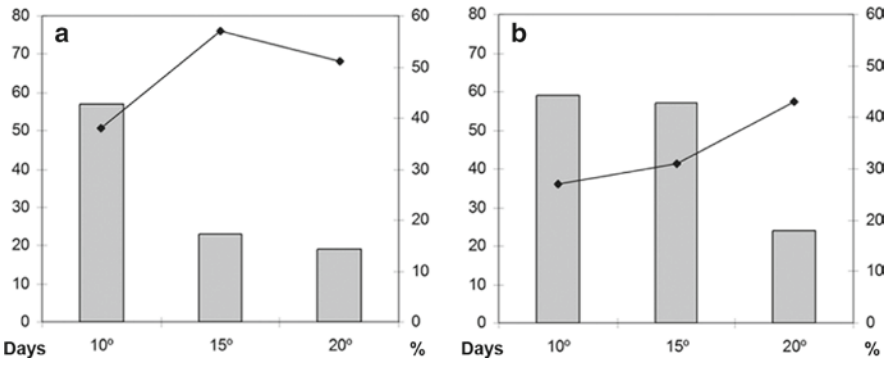

Fig. 14.2 Effect of temperature (in °C) on germination time (columns, measured in days from sowing to first germination) and germination percentage (*lines*) in *Jamesonia imbricata* (**a**) and *Jamesonia rotundifolia* (**b**). There is a clear increase on the germination time when temperature falls and, in general, also a reduction of the germination percentage *Jamesonia rotundifolia* seems to be more sensitive to variation in temperature since the change from 20°C to 15°C implies more drastic effects than in *J. imbricata*

much the natural viability of the species (Pence 2000; Ballesteros et al. 2006; Pence 2008). A remarkable case is that of *Equisetum hyemale* (Whittier 1996). Germination percentage was of ca. 98% with recently collected spores. Spores stored at room temperature were completely inviable even with just 1 month old, but spores stored at −70°C for 16 months yield ca. 28% germination. In conclusion, this method made possible to extend more than 16 times the spore viability of *E. hyemale*.

14.4 Physiology of Fern Spores Viability

The physiology of the spore germination is reasonably studied, although there is much work to be done yet. With respect to the external factors that mediate in the germination, light is by far the best known, including detection, internal signalling and photomorphogenetical responses (Raghavan 1989; Wada 2008). The influence of temperature is also clear but the physiological mechanisms involved have been unravelled to a much lesser extent (Haupt 1992), probably due to a complex interaction with light. The knowledge of pheromones' role is also increasing (Schneller 2008).

All these physiological mechanisms are obviously related to spore viability, because the most clear natural sign of viability is germination. Thus, it becomes difficult to puzzle out which physiological features are of interest to explain viability as a biological process in its own, if it is possible to consider it so. Search should be done in the direction of what factors affect to the establishment, cease and duration of the spore dormancy, that allow to distinguish between spores with short or long viability. In this sense, some research has been done to clarify why green spores present so short viability.

For this task, it seems reasonable to start stating a hypothesis considering the most obvious difference of the green spores, which is the presence of photosynthetically active chloroplasts. When faced with suitable germination conditions in soil or cultures, these spores can rapidly obtain the energy necessary to promote synthetic activities. On the contrary, in non-green spores there is need to previously obtain resources from the hydrolysis of storage material. This fact could probably explain the differences in the germination times of both types of spores (Raghavan 1989).

In *Equisetum*, the short viability has been related to the high respiration rate, water content and catalase (Okada 1929; Hauke 1978), as well as to the lack of a spore wall protective enough against drying (Wollersheim 1957). The water content in green spores is higher that the content in non-green spores: in *Equisetum fluviatile* is about 49% and in *Osmunda* of about 17%, whilst in *Blechnum spicant* and *Pteridium aquilinum*, the content is 4–15% (Gullvag 1968). In addition, chlorophyllous spores have fewer granules of lipids and lipoproteins than non-chlorophyllous (Gullvag 1969; Olsen and Gullvag 1974). These spores are found in an active physiological state of intense breathing in which the storage compounds are used in a short time and, consequently, they lose viability.

Evidence has been presented to show that the effects of age may be partly attributable to the declining ability of the spores to synthesize metabolites essential for germination and gametophyte development.

14.5 Viability Detection Techniques

From the beginnings, fern spores viability has been measured by means of germination percentage, because germination is an easily detected effect of a viable spore. Usually, the measurement is made counting the relative proportion of germinated spores (those in which a rhizoid, sometimes a prothallial cell, is evident) in a random sample of 100 spores. This is a very simple and inexpensive method, but for a majority of species, especially for those with non-green spores, requires a certain period of time: first, there is a time between the sowing and the observation of first germinations; and second, there is a time from first germination to ensure that all or most of the viable quiescent spores that could germinate do so, i.e., to achieve the maximum germination rate. The latter time is greatly variable between species, but for many is in the range of weeks to few months (Fig. 14.3). Usually, an increase in the time to first germination implies an increase in the time needed to

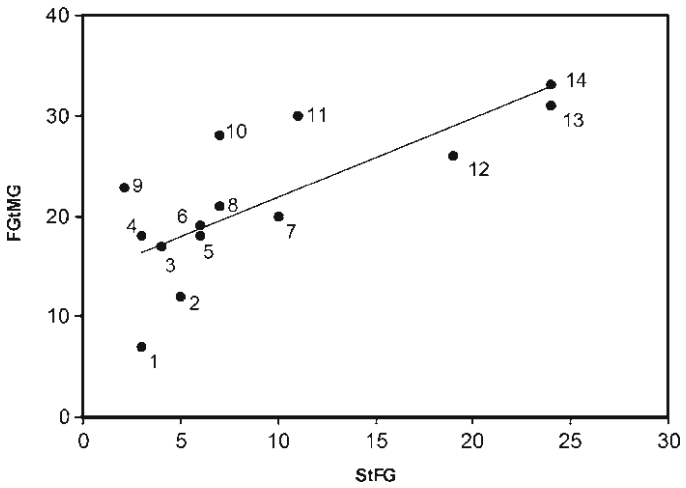

Fig. 14.3 Relationship between days from sowing to first germination (*StFG*) and days from first germination to maximum germination percentage (*FGtMG*) in various fern species: (1) *Pteris vittata*; (2) *Polypodium feuillei*; (3) *Pleurosorus papaverifolius*; (4) *Argyrochosma nivea*; (5) *Blechnum serrulatum*; (6) *Niphidium macbridei*; (7) *Cheilanthes pilosa*; (8) *Polystichum pycnolepis*; (9) *Polystichum polyblepharum*; (10) *Pteris incompleta*; (11) *Cheilanthes glauca*; (12) *Jamesonia imbricata*; (13) *Jamesonia scammaniae*; (14) *Jamesonia rotundifolia*. An increase in the time to first germination implies an increase in the time needed to achieve the maximum germination percentage

achieve the maximum germination percentage, but sometimes there is no such direct relationship: for example, *Cheilanthes pilosa* and *Cheilanthes glauca* showed similar times to first germination, but the latter lasted longer to achieve its maximum percentage (Fig. 14.3). The variation in the time to germinate among a spore population that comes from one or more sporophytes, is assumed to respond to the same individual or intraspecific viability variation yet commented.

The method of measuring viability through germination percentages presents the limitation of the many culture factors that presumably could affect the results (culture medium, temperature, light intensity and composition, pH, etc.). In this sense, another important methodological factor to take into account is the culture spore density, since very high or very low densities affect germination (Ashcroft and Sheffield 2000), thus modifying the evaluation of viability.

Ultimately, some other detection techniques have been proposed, usually based on the capacity of living cells to change the structure of chemicals, yielding some coloured or fluorescent molecules which can be more or less easily detected. One clear advantage is that these methods allow viability estimation in a scale time of hours to few days.

TTC (2,3,5-triphenyltetrazolium chloride) is a redox indicator used to differentiate between metabolically active and inactive tissues and cells, by visually detecting cellular respiration activity. The TTC, which presents a white colour, is enzymatically reduced in living cells or tissues to TPF (1,3,5-triphenylformazan), a chemical form with an easily detected red colour, due to the activity of various dehydrogenases. Thus, in non-living cells or necrotic tissues, in which dehydrogenases enzymes have been denatured or degraded, TPF is no formed and its colour remains white.

The method, more or less modified, has been largely used to detect viability in diverse eukaryotic organisms (Lin et al. 2001), including pollen (Norton 1966). Recently, an adjusted TTC method has been developed for its use in the detection of fern spore viability of *Dryopteris guanchica*, from an environmental toxicity perspective (Catalá et al. 2009). The promising results they achieved open the door to a new, rapid, low cost assay that easily permits the detection of spore viability.

Fluorescein diacetate (FDA), a non-fluorescent derivative of fluorescein, can be used in hydrolysis assays to measure enzyme activity and viability in eukaryotic and prokaryotic cells. Non-specific esterases of living cells will actively convert the non-fluorescent FDA into the green fluorescent compound fluorescein, which, accumulated within cells, allows direct visualization by epifluorescent microscopy. A fluorescent result is, thus, a sign of viability (Steward et al. 1999). The colouring result is strongest when enzymatic activity is greatest, so the method is sometimes suitable for quantitative measurements using a spectrofluorometer. A problem with the use of FDA technique is that some media components, as peptone, tryptone and yeast, and some buffers, as Tris–HCl and sodium phosphate, can promote FDA hydrolysis in absence of living cells (Clarke et al. 2001) thus giving possible false results. For a time, the FDA method has been successfully applied to pollen viability tests (Heslop-Harrison and Heslop-Harrison 1970). Some recent unpublished results are suggesting the validity of the technique for detection of pteridophyte spores viability.

Some other similar chemical methods could be employed as tests of fern spores viability, but to date have not been used for this matter. Among others, we can point out the method called MTT (2,5-diphenyl tetrazolium bromide), that seemed to be most suitable that FDA in testing pollen grains (Vizintin and Bohanec 2004). The MTT technique does not need fluorescence microscopy, which is a clear advantage. A modification of the FDA method is that of SFDA (sulfofluorescein diacetate) which demonstrated to be valuable in detecting living cells within plant tissues (Yamori et al. 2006). Future researches will inform about the applicability of all these techniques to the detection of spore viability.

The nature of the spore wall, which could partly prevent the entrance of external molecules into cytoplasm, is a notable barrier for the implementation of the chemical methods to the study of pteridophyte spores.

14.6 Viability and Conservation

Since spore viability is important for ex-situ conservation purposes, a relatively high amount of studies concerning germination and loss of viability of spores from this perspective have been carried out in the last 2 decades (Pence 2008). Several methods have been assayed to preserve spores of a number of species. Viability of most pteridophyte spores was believed to be retained when stored in dry and cold conditions. However, several exceptions have been found of species that show a higher viability when stored under different conditions (*Cyathea delgadii*, *Psilotum nudum*, *Athyrium filix-femina*, *Blechnum spicant*, *Polystichum setiferum*, *Phyllitis scolopendrium* and *Todea Barbara*, among others). Also of interest is the cryopreservation technique, very useful when dealing with economical interesting plants, as an important component in plant biotechnology programmes.

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Chapter 15
Microsatellites: A Powerful Genetic Marker for Fern Research

Ares Jiménez

15.1 Genetic Molecular Markers

Knowing the genetic configuration of organisms is relevant for many fields of science, from biodiversity conservation to the development of medicines for human diseases, including forensics, taxonomy, evolution, phylogeography, crop enhancement and genetic diversity studies. The most comprehensive and informative method for molecular characterization is complete genome sequencing. However, genome sequencing is still, despite the momentum that bioinformatics and genome technologies are gaining recently, a long and expensive process. Therefore, it appears that sequencing the genome of non-model, non-economically important species is unrealistic even for studies dealing with only one or a few individuals.

Molecular markers are heritable and polymorphic protein or DNA characters which we can use as a tool to explore the genetic diversity within and among organisms. These markers provide a sample of the total genetic variability of each individual under study without the requirement to sequence their whole genome. If variable enough, genetic polymorphisms detected by molecular markers can discriminate among different genotypes, giving us the possibility to assess the degree of similarity or divergence of all individuals under study at several hierarchical levels (intrapopulational, interpopulational, specific, and so on). This discriminatory power serves as the basis of the endless uses of molecular markers in biological studies, such as in population biology, population and metapopulation dynamics, germplasm analysis, differentiation and evolution, relationships among individuals and populations, identification of individuals, genetic mapping, and plant breeding (Bachmann 1994; Powell et al. 1996; Estoup et al. 1998; Chambers and MacAvoy 2000; Sunnucks 2000; Balloux and Lugon-Moulin 2002; Selkoe and Toonen 2006).

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The most popular markers in the pre-PCR years were allozymes and RFLPs (restriction fragment length polymorphisms). Both markers are codominant, that is, each individual of the offspring will inherit one allele of each of its parentals. This type of inheritance allows us to distinguish between homozygous and heterozygous states and can thus give us additional information about the breeding systems of species, inbreeding levels, hybridization frequencies, and heterozygosity.

Although in decline nowadays, allozyme electrophoresis has been a major contributor to our current knowledge of fern evolution, reproduction and population genetics, and numerous examples of the application of the technique to ferns can be found through the literature (e.g. Werth et al. 1985; Soltis and Soltis 1990; Vogel et al. 1999; Quintanilla et al. 2007). The position of allozymes as a big favorite marker was supported mainly by their straightforwardness and comparatively low cost as compared to other alternatives (e.g. Weising et al. 2005). Allozymes have several minor disadvantages, such as a limited number of allozyme loci to study and the requirement of high-quality samples, as damaged tissue may be devoid of enzyme activities (Sunnucks 2000). However, the most important constraint plaguing allozymes is their low sensitivity (Powell et al. 1996; Sunnucks 2000). As allozymes are codified by functional genome regions which are expressed and therefore possibly subject to selection (Koehn et al. 1983; Volis et al. 2003), they are frequently conserved and deleterious mutations are quickly purged. Consequently, allozymes may not be variable enough as to detect genetic polymorphisms in populations with low genetic variation, as frequently seen in clonal, inbreeding or recently founded and bottlenecked populations (e.g. Quintanilla et al. 2007; Jiménez et al. 2009).

On the other hand, RFLPs have been less extensively used than allozymes in fern science, where they have been the marker of choice mostly for genome characterization (e.g. Hasebe and Iwatsuki 1990; McGrath et al. 1994; Nakazato et al. 2006).

The advent of PCR technology (Saiki et al. 1988) supposed a revolution in the field of molecular markers, as it triggered an explosion of new DNA techniques. The most used PCR-based molecular markers have been RAPDs (random amplified polymorphic DNAs), AFLPs (amplified fragment length polymorphisms), SNPs (single nucleotide polymorphisms), and microsatellites. RAPDs were a popular choice during the 1990s (Bachmann 1994; Avise 2004), as they provided a multi-locus, quick and easy way to detect genetic differences among individuals, with no previous requirements of sequence knowledge. However, they have been the target of some criticism, as they are mostly dominant and show a poor reproducibility, which limits them in inter-lab studies (Avise 2004). AFLPs are also a multilocus approach of relative ease that can be performed with limited funds, and counts with the advantage of revealing large numbers of polymorphisms in several anonymous loci, as they provide a number of bands putatively corresponding to alleles (Avise 2004). Therefore, they are a suitable option when wanting to compare genetic diversities among populations. However, as in the case of RAPDs, AFLPs are mostly dominant, so no distinction can be made among homozygous or heterozygous individuals as the output of these techniques is allele presence/absence (Sunnucks 2000; Avise 2004). The nonspecificity of the PCR primers in these techniques is an additional drawback, as sample contamination may lead to the misinterpretation of results (Sunnucks 2000; Avise 2004). Finally, SNP technology is promising as these markers are codominant and

extremely sensitive, but their high cost prevents their application outside model and commercially valuable plant taxa (Weising et al. 2005).

15.2 Microsatellites

15.2.1 What and Where Are They?

Microsatellite markers, also known as simple sequence repeats (SSRs), short tandem repeats (STRs) or simple-sequence length polymorphisms (SSLPs), are tandem repeats of 2–6 nucleotides which are present in most nuclear genomes studied till date (Tautz and Renz 1984; Chambers and MacAvoy 2000; Avise 2004). Microsatellites appeared for the first time in the literature in the 1970s, when Skinner et al. (1974) described a sequence with tetranucleotide repeats in the hermit crab *Pagurus pollicaris*. However, their potential as a PCR-based single locus marker for plant research was not highlighted until two decades later, when Akkaya et al. (1992) described the first locus-specific plant microsatellites in soybean. Since then, an increasingly intense flow of papers reporting plant research using these markers has been published (e.g. Reusch et al. 1999; Boys et al. 2005; Csencsics et al. 2009).

Microsatellites are habitually classified in different categories according to two basic criteria (Chambers and MacAvoy 2000; Weising et al. 2005). Depending on the number of nucleotides constituting the repeat motif, microsatellites can be categorized in di-, tri-, tetra-, penta- and hexanucleotide microsatellites (Table 15.1). On the other hand, on the basis of the regularity of repeat arrays, the most simple microsatellites can be pure, interrupted, compound or interrupted compound (Table 15.1).

Microsatellite polymorphism is based on the length of the sequence where the tandem repeat is contained. Consequently, alterations in the DNA molecule adding or deleting one or more repeat units will originate stretches longer or shorter than the original one, which will be scored as different alleles (Table 15.2). Microsatellite mutation causing polymorphism is considered to occur mostly by DNA polymerase slippage during DNA replication, a phenomenon typical of simple, repetitive sequences (Tachida and Iizuka 1992; Eisen 1999; Schlötterer 2000). Another additional mechanism which can account for microsatellite variation is recombination between DNA strands during cell division (Harding et al. 1992).

Several models of microsatellite evolution have been proposed depending on the number of potential alleles, the probabilities of generating new alleles with different sizes, and the minimum and maximum allele sizes (e.g. Estoup et al. 2002; Sainudiin et al. 2004). However, the microsatellite evolution process seems to be more complex than initially thought. The debate about the exact mechanisms responsible for microsatellite mutation is still open, as no single model completely fits with all microsatellites studied up to date (e.g. Ellegren 2004; Sainudiin et al. 2004; Veytsman and Akhmadeyeva 2007).

Regardless of the mutational model of microsatellites, it is a fact that replication errors causing microsatellite size variability take place frequently. Mutation rates in the range from 10^{-6} to 10^{-2} per locus per gamete per generation have been reported

Table 15.1 Classification of microsatellites

Type of motif	Sequence
Dinucleotide	CTGAGCTGCACCGTGGACCAA**CACACACACACACACACACACAC ACACACACACACA**GATTCGCGGTTAGCG
Trinucleotide	CTGAGCTGCACCGTGGACCAA**CCACCACCACCACCACCACCACC ACCACCACCACCA**GATTCGCGGTTAGCG
Tetranucleotide	CTGAGCTGCACCGTGGACCAA**CAGACAGACAGACAGACAGACAG ACAGACAGACAGA**GATTCGCGGTTAGCG
Pentanucleotide	CTGAGCTGCACCGTGGACCAA**CAGAACAGAACAGAACAGAACAG AACAGAACAGAA**GATTCGCGGTTAGCG
Hexanucleotide	CTGAGCTGCACCGTGGACCAA**CCAGCACCAGCACCAGCACCAGC ACCAGCACCAGCA**GATTCGCGGTTAGCG
Regularity	
Pure	CTGAGCTGCACCGTGGACCAA**CACACACACACACACACACACAC ACACACACACACA**GATTCGCGGTTAGCG
Interrupted	CTGAGCTGCACCGTGGACCAA**CACACACACACA**CTCT**CACACAC ACACACACACACA**GATTCGCGGTTAGCG
Compound	CTGAGCTGCACCGTGGACCAA**CACACACACACAGAGAGAGAGAG AGAGAGAGAGAGAGA**TTCGCGGTTAGCG
Interrupted compound	CTGAGCTGCACCGTGGACCAA**CACACACACACAGAGAGAGAGAG AGAGA**GTGT**GAGAGAGAGA**TTCGCGGTTAGCG

Several categories are established depending the number of nucleotides in the repeat motif and to the regularity of the microsatellite sequence. The microsatellite is marked in bold, and the rest of the sequence corresponds to the flanking regions and to interruptions in the repetitive array

Table 15.2 Microsatellite polymorphisms

Sequence	Allele size (bp)
CTGAGCTGCACCGTGGACCAA **CACACACACACACACACACACACACACACACACACA** GATTCGCGGTTAGCG	72
CTGAGCTGCACCGTGGACCAA **CACACACACACACACACACACACACACACACACACA CACA**GATTCGCGGTTAGCG	76
CTGAGCTGCACCGTGGACCAA **CACACACACACACACACACACACACACACACACACA CACACACACA**GATTCGCGGTTAGCG	82
CTGAGCTGCACCGTGGACCAA **CACACACACACACACACACACACACACACACACACA CACACACACACACACACA**GATTCGCGGTTAGCG	92

The addition of nucleotide repeats to a putative allele results in larger sequence sizes. The microsatellite is marked in bold, and the rest of the sequence corresponds to the flanking regions

through the literature (Jarne and Lagoda 1996; Schlötterer 2000; Zhang and Hewitt 2003). This hypervariability is one of the most relevant traits of microsatellites as a molecular marker. Each locus can potentially have a large number of alleles, thus the combination of just a few loci is often enough for an accurate molecular identification ("fingerprinting") of an individual.

Microsatellites are widespread through the whole nuclear genome of organisms and also in mitochondrial and chloroplastic DNA (Weising et al. 2005). These markers are mostly regarded as selectively neutral (Weising et al. 2005; but see Zhang and Hewitt 2003). The main reason for neutrality is that the mutation of dinucleotide-based microsatellites, which represent the largest proportion of currently described microsatellites (Weising et al. 2005), and also of tetra- and pentanucleotide microsatellites, would cause a frameshift which would be selected against if they had deletereous phenotypic consequences (Field and Wills 1998; Metzgar et al. 2000). Therefore, most of these microsatellites are expected to be located in non-coding DNA regions (e.g. Li et al. 2002; Ellegren 2004). Conversely, tri- and hexanucleotide microsatellites are likely to appear at similar frequencies in coding and non-coding regions because their mutation does not involve a frameshift, which makes them likely candidates to appear in expressed sequence tag (EST) libraries (Pashley et al. 2006; Bouck and Vision 2007).

Regarding the functional role of microsatellites in the genome, it is still unclear. Despite their putative neutrality, there are punctual evidences that microsatellites are involved in, for example, some processes of chromosomal organization, DNA replication, recombination and gene transcription (Li et al. 2002).

15.2.2 How to Use Them?

Many protocols to develop microsatellite markers are available to the scientific community (e.g. Chambers and MacAvoy 2000; Zane et al. 2002; Glenn and Schable 2005; Weising et al. 2005). One of the most popular protocols, although with numerous possible modifications, is microsatellite development from a microsatellite-enriched genomic library, which typically consists of the following steps:

1. DNA extraction from one or a few individuals. This initial DNA will be used to isolate the microsatellites.
2. DNA cutting with restriction enzymes. Cutting the DNA into manageable fragments of 400–800 bp facilitates further steps.
3. Detection of microsatellite-rich fragments. Hybridization of the cut DNA with biotinylated molecular probes consisting of nucleotide repeats and subsequent capture of hybridized fragments with streptavidin-coated beads allows the detection of microsatellite-rich fragments. Although this step is not mandatory, creating a microsatellite-enriched genomic library is convenient as non-enriched libraries result in low efficiencies of microsatellite obtention.
4. Cloning. Microsatellite-rich fragments are transformed into bacterial cultures in order to build the genomic library, which makes available large quantities of each fragment.
5. Positive clone selection and sequencing. The colonies are screened for microsatellite-rich fragments. Those which are positive can be sequenced to obtain the configuration of the microsatellites and their flanking regions.

6. Primer development for PCR amplification. Flanking regions must be chosen to design the primer pair for each microsatellite, which can be done with online, freely available software such as PRIMER3 (Rozen and Skaletsky 2000). These primers will guide the repeated amplification of the microsatellites during the polymerase chain reaction. Short flanking regions may lead to unspecific amplification products, so it is advisable to design primers that are about 20 nucleotides long. Primers should amplify fragments from 100–250 bp to avoid ambiguous allele interpretation.
7. Primer optimization and labeling. Each primer pair must be tested under different PCR conditions (number of cycles, annealing temperature, etc.) until single, sharp bands of PCR products are revealed in agarose gels after electrophoresis. As gel electrophoresis lacks the resolution required to discriminate between alleles with similar sizes, it is convenient to label the optimized primers with fluorescent chromophores to enable the detection of PCR products in an automated genetic analyzer. Using different colored labels facilitates multiplexing (i.e., amplifying two or more microsatellites in the same PCR), thus enabling the simultaneous genotyping of several loci with the subsequent saving in laboratory reagents and consumables.

Once all primer pairs are conveniently labeled and tested, all samples in the study can be processed. Samples usually consist of little pieces of tissue of each individual under study dried in silica-gel, but the high specificity of the PCR allows to use low quality samples such as damaged tissue or old herbarium material. PCR products are run in the genetic analyzer, which translates the electropherogram separation of amplified fragments with different sizes into a peak graph. Some of these peaks are assigned alleles, and individuals can be then genotyped.

As seen above, the development of microsatellite loci is laborious and requires some relatively expensive equipment. A money- and time-saving shortcut for the acquisition of polymorphic microsatellite loci is cross-species amplification (Barbará et al. 2007). The flanking regions of microsatellites are sometimes preserved during speciation events, so microsatellites developed for a given species may be present in closely related species, genera, or even families. Therefore, before starting a microsatellite project on a given species, it is wise to mine the literature and genomic databases such as GenBank for published microsatellite loci developed for related taxa. This approach is especially convenient when studying exotic or endemic species for which developing specific microsatellites *de novo* may result inconvenient. Unfortunately, the success rate of cross-species microsatellite transfer is highly variable among plant taxa (Barbará et al. 2007). The general trend is that the more phylogenetically distant from the focal species, the less likely polymorphism and amplification are (Chambers and MacAvoy 2000). Using cross-species transferred microsatellites has the risk of no amplification due to mutations in the flanking regions (Chambers and MacAvoy 2000). In this regard, EST-microsatellites have greater possibilities for cross-amplification (e.g. Pashley et al. 2006). It must also be kept in mind that microsatellites tend to be shorter in nonfocal species, probably as a consequence of an “ascertainment bias” for long microsatellites during microsatellite development (Weising et al. 2005).

In the case that no microsatellites have been previously developed for the species of interest, new loci must be identified by the procedure described above. Fortunately, having all the laboratory equipment is not mandatory to carry out a microsatellite analysis. With the spread of the technique, a number of companies and research centers have made available the execution of all steps of microsatellite development and even genotyping for reasonable prices and in a few months. Even if it supposes a greater expense than developing microsatellites in the lab, it should be the way to go for research groups not trained in microsatellite development who sporadically need these markers for punctual research.

Finally, choosing the right statistics to analyze microsatellite data is also important. Microsatellite statistics for population genetics are partly shared with those designed for allozymes and will be familiar to those trained in the latter technique, but other statistics specific to microsatellites which consider their mutational processes, such as R_{ST}, have been developed (e.g. Luikart and England 1999; Balloux and Lugon-Moulin 2002). The researcher should thus carefully consider these statistics before analyzing the data. It must also be noted that making comparisons between statistic values obtained for microsatellites and for other markers is not always straightforward, as each marker evolves in a different way. And last, during the design of the study it must be remembered that the high variability of microsatellites may require extensive population sampling to accurately determine allele frequencies (Estoup et al. 2002).

15.3 Microsatellites in Ferns

15.3.1 Previous Microsatellite Works on Ferns

The application of microsatellite technology to fern research started at the beginning of the present decade and, since then, only a few articles have been published. Roughly half of these publications correspond to "primer notes" where microsatellite primer pairs are characterized (Vitalis et al. 2001, 7 loci for *Marsilea strigosa*; Woodhead et al. 2003, 10 loci for *Athyrium distentifolium*; Squirrell et al. 2004, 9 loci for *Athyrium distentifolium*; Kang et al. 2006, 14 loci for *Adiantum reniforme* var. *sinense*; Chen et al. 2008, 8 loci for *Pteridium aquilinum*). The other half of studies report some successful applications of microsatellites to study fern breeding systems and several aspects of fern population genetics such as genetic structure, genetic diversity, and recent demographic and evolutionary processes (Pryor et al. 2001, with the description of 3 loci for *Adiantum capillus-veneris*; Vitalis et al. 2002; Woodhead et al. 2005; Kang et al. 2008; Jiménez et al. 2010, with the description of 8 loci for *Dryopteris aemula*).

Fern microsatellite literature is thus still nascent, but some basic information can already be gathered. Please bear in mind that the three loci developed for *Adiantum capillus-veneris* (Pryor et al. 2001) are not taken into account for these calculations because their repeat motif is not specified. Forty out of the fifty-six microsatellite

loci characterized across the other studies contained pure tandem repeats, whereas 6 of them were interrupted, 7 were compound, and 3 were both interrupted compound. Dinucleotide repeats were the most abundant type with over 50 fragments, followed in decreasing frequency by 5 trinucleotide, 2 tetranucleotide, and 1 hexanucleotide repeats. The most common dinucleotide repeat motif was $(CT)_n$, closely followed by $(AG)_n$; consequently, microsatellite isolation in subsequent studies may find a greater success if their genomic libraries are enriched for these motifs. The number of alleles per locus fluctuated between 2 and 16, and PCR product sizes went from 77 to 263 bp.

Cross-species amplification has been attempted only for microsatellites isolated from *Athyrium distentifolium* (Woodhead et al. 2003; Squirrell et al. 2004) and from *Dryopteris aemula* (Jiménez et al. 2010). Although cross-amplification within the Woodsiaceae family seems fairly successful, microsatellites developed for *A. distentifolium* from anonymous genome regions (Squirrell et al. 2004) showed a more discrete degree of transfer than EST-microsatellites (Woodhead et al. 2003). This difference illustrates the main advantage of using EST-microsatellites, i.e., a greater probability of cross-amplification in other species as compared with anonymous microsatellites (Bouck and Vision 2007). On the negative side, EST-microsatellites are potentially less polymorphic, as expressed sequences are susceptible to be preserved during evolution and some deleterious alleles would be purged (Pashley et al. 2006; Bouck and Vision 2007). Microsatellites developed for *D. aemula* did not show a great potential for cross-family amplification, but did show a remarkable potential for amplification in other *Dryopteris* taxa (Jiménez et al. 2010). What is more, all microsatellite loci tested provided amplification products for almost all hybrids and polyploids containing a copy of the genome of *D. aemula*. This success for amplification in taxa containing at least one genome of the microsatellite focal species may be extremely advantageous for studies on diploid-polyploid fern complexes. Regrettably, only more studies on cross-amplification in these complexes will prove if microsatellite flanking regions are preserved during hybridization and polyploidization processes in all fern clades.

15.3.2 A Particular Study Case: Dryopteris aemula

Jiménez et al. (2010) recently isolated several microsatellites from the diploid fern *Dryopteris aemula* and used them to characterize the genetic diversity of several Iberian and Azorean populations of this species. A previous study (Jiménez et al. 2009) showed a null genetic variation at 13 allozyme loci in Iberian populations of *D. aemula*, so a more powerful genetic marker was needed. We also pursued to know the breeding system of this species, so microsatellites appeared as the most adequate tool to answer our questions. In our case, microsatellites were established by a commercial company, which circumvented all the work from DNA extraction to primer development. If money is not a big constraint but time is, this is the procedure to be taken for researchers not trained in microsatellite development, as the researcher is practically guaranteed to have a set of working microsatellite loci in a few months.

Five polymorphic microsatellite loci were informative enough to reveal that *D. aemula*, a Tertiary relict species, probably found a warm refugium during the Quaternary glaciations in the Azorean archipelago, from where continental Europe would have been recolonized after the ice retreat (Jiménez et al. 2010). Additionally, microsatellites shed light on two completely unexpected phenomena. First, it seems that, despite the potential of ferns to travel long distances by wind spore dispersal, gene flow among *D. aemula* populations is rare. And second, high inbreeding values indicated that outcrossing is uncommon in this species, in contrast to most other diploid ferns (Ranker and Geiger 2008). These interesting traits of the biology of *D. aemula* would have gone unnoticed if we had not gone beyond allozymes, thus illustrating how investing resources in microsatellite studies can be rewarding.

15.4 Advantages and Disadvantages of Microsatellites for Fern Studies

15.4.1 Why to Use microsatellites?

One of the most important advantages of microsatellites is their high variability. In fact, they often reveal the highest levels of polymorphism when compared to other markers (Bachmann 1994; Powell et al. 1996). Therefore, as in the case of *Dryopteris aemula* (Jiménez et al. 2010), they may be of great utility when applied to populations with little genetic diversity difficult to detect with less sensitive markers. This hypervariability is a consequence of their high mutation rates, which makes them particularly suitable to answer questions located in a recent time frame (Chambers and MacAvoy 2000; Selkoe and Toonen 2006).

An additional important advantage of microsatellites is their codominant inheritance, which allows the calculation of the proportion of homozygotes and heterozygotes and the assessment of inbreeding in populations. This is a very relevant feature for biodiversity conservation and management, as endangered species are often threatened by risks such as genetic drift and inbreeding depression (e.g. Ellstrand and Elam 1993; Buza et al. 2000).

Another further advantage of microsatellites is their ease of use once one is familiar with the technique and has the appropriate working primers available. When the primer development stage is over, everything boils down to extracting sample DNA, performing PCRs and running the amplification products in a genetic analyzer. Allele interpretation is also pretty straightforward and should be intuitive to scientists which have previously worked with allozymes (Estoup et al. 1998; Degen et al. 1999).

These traits, together with their putative neutrality, make microsatellites a versatile marker appropriate for describing population genetic structure, performing parentage and relatedness tests, evaluating genetic diversity, and studying recent population history, all of which are important for studying fern biology and designing fern conservation strategies.

15.4.2 Problems and Limitations

Despite its numerous advantages, microsatellite technology also has its weak points. The most immediate one is that applying microsatellites to ferns implies developing them *de novo* for almost every taxon to be studied, as the number of species for which microsatellites have been developed is currently very low. Unfortunately, the only way around it is trying cross-amplification and, as seen above, it may produce poor results. All microsatellite development routines are subject to an "attrition rate", that is, only some of the initial total of cloned sequences will result in useful, interpretable microsatellites (Squirrell et al. 2003). This attrition reduces the number of usable loci at several steps. On average, this attrition rate in plants is about the 83% of the initial total (Squirrell et al. 2003), which is an important matter to consider when starting a microsatellite project. Regarding ferns, it is unfortunate that these plants tend to have large genomes (Nakazato et al. 2008), as microsatellite attrition rate is correlated with haploid genome size (Garner 2002). In addition, it seems that the odds of successful cross-species transfer also decrease with greater genomes sizes (Barbará et al. 2007), which is again a disadvantage when working with most ferns.

Microsatellites also have some intrinsic traits which can prove troublesome for fern research. DNA polymerase slippage during the PCR produces stutter peaks, the most common artifact of the technique (Selkoe and Toonen 2006). These peaks are separated from each other and from the true allele peaks at regular intervals. Stutter peaks can represent a problem for genotyping when they are abundant and alleles in heterozygous individuals are close from each other. In this case, genotyping diploid individuals can become a challenge, let alone trying to do so with polyploids. Allele misassignment can be avoided with careful results interpretation. The peak corresponding to the true allele is frequently the highest one and should correspond to the largest fragment amplified. Also, assigning alleles in troublesome samples can be enhanced by comparing individuals and trying to identify the typical stuttering pattern of each microsatellite locus.

Inherent to microsatellites and their mutation processes is the possibility that alleles with the same size are not identical by descent (Avise 2004; Selkoe and Toonen 2006). This problem, known as size homoplasy, may limit results interpretation and may lead to inflated estimates of gene flow if mutation rates are high (Selkoe and Toonen 2006). Homoplasy should not be a big source of error in population genetic studies, though it could be tricky in studies dealing with highly divergent groups, a feature that limits the utility of microsatellite markers for phylogenetic reconstructions (Selkoe and Toonen 2006).

A last source of concern when planning to study ferns with microsatellites are genome duplications, which is something to bear in mind because polyploidization is thought to have played a major role in fern evolution (e.g. Grant 1981). In polyploid species or in ancient polyploids which have diploidized but still conserve duplicated parts of the genome, duplicated microsatellite loci can be frequent. The amplification of duplicated loci can be easily detected in diploid species if three or more alleles appear in the same locus, but detecting duplications in recent or ancient polyploids can become a challenging task.

Although these limitations may seem overwhelming, they are relatively easy to overcome and will rarely ruin a carefully designed and executed work.

15.5 Future Prospects

15.5.1 Potential Applications

Just a few articles have been published reporting the use of microsatellites in the field of population genetic studies. However, the range of potential uses of microsatellites for fern research beyond population genetics remains largely unexplored. Other applications of this powerful marker for fern research may include, for example, disentangling reticulate hybrid or polyploid complexes, evaluating gene flow and introgression in mixed populations, tracking back mutations in apogamic taxa, studying inheritance patterns from sporophytes to gametophytes, and genetic mapping. It appears that the uses of microsatellites in these plants are only limited by the inventiveness of fern researchers.

15.5.2 Next-Generation DNA Sequencing

The limiting factors when intending to work with microsatellites are mainly, as in most studies, time and money. As seen above, standard protocols to develop microsatellites *de novo* for a new focal species require large amounts of both factors in order to obtain a high number of potentially polymorphic, informative microsatellites. The so-called "next-generation DNA sequencing", which actually includes several different approaches (454, Illumina, etc.), opens the door for massive development of molecular markers in a not very distant future. These techniques can be used to obtain full genomes in relatively short times, which can be subsequently scanned for hundreds of putative microsatellite loci. Of course, primer design and testing will still have to be performed, but this high throughput sequencing technology not only provides many more loci than the ordinary procedure, but also at a much higher speed.

15.6 Conclusion

Fern research clearly needs an update from traditional marker-based studies to the state-of-the-art techniques currently used in other organisms, and microsatellite technology arises as a good alternative to do so. The high resolutive power of microsatellites opens up many opportunities to explore aspects of fern biology which could not be studied with other approaches. Even if developing and using them with no previous experience may be somewhat cumbersome and resource-demanding,

the available examples show that the results are well worth the effort. The rapidly decreasing costs of DNA sequencing and automation of lab procedures further suggest a brilliant future of microsatellites in studies of fern biology.

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Chapter 16
Diversity in Natural Fern Populations: Dominant Markers as Genetic Tools

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Abbreviations

AFLP	Amplified fragment length polymorphism
FIASCO	Fast isolation by AFLP of sequences containing repeats
IRAP	Inter-retrotransposon amplified polymorphism
ISSR	Inter-simple sequence repeats
MSAP	Methylation-sensitive amplified polymorphism
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
REMAP	Retrotransposon-microsatellite amplified polymorphism
SCAR	Sequence-characterized amplified regions
SNP	Single nucleotide polymorphisms
SSR	Single sequence repeat

16.1 Introduction

DNA fingerprinting involves the display of a set of DNA fragments from a specific sample. The use of PCR has allowed the development of a broad variety of DNA fingerprinting techniques. The choice of the appropriate fingerprinting technique will depend, in each case, on the application and the organism under analysis. It is essential to keep in mind that the obtained results will be influenced by the selected technique as each one has advantages and drawbacks. Ideally, a fingerprinting technique should require no prior investments in terms of sequence analysis, primer synthesis, or characterization of DNA probes, being easily transferable from one

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organism to another. The simplicity of the procedures, low cost, and speed are also a plus. The scoring must be unambiguous and susceptible to automatization, and, overall, the obtained genetic profiles must be reliable and reproducible.

DNA fingerprinting techniques in general can be divided on the basis of the ability to distinguish heterozygosis. In dominant markers (RAPD, ISSR, REMAP, IRAP, AFLP, MSAP), the determination of a genetic profile is defined by the detection of a band or peak of a specific size that is scored as allele present (plus/1) or absent (null/0). Changes in DNA sequence and single-base substitutions inducing DNA conformation changes can be detected as shifts in electrophoretic mobility (Orita et al. 1989). So, in the case of diploid or polyploid organisms, a band will be identically scored if it is present in homozygous (1|1) or heterozygous (0|1) state. These dominant techniques are also known as unspecific because anonymous sites are analyzed. The use of universal sets of primers allows multiple band detection in each reaction without previous knowledge of the analyzed genome. However, this a priori advantage is also the cause of their main drawbacks: reproducibility and scoring problems. In contrast, codominant markers (isoenzymes, SNP, SSR, microsatellites; see Chap. 15) are specific as a known site of the genome is analyzed in each reaction. As a range of allelic forms can be determined for each locus, the specific allelic conformation of each individual can be detailed allowing homozygous (AA) and heterozygous (Aa) states to be differentiated. For many applications, particularly in population genetics, codominant markers are more powerful than dominant markers, enabling allele frequencies to be estimated and require smaller sample sizes to achieve equivalent analytical power. To be able to determine the genetic information of a specific site of the genome, previous knowledge of the genome of the species is required. This point used to be a major drawback in the use of microsatellites, as the adjacent areas of the microsatellite need to be determined. In the case of SNPs, the sequencing of each individual was too expensive to be applied as a routine marker. Recently, the increasing number of complete sequenced genomes and the developing of new methods such as pyrosequencing techniques have enabled sequencing, at least, to become increasingly routine and relatively cost effective.

16.2 Why Choose a Dominant Marker?

Although at first sight it might appear obvious that codominant markers are always the best option and it has even been suggested that dominant fingerprinting will become obsolete, Meudt and Clarke (2007) predicted that multilocus fingerprinting techniques will remain useful for the immediate future. Many biologists work on species that present low priorities for whole genome sequencing (e.g. noncommercial species) and, therefore, low-cost techniques that require no prior sequence information, such as AFLP, are particularly attractive. In addition, DNA sequencing and assembly of complex, large, polyploid, and/or repetitive genomes is still technically difficult. Pteridophytes show great genome complexity and high frequency of

polyploid species. The polyploidy confers several advantages at the molecular and population level, including reduced inbreeding depression and an increase in heterozygosity and selfing rates. Soltis and Soltis (2000) revised the estimates of the frequency of polyploidy in plant species, reporting a value for angiosperms of nearly 50% (ranging from 30–35% to 80%) and an even higher one in Pteridophytes. The average chromosome number in homosporous ferns was estimated to be $n = 57.05$, as opposed to $n = 15.99$ in angiosperms. Angiosperm species with chromosome numbers over $n = 14$ are generally considered to present polyploidy. If this rule were applied to homosporous ferns, it would include 95% of the species. Isozyme studies showed that despite their high chromosome numbers, homosporous ferns with the lowest chromosome number in their genus ($n = 27–52$) have gene expression patterns typical of diploid angiosperms (revised in Nakazato et al. 2006). Three types of polyploids with different behavior in the genetic heritage due to the origin of the additional genomes could be distinguished (Stebbins 1947). Allopolyploids result from the combination of divergent parental genomes and are characterized by fixed (nonsegregating) heterozygosity. Autopolyploids' genomes belong to the same species so are characterized by polysomic inheritance. Finally, segmental allopolyploids are produced from parents with some chromosomal regions homologous and others are homoeologous, showing complex heritage patterns. The complex meiosis caused by polyploidy is often associated with the defining features of neospecies as can cause reproductive isolation and morphological differentiation. Otto and Whitton (2000) reported that ploidy changes may represent from 2 to 4% of speciation events in flowering plants and 7% in ferns. Recently published data combining information from cytogenetic and phylogenetic databases have established that 15% of angiosperm and 31% of fern speciation events are accompanied by ploidy increase (Wood et al. 2009).

16.3 Measuring Molecular Diversity in Complex Genomes

Molecular markers can provide solid probes on the origin of polyploids. Jimenez et al. (2009) reported that the Iberian fern *Dryopteris corleyi* was originated by allopolyploidization from the diploids *D. aemula* and *D. oreades* using 10 enzymatic systems. The zymograms defined a unique genotype for *D. aemula* across the three populations sampled all along the North of Spain; two genotypes for *D. corleyi* and *D. oreades* harbored a moderate genetic diversity within its populations. Isozymes are codominant markers that are easily transferred from one specie to another, and their use is invaluable in the advance of Pteridophyte population genetics [e.g. (non exhaustive) Hooper and Haufler 1997; Pajarón et al. 1999; Herrero et al. 2001; Hunt et al. 2009; Jimenez et al. 2009]. Isozymes have some drawbacks like a low number of available loci and, usually, low diversity. So, in some cases, isoenzymes fail in detecting sufficient variation within a species to allow studies at the population level (Murphy et al. 1996). One example of this potentially low diversity was reported for the angiosperm *Tragopogon mirus*, which is a 4n species derived from the 2n species

T. dubius and *T. porrifolius.* Cook et al. (1998) using RAPD markers, dominant and unspecific, were able to find variability and identify a distinct population origin among individuals isozymically identical. Five populations with isozyme multilocus "genotype 1" and two populations with isozyme "genotype 2" were detected. The intra-specific variation caused by polyploidy can, sometimes, be masked by the technique selected to measure the genetic diversity of a population.

Microsatellite markers offer an alternative to isozymes for Pteridophyte population studies due to their higher variability. In the case of *Marsilea strigosa,* lack of isozyme variation at 12 putative loci was reported for the French and Spanish samples analyzed (Vitalis et al. 1998), but microsatellite analysis succeeded in detecting variability among the same populations, concluding that *M. strigosa* reproduces predominantly through selfing. Also, a very high population differentiation at the Mediterranean scale was reported indicating that gene flow was highly restricted (Vitalis et al. 2002). In addition to higher variation rates than isozymes, SSR have the advantages of a comparatively straightforward interpretation and automatization. Some of the common problems associated with the use of microsatellites are the presence of null alleles (failure of amplifications due to changes in the annealing site of the primer) or extra bands (additional annealing sites). Both problems alter the measured genetic parameters, especially the values of heterozygosity and can be more problematic in the case of complex genomes. Microsatellites have low transferability between species, and in some cases, lack of correct amplification is detected even among subspecies (Peredo et al. 2010). So, prior analysis of the genetic structure of a species using specific markers is imperative to be familiar with the expected ploidy and the origin of the genomic doses. In case of allopolyploids, data of the behavior of each locus in the parental lines are crucial. But even if a correct amplification is determined, it is needed to keep in mind that allopolyploid formation may be associated with rapid and extensive genomic changes (Feldman et al. 1997), so the possibility of changes affecting the SSR is a reality. The distinct genomes forming an allopolyploid interact extensively through a variety of molecular mechanisms including inter-genomic concerted evolution of divergent sequences (Volkov et al. 1999) and inter-genomic exchange of chromosome segments (Parokonny and Kenton 1995). Using ISSR in the 23 species of the complex *Aegilops* (Poaceae), Gong et al. (2006) detected that the genome constituents of allopolyploid species changed greatly through evolution compared with their ancestral diploid species. However, the variation was not homogeneous; one specific genome, U, showed minor alterations and others suffered different levels of changes. The direction of the hybridization might also have a specific effect on the stability of the genomes. Song et al. (1995) observed that paternal genomes showed more alterations than maternal genomes in the genus *Brassica* polyploids, while Gong et al. (2006) reported that some maternal genomes displayed more alterations than paternal genomes in allopolyploids of *Aegilops*.

As dominant marker systems are based on frequent target sequences present in all genomes, they produce numerous genome-wide di-allelic loci that are individually less informative than microsatellite loci but derive their statistical power from

their sheer number (revised in Meudt and Clarke 2007). Woodhead et al. (2005) performed an explicit comparison of the utility of EST-SSR, SSR, and AFLPs in the fern *Athyrium distentifolium*. The large numbers of dominant markers available for AFLP outperformed smaller numbers of hypervariable codominant markers (SSRs) for population determination. AFLP correctly assigned 98.7% of individuals to their source population compared to 82.7% for the combined 18 SSR loci. We agree with the authors considering that the AFLP approach should not just be considered a "poor second best" when time prevents SSR primer isolation (Woodhead et al. 2005). Unfortunately, we also agree with the difficulties in adding new data into one's own data sets (e.g., equipment or chemical changes) and in the huge problems of mixing AFLP data from different groups.

16.4 Critical Steps in AFLP Protocols

The reproducibility of the AFLP data, even in the same laboratory, highly depends on the setting up of the protocols. The AFLP protocol was first defined by Vos et al. (1995) as a technique in which sets of restriction fragments will be visualized by PCR without knowledge of nucleotide sequence. AFLP permits the detection of restriction fragments in any background or complexity, including pooled DNA samples and cloned (and pooled) DNA segments. AFLP markers are considered to be far more reliable than other unspecific markers such as RAPD [see Park et al. (2009) for a recent comparison among methods], but there can be problems in reproducibility. A complete and recent revision of the most important steps to complete in order to get reliable data can be found in Meudt and Clarke (2007), but in brief, the setting of the protocol must include:

An appropriate DNA extraction method: 100–1,000 ng of high molecular weight DNA (not degraded) free of contaminants is needed. Samples obtained from different extraction methods might lead to experimental mistakes. Vos et al. (1995) demonstrated that AFLP procedure was insensitive to the template DNA concentration, although aberrant fingerprints were observed at very high template dilutions.

Restriction enzymes: The fragment range produced should be appropriate for amplification and electrophoresis (100–1,000 bp). Two restriction enzymes are used, including a low cut frequency enzyme (EcoR I) and a high frequency one (Mse I). Some alternatives to these enzymes (e.g., Pst I, Msp) can be methylation-sensitive enzymes, which can be interesting for other techniques such as MSAP but can affect the AFLP profiles.

Choice of selective primers: The number of selective nucleotides on the selective primers should be increased with increasing genome size so that the number of fragments is high enough to maximize resolution but low enough to minimize homoplasy. Also, not all the primer combinations will produce clear and easy-to-score profiles (e.g., low quality peaks).

Optimization of digestion, adapter ligation, PCR reactions, and electrophoresis conditions: Due to the sequential nature of these steps, the performance of all of them should be accurate to avoid problems that will lead to low quality fingerprint patters. A careful check of chemical concentrations, reaction protocols, incubation times, and dilutions is usually needed.

Fixing strict criteria for data scoring: Several separation (acrylamide gels, automatic sequencer) and visualization methods (silver staining, radioactivity, fluorescence) are available for AFLP. In each case, a broad number of DNA loci will be scored but problems related to "noise" or ambiguous bands will come across the process. Some grade of scoring mistake is inevitable, but following fixed criteria in all the scoring processes will reduce it to the minimum.

Establishment of internal controls: Replicates are the only objective measure of quality of the AFLP data. Duplicate profiles from separate DNA extractions of a single individual should be generated for at least 5–10% of all samples. Duplicates should follow all treatments (DNA extraction, digestion, ligation, amplifications, electrophoresis, visualization, and data scoring) and should be analyzed for each primer combination. Using replicates, the usual error rate has been estimated between 2–5%.

16.5 Measure of Genetic Diversity Using Dominant Markers in Natural Fern Populations

One problem of capital interest in any sort of life form is the measure of genetic diversity present in a species or population. Genetic diversity is the result of the evolutionary history including recent processes such as habitat fragmentation or reduction, which might lead to a decrease in the levels of genetic diversity, reducing the potential of species or populations to survive. The major habitats of Pteridophytes are wet and seasonably mild mountains and uplands of tropical and subtropical regions. Up to 3,000 species of ferns had been described in tropical America with a level of endemism of 40%. The Asia-Malaysia region includes 4,500 species, with the higher numbers of species per unit area in islands adjacent to continental Asia. In addition to the well-known conservation interest of endemic species and the sensitivity of island ecosystems to human actions, Pteridophytes are of special interest in terms of conservation due to the phylogenetic relations among taxa. Pteridophytes can be grouped in around 45 taxonomic units, of which nearly half include less than 50 species and 25% less than 10 species. Besides, the majority of these groups are regarded as primitive and not closely related to other groups (Given 1993).

Prior to any conservation actions, the possible taxonomic uncertainties in the species of interest need to be clarified. The use of molecular markers can reveal the presence of differentiate manage units, or by the contrary, state that two apparently distinct units are indistinguishable from a genetically point of view. The New Zealand fern species *Asplenium hookerianum* and *A. colensoi* are defined by the morphology of the pinnules: broad (*hookerianum*) and narrow (*colensoi*). Using the

morphology of the pinnules, sequences of chloroplast and AFLP markers, Perrie and Brownsey (2005) reported that although the morphological data were consistent with the idea that two species may be present, the molecular AFLP fingerprinting and chloroplast *trnL-trnF* DNA sequence data sets did not indicate a real separation between species, as the samples of *A. hookerianum sensu lato* were not grouped by pinnule morphology. In fact, the site of origin, irrespective of pinnule morphology, provided a much better explanation of the genetic variation present in the analyzed individuals. According to the authors, the recognition of *A. colensoi* as an independent species was not supported. Korpelainen et al. (2005) analyzed the genetic variability of several species of the homosporous fern *Adiantum* for which there is no comprehensive classification of the genus available yet. For the genetic study, the sporophyte collection was conducted at nine sites at the Kothayar Hills (South India) along a 35 km long transect and included two sites of *A. hispidulum* ($n=171$), two of *A. incisum* ($n=30$), four of *A. raddianum* ($n=114$), and three of the rare in the area *A. zollingeri* ($n=29$). Using ISSR, the authors concluded that the populations of *Adiantum* in the area possessed considerable level of genetic variation and the major amount of variation was detected at the population level (71.4%), while only 18.5% of the genetic variation was attributed to differences among the four species analyzed. However, the genetic data supported the idea of four separate taxa and, according to the authors, provide no support for hybridization events.

In addition to clarifying taxonomic uncertainties, dominant markers can also be applied for measuring genetic diversity. The genetic diversity determines the response to long- and short-term environmental changes and overcomes stochastic factors that could otherwise result in extinction. We selected AFLP as an appropriate method for evaluating the genetic diversity and structure of Cantabrian coast populations of two fern species, the outcrossing *Blechnum spicant* and the apogamic *D. affinis ssp. affinis*. The results indicated that the genetic diversity could be cataloged as very low in the case of *D. affinis* and medium in *B. spicant*. Most of the genetic variance detected in *D. affinis* was attributed to differences among localities indicating high fixation of alleles within each locality. Although two differentiated genetic groups were detected, further analyses are needed to explain this distribution: one cluster included the populations in both limits of the analyzed area while the second population is in the center. In *B. spicant*, we were not able to detect any cluster structure suggesting a continuous genetic flow among the localities. Two of the sampled sites were shared by both species while the third was different in each case, so it is feasible assuming that both species share the abiotic and human perturbations of environment. Our results seems to confirm the importance of the reproduction system in the genetic diversity present in populations, making it essential to carry out special conservation actions on *D. affinis*, as its low genetic variability can make it more susceptible to environmental changes.

Once the genetic diversity within and among populations of a species has been described, the demographic fragmentation can be established. Other decisions can lead to finally avoid the risk of local extinction, such as the selection of appropriate genotypes for introduction in order to maximize genetic diversity without altering the genetic diversity of a population. In the case of the giant fern *Angiopteris chauliodonta,* which is almost certainly one of the rarest *Angiopteris* species both in

terms of its distribution (only Pitcairn Island) and reduced population size (147 mature adults in 1997), a decline has been reported by several of the islanders. Probably, it is caused due to several factors: forest clearance, exploitation, invasive species, substrate erosion, and reduced reproductive potential. So, for its survival *A. chauliodonta* required both in situ and ex situ conservation actions. In situ conservation was carried out in protected areas that were selected to maintain some of the best habitats containing native vegetation and should include most genetically diverse and representative populations. Kingston et al. (2004) reported the existence of six localities in the island and measured the genetic diversity of all of them using RAPD in order to define the genetic structure of the species and the most appropriate conservation actions. The authors reported high within-population diversity, not showing fragmentation, which is common in other rare plant species. The lack of population differentiation is probably caused by the small total area covered by the species and the short distance between the localities, which allows gene flow. One potential reserve area, including the three largest and most genetically diverse populations, was defined with the aim of maintaining viable levels of genetic diversity. The authors proposed conservation actions based on the genetic data consisting in the generation of new populations by mixing individuals from several existing populations as the existing populations are not genetically isolated. These actions are completely different to those proposed for *Dryopteris cristata* or the critically endangered *Isoetes sinensis,* and *Sticherus flabellatus*. Landergott et al. (2001) analyzed 14 populations of the allotetraploid fern *Dryopteris cristata* ($2n = 164$), using RAPD, in the southwestern border of its European distribution (Switzerland, Liechtenstein, and Germany), where it is rare and endangered; they reported that the genetic diversity of the species is extraordinarily low, detecting only 27 multiband phenotypes in 280 individuals. The authors proposed *D. cristata* might be the result of a single or very few colonization events of Central Europe accompanied by considerable inbreeding, maybe caused by intragametophytic selfing, leading to the production of completely homozygous sporophytes. A high degree of differentiation among the sampled populations was detected in the genetic analysis, rejecting the spore dispersal over long distances. According to the molecular data, in western Switzerland there are two genetically distinct populations of *Dryopteris cristata,* so, for the maintenance of regional genetic diversity it would require the conservation of both pairs of populations. Kang et al. (2005) used AFLP to investigate the genetic variation and population structure of seven extant populations of the tetraploid *Isoetes sinensis.* The estimated genetic diversity at the population level was relatively high, according to the authors, probably due to the allopolyploid origin of the species. This implies that the reduction in population size was a relatively recent event and may not have sufficient time to result in a decrease of the intrapopulation genetic diversity. So, *I. sinensis* populations appeared to maintain adequate genetic diversity in terms of neutral genetic variation. Further statistical analysis indicated that a large proportion of genetic diversity was attributed to geographic isolation, suggesting a highly restricted gene flow between and a high degree of differentiation among populations. This absence of gene flow together with the small population sizes, might lead to a rapid genetic erosion due to the genetic drift and increase the extinction risk of local populations. So, a genetic reinforcement measure by translocation

is one of the proposed methods to rehabilitate this species. The author signaled the risk of inter-population outbreeding depression due to the high genetic differentiation detected that could have been caused by different environmental adaptations with implications in the evolutionary history of each population. *Sticherus flabellatus* is an Australian fern of special interest for the horticultural industry. During recent years, the market demand has been fulfilled by bush-harvesting natural populations. Keiper and McConchie (2000) reported a high level of population fragmentation in *S. flabellatus* detected by AFLP analysis. The author analyzed eight natural populations, three of them within minimally disturbed habitats and five in the area usually harvested for the Sydney flower markets. Populations could be grouped according their genetic diversities: a first group included two populations of the non-disturbed habitats that showed the highest levels of diversity (0.10–0.11), a second group included populations with medium values (0.06–0.07), and a third group was formed by two populations with extremely low levels of diversity (0.03–0.04). Differences among populations were detected and two different clusters were reported: one had a moderate level of variation but the second included low genetic diversity. The authors suggested that to retain existing diversity, emphasis should be on conserving as many populations as possible. Genetic diversity could partially be restored to depleted populations through the introduction of individuals carrying novel genes but, as in the case of *Isoetes sinensis,* special care must be taken to avoid reductions in overall population fitness through the introduction of genotypes that have evolved under widely different selective regimens.

The pressure exerted by the environment, which selects some genotypes presenting advantageous features in each habitat, is another important factor affecting the genetic diversity. In some cases, the adaptation is not caused by genetic differentiation of the individuals but by the phenotypic plasticity of the species. It is assumed that Pteridophytes display strong habitat fidelity, being one of its most important components the critical stage of gametophyte which is most likely to be vulnerable to stress (Given 1993). However, there are fern species that grow in a range of different habitats and might have developed adaptive strategies suited to each particular habitat. Molecular markers can also help to determine whether the adaptation to a specific habitat has a genetic origin or if it is caused by phenotypic plasticity. In a recent work, Schneller and Liebst (2007) used dominant markers to test the hypothesis of genetically determined adaptations caused by environmental differences along an altitudinal gradient. The authors analyzed using several markers (phenotypes, isozymes, and RAPD) Swiss populations of *Athyrium filix-femina* ($2n=40$), a fern widely distributed in Europe and Northern Asia, in an altitudinal cline of 1,400 m and in a geographic range of 100 km. It was reported that the genetic variability was mainly found within populations with no correlation along the altitudinal gradient or distance. This lack of genetic and phenotypic differentiation reflected a massive gene flow caused by the long-distance spore dispersal, characteristic of many homosporous fern species. The authors concluded that due to this effective gene exchange, the local genetic adaptations to the strong differences in ecological conditions across an altitudinal range were less probable. However, genetic adaptation, when comparing populations over a larger distribution area in which the isolating mechanisms due to limits of spore dispersal are evident,

was successfully detected by molecular markers: Italian populations were less different from the Swiss than the more distant Spanish ones.

Once divergent populations are present in a species, reproductive barriers can appear and lead to speciation events. Genetic mapping of hybrid incompatibilities has provided insights into the number, location, and effect of the genetic factors underlying intrinsic postzygotic reproductive barriers. One sensitive assay for determining the loci underlying reproductive barriers between parental populations is the study of the transmission ratio distortion (TRD) of marker loci in segregating hybrid populations. TRD has successfully proven the existence of strong, but incomplete, reproductive barriers between two divergent races of the homosporous fern *Ceratopteris richardii* ($n=39$). Nakazato et al. (2007) combined spore germination rates, QTL analysis, and transmission ratio distortion (TRD) of RFLP, AFLP, and isozyme markers distributed across the genome on the basis of hybrid populations. A substantial proportion (26.89%) of markers showed a significant deviation from the neutral expectation of 50%. Further statistical analysis of linkage-group-wide TRD patterns indicated that 31 of 41 linkage groups (75.61%) showed evidence of at least one TRD locus, indicating that genetic factors contributing to reproductive barriers are common and scattered throughout the genome. For the study, the authors utilized the linkage map of the specie developed using molecular markers and double-haploid lines. Nakazato et al. (2006) signaled that linkage mapping with double-haploid lines does not involve repulsion phase, and therefore dominant markers such as AFLP provide the same power as codominant markers.

PCR-fingerprinting methods are also valuable tools for diversity and classification studies of symbiotic organisms of Pteridophytes helping to clarify the co-evolution between, for example, the cyanobacteria and its corresponding host, the aquatic fern *Azolla*. PCR tools provided data on the taxonomy of cyanobacteria that had been difficult to generate by other classification methods and these may also be useful for the taxonomy of *Azolla.* Zheng et al. (1999) reported specific fingerprint patterns from the cyanobionts associated with the different *Azolla* species using short tandemly repeated repetitive (STRR) sequences. Individual fingerprints were obtained from all cyanobacteria isolated from the different *Azolla* species. The specific band patterns of the isolates were characteristic and allowed grouping the cyanobacteria in three groups, which were determined by the host species. One cluster included the cyanobacteria obtained from *Azolla* species from section *Euazolla*, another from section *Rhizosperma,* and a third one from *Azolla filiculoides*. Sood et al. (2008), using the same technique, reported the genetic distinctness of these *Azolla* cyanobionts when compared to cyanobacterial strains of the genera *Anabaena* and *Nostoc*.

16.6 Perspectives: Dominant Markers as an Effective Way to Develop Specific Markers

Although unspecific dominant markers have proven their efficiency in detecting genetic diversity and have successfully identified population fragmentation in many species, their drawbacks such as adding new data to data sets are still a major

problem. However, dominant markers have proved to be a successful method to generate specific markers that can be adapted to a species of which there is no or limited genomic knowledge. A number of methods have been recently used to identify microsatellite markers including the Fast Isolation by AFLP of Sequences Containing repeats (FIASCO) approach (revised in Zane et al. 2002). The FIASCO protocol is based on the AFLP method (restriction, adapter ligation, and PCR amplification) so it has smaller genomic DNA (250 ng) requirements than other SSR-generation methods. The total DNA is restricted with only one enzyme (e.g., *Mse*I), and after the adapter ligation a PCR reaction is carried out to amplify all fragments flanked by *Mse*I sites. To obtain a library enriched in microsatellite regions, methods such as streptavidin-coated magnetic particle beads can be applied. As a whole, FIASCO significantly simplified cloning, transformation, and colony selection prior to SSR identification. Eleven polymorphic microsatellite markers were identified in the endangered shrub *Ammopiptanthus mongolicus*, 24 SSR in the sacred lotus (*Nelumbo nucifera* Gaertn.), and 47 polymorphic perfect SSR primers in allopolyploid white clover (*Trifolium repens* L.) ($2n=4x=32$) (Zhang et al. 2008).

Unspecific methods require high quality DNA, not degraded, to successfully obtain profiles, so that old specimens cannot be included in the studies. The use of specific markers allows us to include these invaluable materials that provide information about the past history of the species. ISSR-based methods can also be used as search tools for variable genetic elements, including microsatellites (Korpelainen et al. 2007). Isolated bands from ISSR fingerprinting can be characterized by sequencing and specific primers designed in order to obtain SCAR markers. Using this method, Korpelainen and Pietiläinen (2008) defined primers for the study of short DNA regions in fern specimens from herbaria that were up to 100 years old. The authors reported low diversity in the Northern European *Blechnum spicant*, although they were able to detect several genotypes among the analyzed samples. One group represented a prevalent genotype that was widely distributed and was also detected in present populations in eastern Finland. The southwestern and northwestern herbarium plants formed distinct groups due their genetic differences. The molecular data provided by the existing herbarium collections are unquestionably useful resources for evolutionary and population studies.

16.7 Conclusion

Dominant markers are useful tools to answer genetic questions concerning diversity, reproductive isolation, or conservation actions. Undoubtedly, working with unspecific markers has disadvantages and it is essential to assume the weak points of each technique to obtain the best results possible. Resource restrictions that include both economic and those related to genomic knowledge have been two main reasons for their present application. These resource restrictions together with new functions like economical ways of generating specific markers will, in our opinion, make dominant markers occupy a frontline position in answering important scientific questions, especially in non-model organisms, such as ferns.

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Part III
Environmental Biotechnology: Ecotoxicology and Bioremediation in Ferns

Chapter 17
Mitochondrial Activity of Fern Spores for the Evaluation of Acute Toxicity in Higher Plant Development

Myriam Catalá, Marta Esteban, and Luis García Quintanilla

17.1 Introduction

Ecotoxicology can be described as a vast and complex discipline that employs various approaches for investigations, including residue analyses, field surveys, and toxicity testing (Wang and Freemark 1995). In order to evaluate the real effect of chemicals on the environment or the efficacy of decontamination technologies, chemical quantifications must be completed with toxicological studies on ecologically relevant organisms (Smolders et al. 2004; Dorne et al. 2007). Higher plants are an essential part of a healthy and balanced ecosystem, and new plant models are essential in the evaluation of potential impacts of chemicals on nontarget species (Gong et al. 2001). Many important environmental legislation and guidelines developed under different authorities during the past 30 years have included phytotoxicity tests as a part of chemical safety evaluation (FDA, USEPA, OECD, Environment Canada). Recently, the EU has produced an ambitious regulation that requires the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH). REACH requires the submission of safety data in support of the protection of human and environmental health of all chemicals produced or imported above 1 ton (around 30,000 substances).

Besides chemical safety evaluation, phytotoxicity testing is also warranted in the environmental assessment of complex effluents or contaminated sites. Toxic components that may be practically nontoxic to fish, crustaceans, and daphnids can injure and kill aquatic or riparian vegetation endangering a whole ecosystem. Algal toxicity has often been used as a surrogate for higher plant toxicity testing. However, the sensitivity to toxicants of algae and plants is qualitatively different. For example, algae are more sensitive to metal ions but less sensitive to herbicides than vascular plants (Wang and Freemark 1995).

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17.2 Characteristics of Toxicity Tests

Landis and Yu (1998) in their classic *Introduction to Environmental Toxicology* already point out that one of the most crucial aspects of a toxicity test is the suitability of the test organisms. Toxicity tests, they argue, are performed to gain an overall picture of the toxicity of a compound for a variety of species. They listed some of the criteria when choosing a species for a toxicity test:

1. The test organism should be widely available through laboratory culture, procurement from a hatchery or other culture facility, or collection from the field.
2. The organism should be successfully maintained in the laboratory environment and available in sufficient quantities.
3. The genetics, genetic composition, and history of the culture should be known. However, the majority of toxicity tests in environmental toxicology are conducted with organisms of unknown origin or field collection.
4. The relative sensitivities to various classes of toxicants of the test species should be known relative to the endpoints to be measured. This criterion is not often realized in environmental toxicology. The invertebrate *Daphnia magna* is one of the most commonly used organisms, yet only the results for approximately 500 compounds had been published by 1999. In contrast, by that year, already 2,000 compounds had been examined using the Norway rat as the test species.
5. The sensitivity of the test species should be representative of the particular class or phylum that the species represents. Again this is an ideal criterion, not often met in the case of most test species. The limiting factor here is often the lack of information on the sensitivity of the organisms not routinely used for toxicity testing.

Test protocols of most terrestrial plants are developed for crop species (cabbage, lettuce, oat, ryegrass, etc.) and evaluate seed germination, root elongation, and early seedling and vegetative growth. These tests require special facilities, like greenhouses, and the measurement of the aforementioned endpoints is laborious and cannot be easily automated resulting in high costs. This is an important drawback for extensive use in toxicology screening of new substances by the chemical industry or environmental monitoring of contaminated sites or discharges.

17.3 Current Challenges of Ecotoxicology

Ecotoxicologists express several concerns about standard phytotoxicity bioassays: low number of represented taxa, low ecological relevance, and low sensitiveness of used endpoints. The use of an adequate range of taxa is a key point for the achievement of ecologically relevant results, and regulatory agencies are increasingly more interested in requiring nontarget phytotoxicity testing. The selection of more ecologically relevant species and robust test endpoints remain to be important

challenges for ecotoxicologists. Moreover, the sensitiveness of a species to a given toxicant may vary along the life cycle. The occurrence of a toxic stress during the critical stages such as development may cause the failure of the organism to mature correctly and a delayed death or infertility. The evaluation of lethality as unique endpoint has also aroused criticism. The use of biological markers (biomarkers), as for human health, could make evident early deleterious effects leading to death or biological inefficacy of the organism. Biochemical and physiological biomarkers are especially adequate for automation, which is an important goal for massive bioassay application in toxicity screening or environmental monitoring.

Environmental monitoring and assessment, therefore, faces at present the challenge to develop new tools, more sensitive and reliable, with increased biological and ecological relevance than those currently used, able to detect, in a cost-effective way, early impacts before an irreversible disturbance of the ecosystem may occur.

The respiratory chain, that takes place in mitochondria, is the main energy source in eukaryotic cells. Alterations in this process can yield important consequences and any toxicant affecting mitochondrial activity and cell energy budget will alter normal plant development, even jeopardizing plant survival. Measurements of reduction rates of the respiratory chain can be employed as viability assays in eukaryotic cells. The reduction of tetrazolium salts to water-insoluble colored formazan salts by the respiratory chain has been used in the last 50 years for this aim. Several tetrazolium salts are available for this assay. The reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to the corresponding triphenyl formazan salt (TPF) has successfully been used in the analysis of the viability of seeds or vegetal cells since 1951 (Smith 1951; Kalina and Palmer 1968).

17.4 Ferns and Ecotoxicology

Ferns are important components of numerous plant communities. With about 9,000 living species (Smith et al. 2006), ferns are the second largest group of vascular plants. Fern spores, and spore-developed gametophytes, have long been recognized as useful models for plant research in important areas, namely: plant development, sex determination, gamete production and fertilization, response to environmental factors, and evolution of plant complex traits (Banks 1999). One of the main advantages of this model is its naturally miniature size. Fern spores are single meiotic cells, which develop into gametophytes, which are miniature structures (Fig. 17.1). In this case, the use of microtubes and microplates is imposed by the natural model. The development of rapid and reliable methods to test phytotoxicity with fern spores and gametophytes could dramatically reduce standard tests costs, maintaining the biological relevance of whole plant testing.

We have recently published the first bioassay of acute phytotoxicity based on fern spores (Catalá et al. 2009). The tetrazolium salt we used was TTC. The detailed scheme of the assay protocol is described in Annex 17.1. Here we will

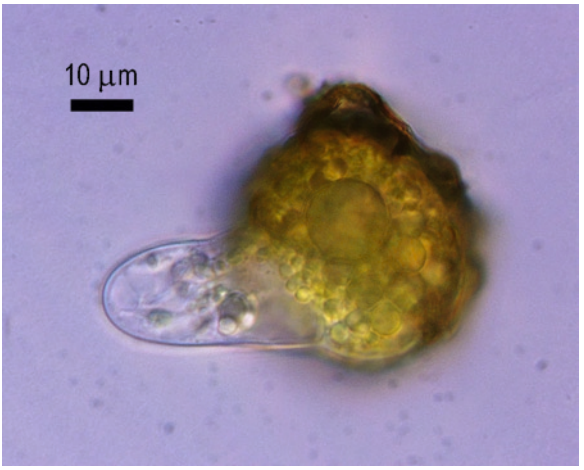
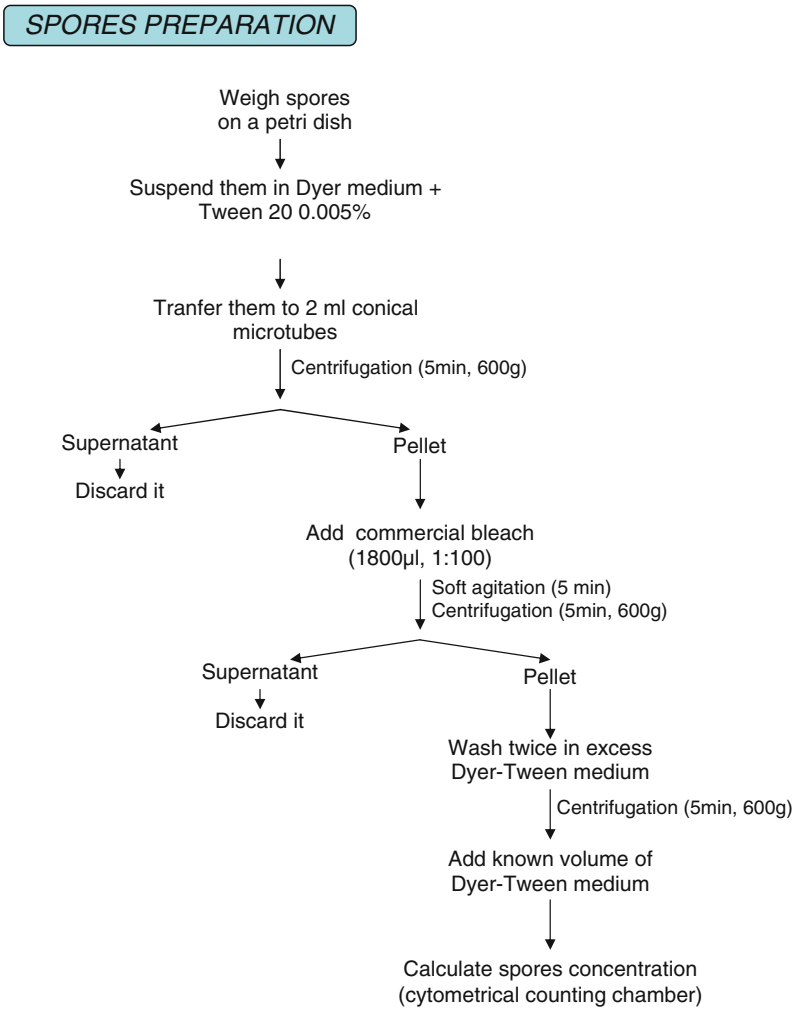
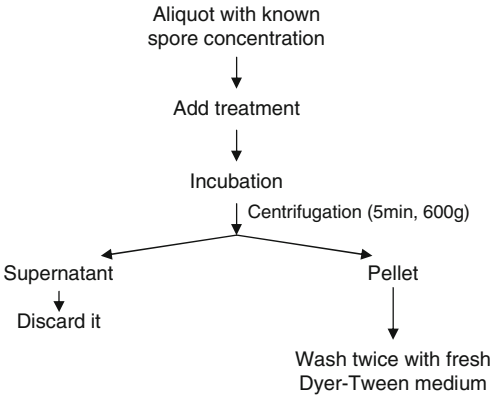


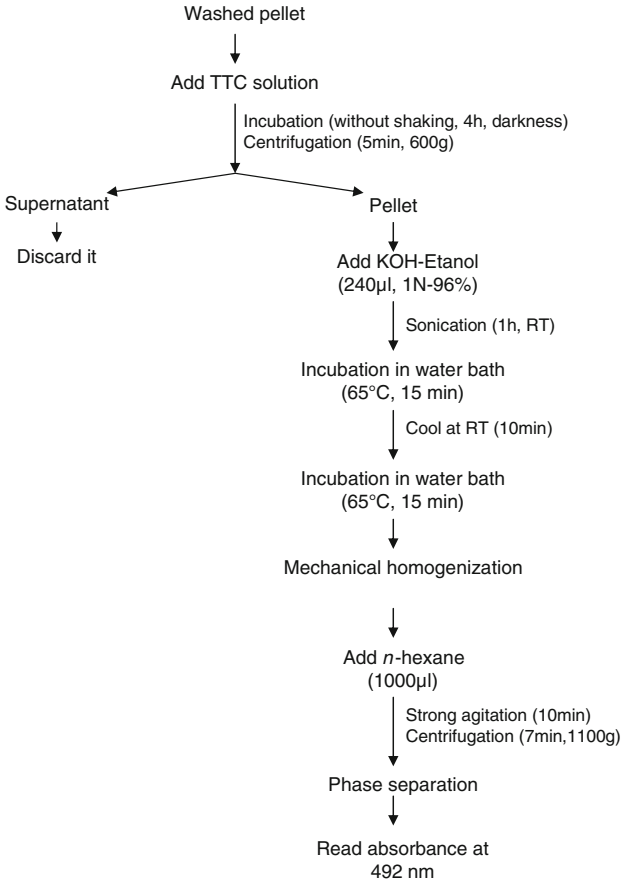
Fig. 17.1 Germination of a *Dryopteris oreades* spore. In wood ferns (genus *Dryopteris*), as in most fern species, spores lack chlorophyll and thus all energy for germination comes from respiration. The oil drops that can be observed in the rhizoid and inside the spore are the main energy storage



EXPOSURE



TTC ASSAY



focus on some practical issues of our bioassay. This testing method based on fern spores is naturally miniaturized and combines biological and ecological relevance together with sensitivity and simplicity, thus making it a promising cost-effective tool for high-throughput toxicity screening and monitoring.

Fern spores show physicochemical characteristics that hamper the adaptation of the TTC method for their study. For example, the presence of highly hydrophobic substances in the cellular wall makes the spores difficult to suspend in aqueous media. Other difficulty is that the spore wall is extremely resistant, and therefore the methods used for sample homogenization and TTC extraction must be especially vigorous. Moreover, pigments from plant tissues (i.e., chlorophyll) can interfere in the spectrophotometric measurement of TPF when is extracted with ethanol. All these subjects must be taken into account and imply modifications of the TTC method used for the study of spores.

Probably the most problematic adaptation of the method for studying fern spores is the aforesaid extraction of the formazan red. In that sense, different homogenization methods can be employed but results in extraction ratios are different even when the order of the treatment changes. In the case of *Dryopteris guanchica*, best results are obtained with a first chemical treatment with KOH/EtOH followed by thermal incubation at 65°C and mechanical homogenization. Mechanical homogenization is a bottleneck step since most of the methods are designed for single samples and are time consuming. We have developed an efficient method using strong agitation of the sample with a small volume of glass fragments, i.e., smashed Pasteur pipettes. This allows the automated simultaneous homogenization of a high number of samples.

Other parameters of this bioassay must be optimized in order to achieve optimal results. Spores of different fern species have different characteristics, such as presence or absence of chlorophyll, wall structure, etc. Thus, the number of spores per sample, pH, TTC concentration, incubation time, homogenization, and extraction should be tested to obtain optimal conditions of the bioassay with different species. We have applied the TTC assay to several species: *Osmunda regalis* (Osmundaceae), *Dryopteris aemula*, *D. affinis*, *D. corleyi*, *D. filix-mas*, *D. oreades*, and *Polystichum setiferum* (Dryopteridaceae). The most appropriate species were *D. guanchica*, *P. setiferum*, and *O. regalis*.

17.5 Candidate Ferns for Toxicity Testing

D. guanchica is located in some Canary Islands and in northwestern Iberian Peninsula and grows in several habitats: laurel-forest (Canary Islands), oak-forest, alder-forest, and heathland (Iberian Peninsula). This species is an allotetraploid derived from *D. aemula* and *D. intermedia*. We choose *D. guanchica* as model species for the initial studies on mitochondrial activity of fern spores and gametophytes based on several advantages. First, leaves and spores are produced throughout the year, and thus mature spores can be collected in different seasons (Fig. 17.2).

Fig. 17.2 Recently expanded leaves of *Dryopteris guanchica* in winter. This is the only wood fern species of Europe. This is the only European wood fern which produces both leaves and spores throughout the year

Second, the germination rate of *D. guanchica* is faster than those of related diploids (Quintanilla and Escudero 2006), which shortens assay times. Third, spore viability can be maintained for at least 1 year with a simple technique (dry storage at 5°C; Quintanilla et al. 2002). In addition, despite the hybrid (allopolyploid) origin of *D. guanchica*, spore abortion percentages are low and similar to those of related diploids (Quintanilla and Escudero 2006).

P. setiferum is mainly present in western and southern Europe. Its ecological amplitude is narrower than that of *D. guanchica. P. setiferum* occurs in river-valley forest. Thus, this fern is a very good option for environmental monitoring of fluvial ecosystems. Moreover, its spore production per leaf is very high, albeit spore maturation is limited to a short period of time (early summer). As an additional advantage, the spores are smaller than those of *D. guanchica*, which is convenient for manipulation. Small spore size allows more spores per volume unit, and thus sample size can be increased without increasing assay cost. Moreover, smaller spores sink slower and give more homogenous liquid suspensions for longer times. This characteristic is important in our TTC assay, as spore suspensions are used in several steps of the protocol. *P. setiferum* is commercialized as garden plant in temperate climates, providing an alternative source of spores.

O. regalis is cosmopolitan and, like *P. setiferum*, inhabits riparian forests. *Osmunda regalis* lives in riverbanks and is sensitive to water pollution. This species produces green (i.e., chlorophyll-containing) spores. Green spores show an elevated metabolic activity from the very moment of collection, without a latency period. The main advantages we have found with these spores are easiness of suspension

in aqueous solution, even without detergents, elevated mitochondrial activity levels, and high sensitiveness to environmental stress. Despite the aforesaid advantages, *O. regalis* spores also show some drawbacks since they must be either used immediately after collection or kept in an ultrafreezer (−86°C) to maintain a high viability (D. Ballesteros, personal communication).

17.6 Application of Fern Spores to Toxicity Testing

The fern spore bioassay for acute toxicity testing may be used to produce valuable toxicological data. Polycyclic aromatic hydrocarbons (PAHs) constitute a family of toxic pollutants derived mainly from fuel use, transport, or storage. The evaluation of the toxicity of contaminated soils or surface waters is of environmental concern. The spore mitochondrial activity bioassay is sensitive to very low concentrations of PAHs, either in known mixtures or in complex PAH-contaminated soil extracts as shown in Fig. 17.3.

As we mentioned above, the utility of bioassays is not limited to the determination of the toxicity of pure compounds or environmental samples, but can also be extended to the assessment of environmental technologies. The assessment of developing technologies for industrial or urban effluent decontamination is especially important. Traditionally, the efficacy of these technologies has been measured by means of chemical quantification of the reduction of specific toxicants. Nonetheless, the products resulting from a decontamination treatment may also be toxic.

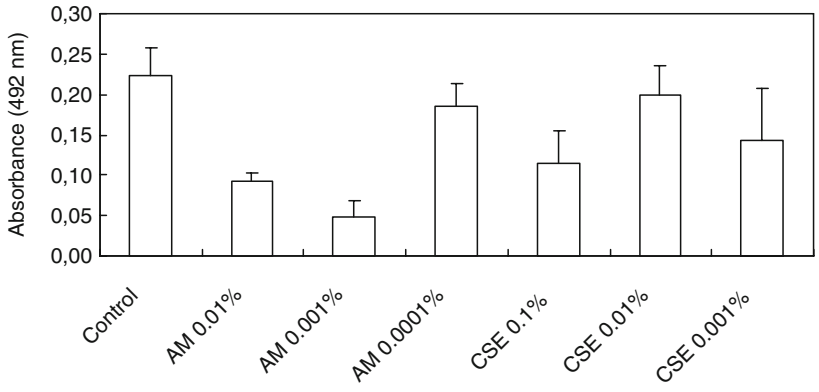


Fig. 17.3 Effect of different PAH-related pollutants on *Dryopteris guanchica* spore mitochondrial activity. Cells were incubated during 24 h in autocleaved Dyer-Tween medium containing different concentration of pollutants (AM aromatic hydrocarbons mixture, CSE contaminated soil extract), at 20°C, PAR 35 μmol m^{-2} s^{-1} and 8 h/16 h photoperiod. Then, mitochondrial activity was evaluated in 2.5×10^4 spores, incubated with TTC 0.6%, pH 8 during 1 h in darkness (From Catalá et al. (2009))

Cyanide is a highly toxic substance found in some industrial effluents that must be eliminated before their discharge into the environment. The analytical results of the chemical monitoring of a process of advanced oxidation for cyanide elimination are shown in Table 17.1. After 18.5 h in the reactor, cyanide concentration has reached concentrations lower than 0.2 mg L^{-1}. From the chemical point of view, the treatment has been a complete success. However, when a toxicity test based on the mitochondrial activity of fern spores is performed, a strong inhibition caused by the 18.5 h-sample can be observed (Fig. 17.4, D. Bru, unpublished results). It becomes evident that cyanide degradation products are still toxic, and this must be taken into account for the complete development of this decontamination process.

Table 17.1 Degradation of cyanide in a titania photoreactor

Time (h)	Cyanide (mg L^{-1})
0	443
5	248
7	169
18.5	0.176

A solution containing KCN was treated in a photoreactor using a titania catalyst. Samples were withdrawn at regular intervals from the upper part of the reactor. The extent of the photodegradation was determined by measuring the remaining cyanide spectrophotometrically

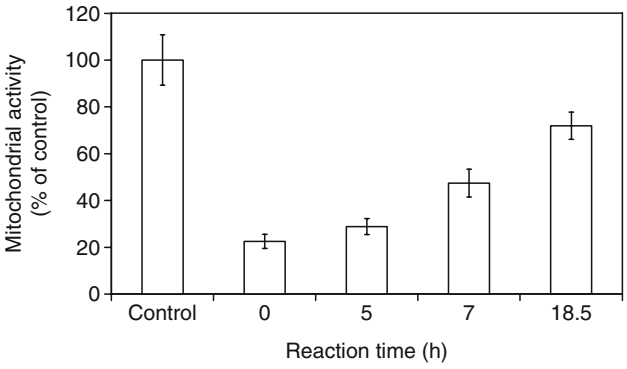


Fig. 17.4 Inhibition of mitochondrial activity in *Polystichum setiferum* spores along a process of advanced oxidation of cyanide. The acute toxicity of samples obtained from titania photoreactor during oxidation of cyanide at different times was assayed with *P. setiferum* spores. Sample pH was adjusted to 7 before treatment and added to microtubes containing 8×10^{-4} cells. Spores were incubated during 20 h in controlled conditions (20°C, PAR 35 μmol m^{-2} s^{-1} and 8 h/16 h photoperiod) and mitochondrial activity evaluated. Control samples were performed with culture medium

17.7 Conclusion

Our bioassay presents several practical advantages: easy collection and manipulation of test organism, low cost, only basic lab equipment needed, relatively short execution time, and easiness of adaptation to high-throughput technologies (i.e., automated analyser, microplate reader, etc.). Despite the simplicity of the method, when we compare the fern spore bioassay based on mitochondrial activity with other bioassays, we find further advantages:

1. Biological relevance: Ferns are higher plants. The conclusions obtained are highly relevant for eukaryotic organisms, especially other higher plants such as crops or wild plants. Comparable bioassays based on prokaryote organisms (e.g., *Vibrio fischeri*) render results that are not easily extrapolated to eukaryotic organisms.
2. Ecological relevance: We can use spores of ferns belonging to different habitats.
3. Versatility. With the same organism and methodology, different types of bioassays would be possible: acute toxicity (24–72 h) or chronic toxicity (weeks). This is also so regarding the nature of the sample to be tested: aqueous, soil, or gas samples. Incubation of gametophytes can be performed in liquid samples: environmental waters, and soil or gas leachates. Spores could also be sown on solid substrates such as soils or directly exposed to gaseous samples.
4. Fern spores can be easily transported and some species preserved at 5°C for months or even years and do not need continuous growth or feeding such as other organisms.
5. Very low volume of test sample needed. If the samples can be analyzed in a microplate reader, less than 1 mL of test sample is needed and automation of the procedure requires only punctual adjustments.
6. The fern spore bioassay allows the screening of a high number of samples at a minimum cost. Plant material and chemical substances are inexpensive, the latter not being especially toxic; therefore, not extraordinary health security measures need to be adopted. The laboratory equipment needed is usually found in regular laboratories.
7. Spores and gametophytes of some species tolerate wide ranges of pH. This characteristic minimizes the problems related with sample pH or acidification during bioassay realization. In the case of *D. guanchica,* we have shown that mitochondrial activity is not altered in a pH range of 5–10 (Catalá et al. 2009).

Finally, this model sums up the advantages of methods based on single cells with those of pluricellular organisms. In a short time, spores give rise to gametophytes that may be used as a pluricellular plant model with no further modifications on the method. These features make this method suitable for commercialization in the shape of a kit.

The method developed for the measurement of mitochondrial activity in fern spores allows obtaining results of cell viability, development, and physiological

state in short periods of time. Then, besides its use in bioassays, it can be applied to multiple fields: (a) conservation programs, as an alternative to germination assays; (b) research in plant physiology and biochemistry, i.e., for the investigation of oxidative pathways; (c) as a tool in the study of biotic and abiotic stress; and (d) as a tool in the study of genetic and genomic factors affecting spore germination and gametophyte development.

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Chapter 18
Chronic Phytotoxicity in Gametophytes: DNA as Biomarker of Growth and Chlorophyll Autofluorescence as Biomarker of Cell Function

Myriam Catalá and José Luis Rodríguez-Gil

18.1 Introduction

For many years acute lethality tests have been the dominant method for both, single compound and whole effluent toxicity studies due to its simplicity and economic and temporal efficiencies. The United States Environmental Protection Agency (USEPA) defines chronic toxicity as "the ability of a substance or mixture of substances to cause harmful effects over an extended period, usually upon repeated or continuous exposure sometimes lasting for the entire life of the exposed organism" (USEPA 1997). By looking at such a definition, most of us can agree that regardless of a few punctual events, organisms in nature are mainly subjected to chronic exposures. With acute exposure being a rare event and lethality a most certainly unwanted outcome it soon became obvious to toxicologists that new relevant chronic toxicity tests needed to be developed. More accurate, direct and relevant estimates of the toxicity of a studied chemical could be obtained by lengthening tests' duration and evaluating subtler endpoints like growth or reproduction. The most conservative tests of this kind and those that offer a better confidence on the obtained data are the full life-cycle tests. However, these are long and costly, and have been limited to animal toxicology testing of compounds with a direct human health association such as drugs. More recently, sub-chronic studies evaluating early life stages (ELS tests) of the organisms have been suggested to provide comparable results to full life-cycle test for a fraction of the time and cost (Landis et al. 1993).

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This is however not such a novel approach in the field of phytotoxicity. Ever since the beginning of the development of the pesticide industry, early life stages of both crops and weeds have been tested in the way of relatively long-lasting germination and seedling growth tests to evaluate the success of the new products.

18.2 Current State of Phytotoxicity Testing

Currently a number of standard protocols are available for the evaluation of subchronic terrestrial plant toxicity (Table 18.1). Due to their primarily agronomic origin (i.e., pesticide validation), the species routinely subjected to these tests are usually limited to a number of common crops (Table 18.2). Until recently, USEPA only required testing on ten annual crop plants (two monocot families and four dicot families). In the drafts of the new harmonized testing scheme created between the USEPA and the Canadian Pest Management Regulatory Agency (PMRA) (USEPA 2010), the addition of one monocot family, eight dicot families, four woody plants, one life-cycle test (reproductive test), and one partial life-cycle test

Table 18.1 Examples of the most commonly applied standard tests for terrestrial plant toxicity testing (OECD 2007; USEPA 2010)

Issuer	Code	Name
OECD	OECD 208	Terrestrial plants (seedling emergence and growth)
OECD	OECD 227	Terrestrial plants (vegetative vigor)
USEPA	OPPTS 850.4100	Terrestrial plant toxicity, Tier 1 (seedling emergence)
USEPA	OPPTS 850.4150	Terrestrial plant toxicity, Tier 1 (vegetative vigor)
USEPA	OPPTS 850.4225	Terrestrial plant toxicity, Tier 2 (seedling emergence)
USEPA	OPPTS 850.4250	Terrestrial plant toxicity, Tier 2 (vegetative vigor)
USEPA	OPPTS 850.4230	Early seedling growth toxicity test
USEPA	OPPTS 850.4200	Seed germination/root elongation toxicity

Table 18.2 Summary of the most common species used for terrestrial plant toxicity testing (OECD 2007; USEPA 2010)

Family	Species	Common name
Solanaceae	*Lycopersicon esculentum*	Tomato
Cucurbitaceae	*Cucumis sativus*	Cucumber
Compositae	*Lactuca sativa*	Lettuce
Leguminosae	*Glycine max*	Soybean
Cruciferae	*Brassica oleracea*	Cabbage
Umbelliferae	*Daucus carota*	Carrot
Gramineae	*Avena sativa*	Oat
Gramineae	*Lolium perenne*	Perennial ryegrass
Gramineae	*Zea mays*	Corn
Amaryllidaceae	*Allium cepa*	Onion

has been proposed. These changes mainly come to address the issue of variability of plant responses to chemical exposures and thus, the need for an increase in the number of test species, but also they represent an attempt to separate phytotoxicity testing from its original agronomic roots and broaden its application to the field of environmental toxicology. This last perspective is well approached by the Organization for Economic Cooperation and Development (OECD) in their guidelines for testing of chemicals (OECD 2007) where a list of possible non-target species to be tested during the hazard characterization phase of environmental risk assessment is provided, under their test number 208 for seedling emergence and growth in terrestrial plants.

The situation of phytotoxicity testing in riverine and aquatic ecosystems is not better than that of their terrestrial counterparts. The presence of phytotoxicity tests in aquatic toxicity guidelines is limited to a number of selected algae species like *Pseudokirchneriella subcapitata* or *Chlorella vulgaris*, and more recently to a number of aquatic macrophytes, especially duckweed (*Lemna* spp.). Wang (1991) pointed out a decade ago that the archaic view of algae and plants being members of the same kingdom should not lead us to the error of accepting algae toxicity data as a surrogate of the potential effects on plants; however, and as an example, only a growth inhibition test in *P. subcapitata* is required under the USEPA Whole Effluent Toxicity (WET) (USEPA, 1972) test guidelines. Particular attempts to apply aquatic macrophytes and aquatic environment-related species can be found in the literature (Doust et al. 1994; Lewis 1995; Mohan and Hosetti 1999; Ferrat et al. 2003); however, their inclusion in regulatory documentation is far from ideal.

In both cases, terrestrial and water-related ecosystems, ferns are consistently ignored, even though when, with more than 10,000 living species, ferns are the second-largest group of vascular plants, being in many cases, key components of numerous plant communities.

18.3 Limitations of Traditional Acute Toxicity Tests

Acute toxicity refers to the effects of a single exposure to a toxicant or mixture of toxicants over a short period of time and it is commonly presented as the Lethal Concentration 50 or LC_{50}, which represents the dose of a substance that is lethal to 50% of the tested organisms. They have been the predominant toxicity tests for many years due to their multiple advantages such us lower costs, shorter test times, and easy quantification of the endpoints (usually mortality) in comparison with the commonly available chronic tests. However, they present numerous disadvantages as well. As mentioned in the introduction, most of the potentially toxic compounds that could end up in natural ecosystems will do so under chronic exposures, especially in the case of plants whose lack of motility prevent them from reducing exposure by avoidance. Despite singular dramatic events, pollution usually occurs in non-lethal concentrations. Sublethal effects such as reproduction inhibition or slow growth/development are to be the most relevant in this kind of scenarios, and yet LC_{50}s are still the standard measurement in toxicology.

18.4 Limitations of Traditional Terrestrial Plant Toxicity Tests

The popularity of acute toxicity testing was not induced by the ignorance of its associated problems; however, chronic toxicity was a logistically difficult alternative to apply. Traditional sub-chronic phytotoxicity evaluations are based on seed germination or root elongation tests (Benenati 1990) and, as presented in the standard test guidelines, are to be performed in pots filled with soil to which the tested compound is applied (unless testing the effects of direct plant spray) (OECD 2007; USEPA 2010). Seeds of the selected test species are planted on the treated soil with a maximum density of 3–10 seeds per 100 cm² of potted soil depending on the size of the seed. After planted, the emerging plants should be maintained under good horticultural practices in controlled environment chambers, phytotrons, or greenhouses (OECD 2007) for a period of 14–21 days after at least 50% of the control plants have emerged. Germination is monitored daily and plant biomass is recorded at the end of the experiment.

These traditional tests, in consequence, are not only long but also require specialized facilities able to host large numbers of pots in specific environmental conditions and where the handling and disposal of large volumes of treated soil should be possible. Together with this, the large amounts of soil to be treated increase the need for large volumes of the compound or environmental sample to be tested. These characteristics not only increase the test cost dramatically, but can, in many cases, make the test unfeasible. Methodologies to reduce sample volume and space requirements have been attempted, from the traditional germination tests in petri dishes to more elaborated phytotoxicity kits (Czerniawska-Kusza et al. 2006). However, all of them are still based on seed germination, and thus, space and sample volume reductions are limited to seedling size and water requirements of the tested plant.

18.5 Usefulness of Ferns in Phytotoxicity Testing

A series of criteria are provided for the selection of non-target species to be tested under the OECD guidelines, in their test number 208 (Terrestrial Plants [seedling emergence and growth]) (OECD 2007). Among these:

- The species selected should be reasonably broad, e.g., considering their taxonomic diversity in the plant kingdom, their distribution, abundance, species-specific life-cycle characteristics, and region of natural occurrence.
- The species have uniform seeds that are readily available from reliable standard seed source(s) and that produce consistent, reliable, and even germination, as well as uniform seedling growth.
- They have been used to some extent in previous toxicity tests and their use in, for example, herbicide bioassays, heavy metal screening, salinity or mineral stress tests, or allelopathy studies, indicates sensitivity to a wide variety of stressors.

- Plant is amenable to testing in the laboratory and can give reliable and reproducible results within and across testing facilities.
- The sensitivity of the species tested should be consistent with the responses of plants found in the environment exposed to the substance.

As mentioned earlier, ferns, with more than 10,000 living species, are the second-largest group of vascular plants, and are main components of numerous plant communities. Therefore, the inclusion of this group in standard ecotoxicological tests is an important goal regarding ecological relevance. Fern spores are produced in large quantities from species, in many cases, such as *Polystichum setiferum*, *Osmunda regalis,* or many *Dryopteris*, commonly found in plant nurseries around the world. Produced spores are uniform, with very low abortive particles which present even germination, above 90% in many cases (Quintanilla and Escudero 2006). Fern spores, and spore-developed gametophytes, have long been recognized as useful models for plant research in important areas, namely, plant development, sex determination, gamete production and fertilization, response to environmental factors, and evolution of plant complex traits (Banks 1999), in part for the convenience of their small size and ease to use in aqueous suspension, which greatly simplifies laboratory procedures.

Our team is currently working on the study of the response of the spores of the riparian *Polystichum setiferum* to different classes of toxicants. Preliminary results will be discussed below. However, the relationship between the sensitivity of the species tested in vitro with the responses of plants in the field is quite difficult to assess. The application of such an approach to non-target wild species is, therefore complex, and will involve basic plant research with the participation of experts in botany and plant ecology together with environmental toxicologists.

18.6 DNA as a Measurement of Cellular Proliferation

The spore germination and development, as for any other pluricellular organism, is characterized by a rapid succession of cell cycles where embryonary cells duplicate their genetic material and divide giving birth to two daughter cells. Microscopic assessment is laborious and present technical difficulties in the initial phases due to colored cell walls. In every cell division, DNA content augments twofold, consequently, gametophyte DNA quantification can clearly indicate the stage of plant development of a certain sample of spores.

Fluorescence is the result of a process that occurs in certain molecules (generally polyaromatic hydrocarbons or heterocycles) called fluorophores. A fluorescent probe is a fluorophore designed to respond to a specific stimulus or to localize within a specific region of a biological specimen. The fact that a single fluorophore can generate many thousands of detectable photons is fundamental to the high sensitivity of fluorescence detection techniques. Fluorescence intensity is quantitatively dependent on the same parameters as absorbance – defined by the Beer–Lambert law as

the product of the molar extinction coefficient, optical path length, and solute concentration – as well as on the fluorescence quantum yield of the dye and the excitation source intensity and fluorescence collection efficiency of the instrument.

The use of epifluorescent probes specific for nucleic acids is common in the field of cell biology and embryology as a means of an estimate of cell number. Unlike other nucleic acid stains (etidium bromide, propidium iodide, etc.) the blue fluorescent Hoechst dyes are cell permeable and bind preferentially to DNA in the presence of RNA (Mocharla et al. 1987). Together with DAPI, Hoechst is included in the class of DNA minor-groove binders. The dye, weakly fluorescent itself in solution, binds to all nucleic acids, but AT-rich double-strand DNA enhances fluorescence twofold greater than GC-rich strands, resulting in an increase in fluorescence and a shift in the emission maximum from 500 to 460 nm (Daxhelet et al. 1989; Labarca and Paigen 1980). Recently, a similar behavior has been fully demonstrated for the interaction of this probe either with animal or plant DNA (Maiti et al. 2009).

18.7 Chlorophyll *a* Autofluorescence as Surrogate of Plant Physiological State

Environmental pollutants can easily interact with key plant metabolic processes, leading to impairment in chlorophyll *a* (Chl *a*) production or function and thus, in the whole photosynthetic activity and in consequence, in plant fitness. In such scenario variations in Chl *a* can easily serve as a simple and sensitive surrogate of the physiological state of the plant, in toxicity testing protocols. Precise quantification of chlorophyll, as applied in many algal toxicity tests, is a destructive method that requires the homogenation of the sample and solvent-extraction of the pigment prior to the measurement.

Chlorophyll is itself a fluorophore and emits red fluorescence when excited at certain wavelengths. In vivo fluorimetric methods allow the measurements of potential quantum efficiency of photosynthesis, providing detailed pictures of the photosynthetic capacity of the studied plant. Studies of chlorophyll *a* (Chl *a*) fluorescence modulation in vivo have been proposed as a useful non-invasive method to evaluate the physiological state of plants (Krause and Weis 1991). However, these methods are complex and not practical for a toxicity bioassay approach. Nonetheless, it has been long demonstrated that the direct measurement of the red autofluorescence from plant cells can be used to report the total amount of the cellular chlorophyll pigment with reasonable accuracy (Galbraith et al. 1988). This approach allows rapid determinations of total chlorophyll by flow cytometry or fluorometry. Optimal fluorometric parameters for Chl *a* detection are $\lambda_{exc} = 420$ nm and $\lambda_{em} = 685$ nm (Agati 1998), but thanks to the width of excitation and emission peaks, standard blue and red filters (e.g., $\lambda_{exc} = 485–8$ nm and $\lambda_{em} = 635$ nm) can be used in the fluorometric estimation of Chl *a* content in plant material with satisfactory sensibility.

The method for in vivo fluorometric quantification of Chl *a* presented in this chapter allows for a significant reduction in sample preparation time, as spore dilutions can be measured directly in the growth medium or test solution. At the same time, the use of high throughput techniques such as multi well plate fluorescence reading allows for simple detection of relative variations in Chl *a* content between treatments as both, controls and samples, are measured virtually at the same time, and thus in the same photosynthetic conditions.

18.8 Applications of the Bioassay of Chronic Toxicity Based on Fern Spores

The development of a bioassay to become a standard method requires the collection of a solid range of previous data on different classes of toxicants, for example, herbicide bioassays, heavy metal screening, salinity or mineral stress tests. In order to find out the sensitivity of a chronic toxicity bioassay based on fern spores we have performed studies on different substances and environmental samples.

In recent years, a reduction in the amount of "conventional" pollutants in surface water (nitrates, heavy metals, sulfur and nitrogen oxides, etc.) has occurred worldwide, but we are witnessing the occurrence of the "emerging" pollutants, which include, among others, pharmaceuticals and their metabolites (Fent et al. 2006). Very low concentrations of human pharmaceuticals have been detected in several countries, either in sewage treatment plants (STPs), surface waters, sea water, groundwater, sediments, or drinking water. Some of the studies published state that such low concentrations of the pharmaceutical drugs found do not constitute an imminent risk for public health but also point to probable ecotoxicological chronic toxicity for aquatic organisms due to a continuous exposure to low doses (Khetan and Collins 2007). However, the information of the toxicity of human pharmaceutical on non-target species at environmental concentrations is almost non-existent, in part due to low sensitivity of standard tests. We have recently obtained some interesting results on the chronic toxicity of the anti-inflammatory diclofenac on fern spore germination (unpublished results). Figure 18.1 shows that diclofenac at all concentrations studied causes a strong decrease on DNA in exposed gametophytes. This decrease can be either due to an inhibition of growth of all gametophytes or to the death of a part of the population. The decrease in chlorophyll autofluorescence is not so dramatic (Fig. 18.1b) which could point to an increased synthesis of chlorophyll aimed at the increase of photosynthesis by the reduced population of cells. These results are of especial concern since detected environmental concentrations of diclofenac frequently fall within the studied range and could have a negative impact on the germination and development of fluvial or riparian higher plants. Additionally, the biomarkers assessed as endpoints of toxicity show an elevated sensitivity and biological relevance. This is especially so for DNA, since this biomarker combines the advantages of detecting lethal as well as sublethal toxicity in developing embryos.

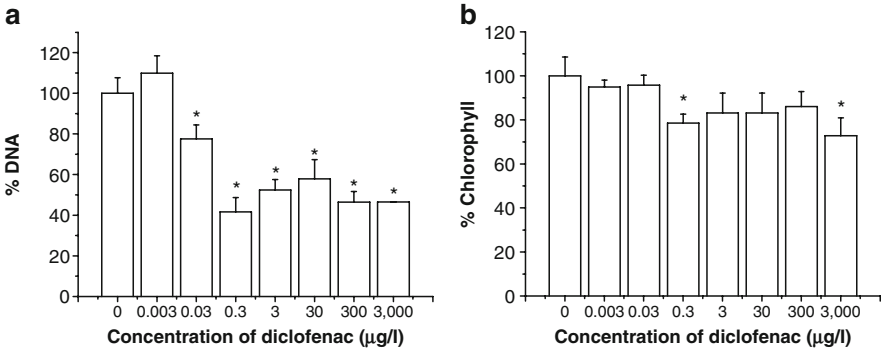

Fig. 18.1 Chronic toxicity test of the anti-inflammatory diclofenac**.** *Polystichum setiferum* spores (8×10^4) were exposed during 1 week to different concentrations of diclofenac in controlled conditions (20°C, PAR 35 µmol $m^{-2} s^{-1}$, 16 h photoperiod). The results are referred to non-treated control spores. (**a**) Chlorophyll autofluorescence ($\lambda_{exc} = 485–8$ nm and $\lambda_{em} = 635$ nm), (**b**) DNA content (unpublished results of Raquel Feito)

Diclofenac, together with some other human pharmaceuticals seem to be quite refractory to conventional operations in sewage treatment plants (STPs). Due to the ecotoxicological risks of these substances in surface waters, the application of non-biological processes, such as advanced oxidation processes, has been increasingly assessed. In order to evaluate the effect of pharmaceuticals on the environment and the efficacy of water treatment technologies, we have recently performed acute and chronic toxicity tests with *Polystichum setiferum* spores (Rodriguez-Gil et al. 2010) in environmental samples. Chemical analysis detected 56 pharmaceuticals in four selected rivers of Madrid (Spain). No relevant acute toxicity of the water samples (as evaluated by alteration of mitochondrial activity, see Chap. 17) was observed but a significant hormetic effect existed in chronic exposure. Hormesis is a compensatory effect caused by a moderate toxic insult. Fluvial water samples induced significant increases in DNA as well as in chlorophyll autofluorescence in developing gametophytes (Fig. 18.2). This kind of response, in chronic exposure, can lead to an early depletion of resources of the gametophyte that could eventually lead to death. Photo-Fenton oxidation provided a high degree of total organic carbon (TOC) mineralization with up to 70% reduction, and a 99% elimination of the studied pharmaceuticals. The chronic bioassay with *Polystichum setiferum* demonstrated a significant reduction of the hormetic effect for DNA in all the samples and for chlorophyll in only two of the samples. Despite chemical elimination of pharmaceuticals and high reduction in TOC, a significant hormetic effect persisted when the gametophytes were exposed to “decontaminated” water samples. This fact seems to be related to the presence of other toxicants in the water matrix and warns against the sole application of chemical analysis for the monitoring of environmental pollution.

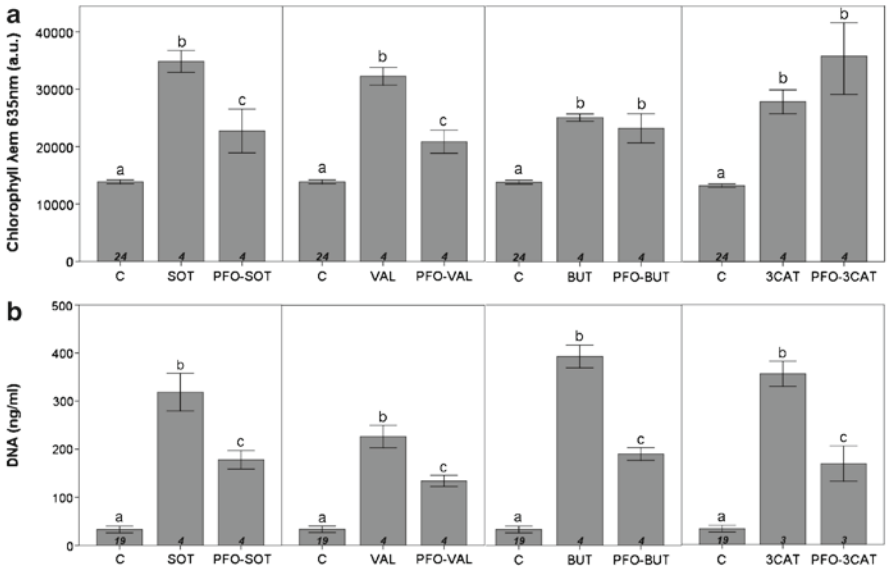

Fig. 18.2 Chronic toxicity test for original river samples and photo-Fenton oxidized (PFO) samples. *Polystichum setiferum* spores (10^5) were exposed during 1 week to fluvial samples and treated water samples in controlled conditions (20°C, PAR 35 μmol $m^{-2} s^{-1}$, 16 h photoperiod). Columns represent average values of the number of replicates (shown in bold at the bottom), bars represent standard error. The letters a, b, and c represent statistically significant groups defined by the Tuckey-b test

18.9 Protocols

The protocols used in the aforementioned experiments are briefly described below

18.9.1 Toxicity Tests

18.9.1.1 Plant Material

Polystichum setiferum spores, a typical fern of riparian habitats, were sampled in NW Spain, A Coruña province, San Xusto River. Fragments of leaf were collected with mature but closed sporangia. Spore release was promoted by drying the fragments on smooth paper for a week in the laboratory. Spores were stored dry at 4°C in darkness until use. Spores were sieved and suspended in gametophyte culture medium, sterilized and counted as detailed in Catalá et al. (2009).

18.9.1.2 Chronic Toxicity Bioassay

A completely new bioassay was developed for the evaluation of chronic toxicity in the development of spore-born fern gametophytes. Aliquots containing 1×10^5 spores were prepared and 1.5 mL of each of the neutralized samples was added ($n = 4$). Control treatment samples contained 1.5 mL of Dyer medium supplemented with Tween 20, 0.005% ($n = 24$) (Catalá et al. 2009). Spores were incubated in suspension in controlled conditions (20°C, PAR 35 μmol m^{-2} s^{-1} 16 h photoperiod) producing green fern gametophytes. After 1 week, samples were frozen (−20°C) until DNA or chlorophyll quantifications were performed.

DNA Quantification

One milliliter aliquots of the pre-incubated spores were sedimented by 5 min centrifugation at 600× *g* in a Heraeus Fresco Biofuge (Thermo Scientific, Waltham, MA, USA). Supernatant was discarded and spores were resuspended in 1 mL of a 1:1 mixture of TNE buffer (Tris base 100 mM, EDTA-Na2 10 mM, NaCl 1 M, pH 7.4) with lyses solution (0.2% Triton X − 100 and 1% NaOH 1 N). Homogenation was performed by 30 min agitation (3,000 rpm, Labnet, Edison, NJ, USA) with approximately 200 μL of sieved glass fragments (0.2–1 mm).

After incubation at 37°C for 1 h in a water bath, 10 μL of the homogenates were transferred to black 96-well flat bottom Greiner plates. One hundred and ninety microliters of either 0.1 μg mL^{-1} bisbenzimide Hoechst 33258 (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) in TNE buffer or TNE buffer alone (for autofluorescence blanks) were added to the wells prior to measurement of fluorescence (λ_{exc}: 360 nm, λ_{em}: 465 nm) in a SPECTRAFluor Plus microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

Chlorophyll Quantification

Two hundred microliter aliquots of the exposed gametophytes were transferred to black 96-well flat bottom Greiner plates, and chlorophyll autofluorescence was measured (λ_{exc}: 485 nm, λ_{em}: 635 nm).

18.10 Conclusions

Higher plants are an essential part of a healthy and balanced ecosystem and new plant models are essential in the evaluation of potential impacts of pollutants. In this context, ferns must be included within relevant taxa in standard bioassays. The newly developed bioassay of chronic toxicity based on fern spores and gametophytes is a

promising cost-effective tool for high throughput toxicity screening and monitoring. This higher plant testing method is naturally miniaturized and combines biological and ecological relevance together with sensitivity and simplicity.

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Chapter 19
Arsenic Hyperaccumulator Fern *Pteris vittata*: Utilities for Arsenic Phytoremediation and Plant Biotechnology

Bala Rathinasabapathi

19.1 Arsenic – A Toxic Metalloid Widespread in the Environment

Arsenic is a widely present toxic metalloid. Arsenic cycling in nature contributes to the contamination of aquifers in many parts of the world (Oremland and Stolz 2003). Millions of people in Bangladesh, India, China, Argentina, Chile, Hungary, Mexico, Peru, and Taiwan are affected by chronic arsenic poisoning (Ng et al. 2003). People in these regions suffer from numerous medical problems induced by arsenic including cancers of the internal organs. The most common route of arsenic poisoning is by drinking arsenic-contaminated water or eating food grown in arsenic-contaminated environments (Zhao et al. 2010). Arsenic poisoning is one of the greatest public health hazards of the century, begging for cost-effective scientific, engineering, and technological solutions.

Arsenic contamination of the environment also happens by human activities. Because arsenic often occurs in association with the ores of copper, lead, and gold, mining activity spreads the metalloid into the environment. Arsenical pesticides had been widely used in agriculture, contaminating large areas of arable land. Organic arsenicals (e.g., roxarsone) used as an intestinal palliative for swine and poultry bring arsenic into the food chain and the environment. Production and storage of chemical weapons and industrial uses of arsenic also contaminate the environment. Several engineering solutions to remediate arsenic-contaminated environment are available. However, these solutions are costly, energy-intensive, and cumbersome (Mohan and Pittman 2007).

Arsenic occurs in many forms which differ in their toxicological properties. From highest to lowest toxicity the forms can be ranked as arsines, inorganic arsenites, organic trivalent compounds (arsenooxides), inorganic arsenates, organic

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pentavalent compounds, arsonium compounds, and elemental arsenic (Dembitsky and Rezanka 2003). Inorganic forms of arsenic – arsenate and arsenite – are the most commonly found in the contaminated soil and water.

19.2 Discovery of Arsenic Hyperaccumulation Trait in Ferns

Arsenic is toxic to plants too. Some of the plant species identified to be tolerant to arsenic had reduced abilities to take up arsenic (e.g., Hartley-Whitaker et al. 2001; Bleeker et al. 2003; Sharples et al. 2000). In contrast, Chinese brake fern *Pteris vittata* had an unusual ability to hyperaccumulate arsenic (Ma et al. 2001). *P. vittata* accumulated arsenic in the fronds up to about 2% of dry weight (Ma et al., 2001). This is a pioneering finding because it opened up new opportunities to develop cost-effective green technologies to remediate arsenic-contaminated soils and water.

Among plants, arsenic hyperaccumulation (i.e., >1,000 μg/g dry wt.) appears to be unique to ferns. Gametophytes of *P. vittata* were also tolerant of high levels of arsenic and showed arsenic hyperaccumulation (Gumaelius et al. 2004; Kamachi et al. 2005). In a study by Kamachi et al. (2005), gametophytes of *P. vittata* and *Athyrium yokoscense* were shown to tolerate and accumulate lead. Possible relations between arsenic and lead accumulation traits in *P. vittata* (Soongsombat et al. 2009) are not known. Even though the chemical nature of arsenic and lead are different, it is possible that these two heavy metals share some of the metabolic targets in the plant.

A number of additional ferns have been reported to have arsenic hyperaccumulation abilities also (Francesconi et al. 2002; Meharg 2003; Sridokchan et al. 2005; Zhao et al. 2002). When 18 non-*Pteris* ferns were screened, three other species were identified as arsenic hyperaccumulators (Srivastava et al. 2010). Table 19.1 shows a list of species with their arsenic accumulation levels in a controlled experiment (Srivastava et al. 2010). Lack of arsenic tolerance and hyperaccumulation in the most primitive fern taxon *Osmunda* and the presence of a gradation within the natural variability for arsenic accumulation among the species (Table 19.1), suggest that arsenic accumulation trait could be an evolutionarily a more recent event in ferns. It is possible that arsenic tolerance and hyperaccumulation have evolved in a stepwise manner depending upon the arsenic levels of the habitats and the selective advantages for the trait. Detailed studies are needed to map the arsenic tolerance and arsenic hyperaccumulation traits into a molecular phylogenetic tree of fern taxa (Meharg 2002).

Several of the ferns including *P. vittata* had been shown to accumulate selenium to levels comparable to some of the selenium-accumulating angiosperms (e.g., *Brassica juncea*) (Srivastava et al. 2005a, b). Potential relationships between arsenic and selenium accumulation traits in the ferns are not known at present.

Table 19.1 Arsenic levels found in fern fronds, in an experiment in which ferns were exposed to 300 μM sodium arsenate in the hydroponic medium for 3 days (Srivastava et al. 2010)

Species (family)	Mean arsenic level in the tissue (μg/g dry weight)
Pteris vittata	404
Chelanthes sinuta	341
Adiantum raddianum	326
Polystichum acrostichoides	271
Actinopteris radiata	244
Pellaea rotundifolia	254
Nephrolepis cordifolia	228
Dennstaedtia punctilobula	220
Athyrium filix-femina	144
Didmochlaena truncatula	120
Hemionitis arifolia	<100
Microlepia strigosa	<100
Davallia griffithiana	<100
Onoclea sensibilis	<100
Osmunda regalis	<100
Rumohra adiantiformis	60
Microsorium	60
Blechnum spicant	29

Note that the most primitive fern *Osmunda regalis* is not an arsenic hyperaccumulator

19.3 Biological Roles for Arsenic Hyperaccumulation

Why would certain ferns hyperaccumulate arsenic? It was proposed that metal hyperaccumulation in plants could be a defense strategy against their natural enemies (Boyd 2007). In a test whether arsenic hyperaccumulation in *P. vittata* could deter herbivores, grasshoppers (*Schistocerca americana*) were supplied with fronds from plants grown in arsenic-containing medium or control conditions. While the insects ate the fronds from control plants, they did not consume arsenic-containing fern fronds, indicating feeding deterrence by either arsenic or arsenic-induced changes in the frond (Rathinasabapathi et al. 2007). Mathews et al. (2009) showed that arsenic accumulated in *P. vittata* could deter scale insect *Saissetia neglecta*, a natural pest of this fern. It is not yet known whether some of the natural pests of the arsenic hyperaccumulating ferns could have evolved resistance to arsenic and could thus carry the arsenic in to the ecosystem.

Studies on selenium accumulation in plants have shown a defense role for selenium against herbivory (e.g., Hanson et al. 2004). It is possible that selenium accumulation in *P. vittata* and other ferns could have a similar role in nature. In controlled experiments, the addition of selenium improved arsenic uptake in *P. vittata* (Srivastava et al. 2008). It has been suggested that selenium accumulation

may perhaps be an adaptation to protect the plant from arsenic-induced oxidative stress (Srivastava et al. 2008).

Although defense against herbivory is a compelling benefit for arsenic hyperaccumulating species, there could also be additional functions for arsenic. *P. vittata* plants were stimulated when the soil contained low levels of arsenic compared to plants in the no arsenic control medium (Tu and Ma 2005), suggesting a growth advantage due to arsenic.

19.4 Arsenate Uptake

Arsenate is a chemical analogue of phosphate and hence one can expect that it would be taken up by plants *via* phosphate transporters. In support of this hypothesis, a number of studies have shown that increasing phosphate supply in the medium decreased arsenate uptake in *Pteris* species (Wang et al. 2002; Poynton et al. 2004). In kinetic experiments, phosphate competed with arsenate (Poynton et al. 2004). Despite these observations, soil-grown *P. vittata* was stimulated by phosphate addition over a period of several weeks, both for growth and arsenic uptake, especially at high levels of arsenate (Tu and Ma 2003). It is possible that *P. vittata* had evolved specialized arsenate and phosphate transporters highly regulated by external concentrations of these ions. Identification of phosphate and arsenate transporters in *P. vittata* and studies on their regulation should shed light on how the fern takes up arsenate at the same time maintaining growth.

19.5 Arsenate Reduction

When *P. vittata* was supplied with arsenate in the medium, a large fraction of arsenic accumulated in the fern frond was in the form of arsenite (Ma et al. 2001). Even excised frond tissue exhibited an ability to reduce arsenate to arsenite (Bondada et al. 2004; Tu et al. 2004; Rathinasabapathi et al. 2006). Reduction of arsenate to arsenite and the export of arsenite out of the cell are the most commonly found strategies in arsenic resistant microorganisms (Mukhopadhyay and Rosen 2002). Based on comparative biochemistry, an arsenate reductase was expected to function in *P. vittata*. An arsenate reductase was identified in *P. vittata* and cDNAs were cloned (Ellis et al. 2006) by using functional complementation of yeast strain deficient for arsenate reductase. Although root tissue does not accumulate arsenite, the presence of arsenate reductase activity in that tissue (Duan et al. 2005) suggests that different tissues of the brake fern may have tissue specific arsenate reductases.

Using a functional cloning strategy, we had identified that cytosolic triose phosphate isomerase could function in arsenate reduction in *P. vittata* (Rathinasabapathi et al. 2006). When *P. vittata* cDNA for triosephosphate isomerase was expressed in an *E. coli* strain deficient for arsenate reductase, the cells acquired improved arsenate tolerance and arsenate reduction, suggesting that *P. vittata* triosephosphate isomerase functioned as an arsenate reductase (Rathinasabapathi et al. 2006).

19.6 Arsenite Uptake

P. vittata took up both arsenate and arsenite forms of arsenic from the substrate. Arsenite uptake in plants is known to be via aquaporin of the NIP (nodulin26-like intrinsic protein) subfamily (Ali et al. 2009). However, experimental evidence for the operation of NIP type aquaporins in *P. vittata* is not available (Wang et al. 2009). It will be most interesting to find out whether aquaporins are involved in arsenite transport in this fern.

19.7 Arsenic Transport within the Plant

Kertulis et al. (2005) found that arsenic is transported from the roots to the frond via the xylem in the form of arsenate. This was also confirmed by Su et al. (2008) except that the arsenic in the xylem sap was reported to be mainly in the form of arsenite (Su et al. 2008). However, there were clear methodological variations between the two studies, especially about how the tissues were sampled and defined in the latter study. More studies are needed to define where arsenate is reduced and how it is transported to the fronds.

Proteins or small molecular weight ligands that may have roles in arsenic translocation within the fern are not yet known. It has been proposed, with some experimental support, that arsenite in the fern frond was localized within the vacuoles (Lombi et al. 2002; Pickering et al. 2006; Yang et al. 2009). A recent study reported the identification of a vacuolar arsenite transporter from P. vittata (Indriolo et al. 2010)

19.8 Oxidative Stress Tolerance

Arsenic causes oxidative stress in cells. Arsenite binds to vicinal thiols in proteins and inhibits iron sulfur proteins, inducing oxidative stress. Early investigations showed that *P. vittata* is more tolerant to arsenic-induced oxidative stress compared to *P. ensiformis*, an arsenic-sensitive fern in the same genus (Srivastava et al. 2005b; Singh et al. 2006). *P. vittata* fronds contained increased amounts of ascorbate and glutathione and several antioxidant enzymes (Srivastava et al. 2005b; Singh et al. 2006). In a study to identify oxidative stress tolerance-related genes in *P.vittata*, we have expressed *P. vittata* cDNAs in *E. coli* and selected recombinant *E. coli* for arsenate tolerance. This approach identified a cDNA for glutaredoxin (PvGRX5) to be important for arsenate tolerance (Sundaram et al. 2008). Glutaredoxins are glutathione-dependent oxidoreductases whose functions in plants are not well understood.

Transgenic overexpression of PvGRX5 in model plants increased arsenic resistance but reduced arsenic accumulation (Sundaram et al. 2009). Although glutaredoxin is an electron donor for arsenate reductase, PvGRX5's role in arsenic tolerance appears to be more than this, because its expression in *E. coli* deficient of arsenate reductase also increased arsenic resistance (Sundaram et al. 2008).

19.9 Utilities of Brake Fern for Phytoremediation

Since the discovery of brake fern's arsenic hyperaccumulation, intensive efforts are underway to use *P. vittata* for arsenic phytoremediation. Demonstration phytoremediation experiments have succeeded to remove arsenic from contaminated water (Natarajan et al. 2009) and soil (Gonzaga et al. 2008) to acceptable levels by repeated biomass harvests of *P. vittata*. Edenspace (http://www.edenspace.com/) in the USA sells *P.vittata* for field phytoremediation applications. Harnessing the beneficial effects of mycorrhizal fungi (Al Agely et al. 2005; Trotta et al. 2006; Wu et al. 2007) and arsenic resistant bacteria (Huang et al. 2010) have been proposed as ways to improve the efficiency of arsenic phytoremediation by *P. vittata*. Current research is also focused on methods to process the arsenic-containing fronds for hazardous waste storage, including incineration and composting.

19.10 Brake Fern as a Source of Genes for Biotechnology

Because much of fern evolution has happened in parallel to angiosperm evolution – in the shadow of the angiosperms (Schneider et al. 2004) – ferns were exposed to extreme environmental conditions including nutrient-poor environments, soils contaminated with toxic material, extremes of temperature, and high altitudes. This has resulted in a rich diversity of fern species with natural adaptations to stress. The author therefore proposed that ferns could be sources of stress-tolerance genes for biotechnology (Rathinasabapathi 2006).

Among the stress tolerant ferns, *P. vittata* is unique with extraordinary adaptations to arsenic and other stress factors. It may be possible in the future to utilize genes from *P. vittata* to engineer fast-growing trees to hyperaccumulate arsenic thus improving the efficiency of the phytoremediation process. Additionally, *P. vittata* and other ferns could provide us with insights for metabolic engineering of crops for improved stress tolerance. Our success in using the glutaredoxin cDNA from *P. vittata* (Sundaram and Rathinasabapathi 2010) for improving high temperature stress tolerance in model species suggest that such strategies are indeed viable.

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Chapter 20
Aerobiology of Pteridophyta Spores: Preliminary Results and Applications

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20.1 Introduction

Aerobiology is the science of airborne biological and non biological particles (viable or non viable), of their origin, transport and deposition in relation to meteorological conditions and their impact on human beings, animals or plants (Lebowitz and O'Rourke 1991; Spieksma 1992).

The field of aerobiology is interdisciplinary with applications in public health, agriculture, plant pathology, palynology and many others. Biology, plant physiology, mycology, meteorology and aerosol physics are the basic sciences for studying production, release, transport and deposition of airborne particles.

Some of airborne non biological particles could be considered as atmospheric pollutants, whose harmful effects on human health are well known (Kim et al. 2001; Cariñanos et al. 2007). The most studied airborne biological particles are pollen grains and fungal spores, with their implications on allergy, phenology, farming productivity, environment or even criminology widely addressed in the literature (Chuine et al. 1999; D'Amato 2000; Cunha et al. 2003; Bryant and Jones 2006; García-Mozo et al. 2006; Sánchez et al. 2009). Nevertheless, little is known about fern spores airborne behaviour and their possible applications on other fields, probably due to the scarce presence of Pteridophyta spores in the atmosphere in comparison with airborne levels of pollen grains and fungal spores (Favali et al. 2003).

On the basis of recent phylogenetic studies within vascular plants (Renzaglia et al. 2000; Pryer et al. 2004), lycophytes (including Lycopodiales, Selaginellales and Isoetales) have been separated from other groups included in the traditional group of Pteridophyta, such as Equisetales, Psilotales, Marattiales and Polypodiales. The latter ones, included in monilophytes group, are now related to seed plants (spermatophytes), both constituting the euphyllophytes clade. In this chapter, we follow the recent classification realised by Smith et al. 2006 for extant ferns, taking

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into account the historical assemblage of pteridophyte that includes together lycophytes and monilophytes due to their common features, i.e., spore-bearing.

We aimed to examine the main works carried out about fern spores aerobiology, in order to gain a better knowledge along with the current and future implications derived of these studies.

20.2 Methodology in Aerobiology Studies

There are different methods used to identify and quantify airborne pollen and spores content, being gravimetric and volumetric methods and their corresponding samplers, the most employed in aerobiological literature. The first one collects mainly airborne particles that fall by their own weight, whereas the volumetric traps capture these particles directly from the atmosphere.

The gravimetric method uses non-volumetric sedimentation traps, such as Tauber type (Tauber 1967), in which the number of grains obtained depends on the area of sedimentation, by means of adding a known spike of *Lycopodium* spores for estimation of absolute pollen and spore values. The samples are obtained monthly, weekly or even daily. Then, these samples are processed by acetolysis techniques and mounted in medium of gelatin-glycerol, counting after a fixed number of biological particles (Hall 1994). The results are expressed as particles trapped per temporal unit (day, week or month) and per square centimetre.

The volumetric method works mainly by volumetric suction samplers based on the impact principle (Hirst 1952). These samplers have two essential components: a vacuum pump whose suction flow rate is 10 L air/min, similar to the volume of air inhaled in humans; and a circular drum driven by a clockwork mechanism that rotates 2 mm every hour, ensuring continuous air sampling and the provision of hourly data. Thus, samples could be obtained weekly, daily or even hourly, and mounted in traditional-used medium of fuchsin-stained glycerine gelatin. The airborne data are expressed as particles per cubic metre of air and per day, after microscopic reading of samples and some standardised mathematical operations based mainly on the size of microscope field of vision used (Domínguez et al. 1991).

Both methods give similar quantitative and qualitative results for weekly total biological particles levels (Levetin et al. 2000), but volumetric method has more sensitive detection of these particles at low concentration (Latorre et al. 2008), an important aspect in allergic studies.

20.3 Aerobiological Notes of Pteridophyta

Pteridophyta includes more than 9,000 species in about 215 genera, distributed worldwide (Strasburguer et al. 2004) and attaining their greatest number and main development in the tropics and subtropics. The same pattern was registered in airborne spore content of Pteridophyte, where of the 49 taxa that have been

distinguished in the few papers that reported data about aerobiology of ferns (Table 20.1), 42 belonged to researches carried out in tropical and subtropical zones of Asia. In that sense, we must point out the work of Yasmeen and Devi (1988) that informed about the airborne presence of 28 types of fern spores. There were only two studies dealing about atmospheric fern spores content in Africa (La Serna and Domínguez 2003; Njokuocha 2006), reporting 16 Pteridophyte spores. The number of spore types could be higher if more researches have been developed in that area, as also occurred in America, with three papers, especially in Buenos Aires and their surroundings (Majas and Romero 1992; Noetinger et al. 1994). On the other hand, five taxa were identified in the six works carried out in Europe. In other published papers about different aspects of palynology (Carson and Brown 1976; Calleja et al. 1993; Cabrales et al. 2003), fern spores were cited in the atmosphere of different tropical areas of Africa, Asia and America without any qualitative analysis.

The total number of fern spores depended on the method used and the spore type. According to annual total data reported in different years and different zones by means of gravimetric method (Fig. 20.1a), *Alsophila* and *Dicranopteris* were the main genera, with 300 and 140 spores per cm^2, respectively, between June 1974 and May 1975 in Taipei Basin (Chen and Huang 1980), followed by *Nephrolepis*, *Pteris* and Polypodiaceae with 49, 9 and 5 spores per cm^2. In Buenos Aires area, 107 spores per cm^2 of *Pteridium* were counted through year 1987 (Majas and Romero 1992), and 53 spores of *Equisetum* from July 1989 to June 1990 (Noetinger et al. 1994).

Volumetric method gave data about some fern taxa (Fig. 20.1b), like *Dicranopteris* with a total of 3,530 spores during year 2003 in Kinmen Island, Taiwan (Huang et al. 2008), the main fern taxa counted in Asia, followed by *Alsophila*, *Pteris* and *Lycopodium* with 40, 10 and 5 spores, respectively. A great number of spores of *Pteridium* were registered in an experimental farm of Great Britain (Lacey and McCartney 1994) between 20 August and 20 October, with around 40,000 spores. A lower annual average number of *Pteridium* spores were counted in other urban areas of Europe, such as Edinburgh (Caulton et al. 2000), with 118 spores during 1988–1997 period, and Salamanca (Rodríguez et al. 2009) with 67 spores between January 1998 and December 2007. In Rzeszów (Kasprzyk 2004) along a 6-year period (1997–2002), a mean annual total of 42, 12 and 5 spores of Polypodiaceae, *Equisetum* and *Lycopodium*, respectively, were registered.

The seasonal variations of airborne Pteridophyta spores levels were different depending on the area analysed (Fig. 20.2). In Asia, fern spore concentrations were continuous over the year in India (Yasmeen and Devi 1988), having higher levels between late Spring and early Autumn in Taiwan (Chen and Huang 1980; Chen and Chien 1986). In Nigeria (Njokuocha 2006), lower spore counts were registered between February and April, whereas in Buenos Aires (Majas and Romero 1992), lower levels were counted in January, March and between May and July. The presence of fern spores over all the months could be related with the high diversity and abundance of Pteridophyte species in tropics and their sequential development of sporangia, because in temperate areas (Nilsson and Praglowski 1974) there were

Table 20.1 Identified airborne taxa of Pteridophyte following classification proposed by Smith et al. (2006)

Division Tracheophyta								
Subdivision	Class	Order	Family	Genera	Africa	America	Asia	Europe
Lycophytina	Lycopsida	Lycopodiales	Lycopodiaceae	*Lycopodium*			2,4,5	7,13
	Selaginellopsida	Selaginellales	Selaginellaceae	*Selaginella*			17	
Euphyllophtina	Psilotopsida	Ophioglossales	Ophioglossaceae			14		
	Equisetopsida	Equisetales	Equisetaceae	*Equisetum*		10,14		7
	Polypodiopsida	Gleichniales	Gleichniaceae	*Dicranopteris*			2,3,4,5	
				Diplopterygium			2	
		Schizales	Anemiaceae	*Anaemia*			17	
			Lygodiaceae	*Lygodium*			2	
		Salviniales	Salviniaceae	*Azolla*		10		
		Cyatheales	Cyatheaceae	*Alsophila*			2,3,4,5	
				Cyathea			16	
		Polypodiales	Lindsaeaceae	*Lonchitis*	12			
				Tapeinidium			4	
			Dennstaedtiacaee	*Dennstaedtia*			2	
				Hypolepis	12		4	
				Microlepia			4,17	
				Pteridium	8	10	2,3	1,9,11,15
			Pteridaceae	*Acrostichum*			17	
				Adiantum	8	14	17	
				Cheilanthes			17	
				Notholaena			17	
				Pteris	12		4,5,17	
			Aspleniaceae	*Asplenium*	8,12		4,17	
			Thelypteridaceae		12		4,17	
				Cyclosorus	12		2,17	
			Woodsiaceae		12	14	4,17	
				Diplazium			2	

Blechnaceae		12	10,14	17	
	Stenochlaena			17	
Dryopteridaceae				2	
	Bolbitis			17	
	Cyrtomium			17	
	Dryopteris	8,12		17	13
	Elaphoglossum	12			
	Lithostegia			17	
	Polystichum			17	
Lomariopsidaceae	*Nephrolepis*	12	6,14	4,17	
Tectariaceae	*Tectaria*			17	
Davalliaceae	*Davallia*	8,12		4	
Polypodiaceae	*Colysis*			2,17	
	Drymoglossum			17	
	Drynaria			17	
	Elaphoglossum	12			
	Goniophlebium			17	
	Lemmaphyllum			2	
	Lepisorus			17	
	Mircrogramma	12			
	Microsorum			17	
	Polypodium	8	6,10	4,17	7,13

Caulton et al. (2000), Chen (1984), Chen and Chien (1986), Chen and Huang (1980), Huang et al. (2008), Hurtado and Riegler-Goihman (1986), Kasprzyk (2004), La Serna Ramos and Domínguez Santana (2003), Leitão et al. (1996), Majas and Romero (1992), Newson et al. (2000), Njokuocha (2006), Nilsson and Praglowski (1974), Noetinger et al. (1994), Rodríguez de la Cruz et al. (2009), Yang et al. (2003), Yasmeen and Devi (1988) (Underlined references correspond to studies carried out by means of gravimetric methods)

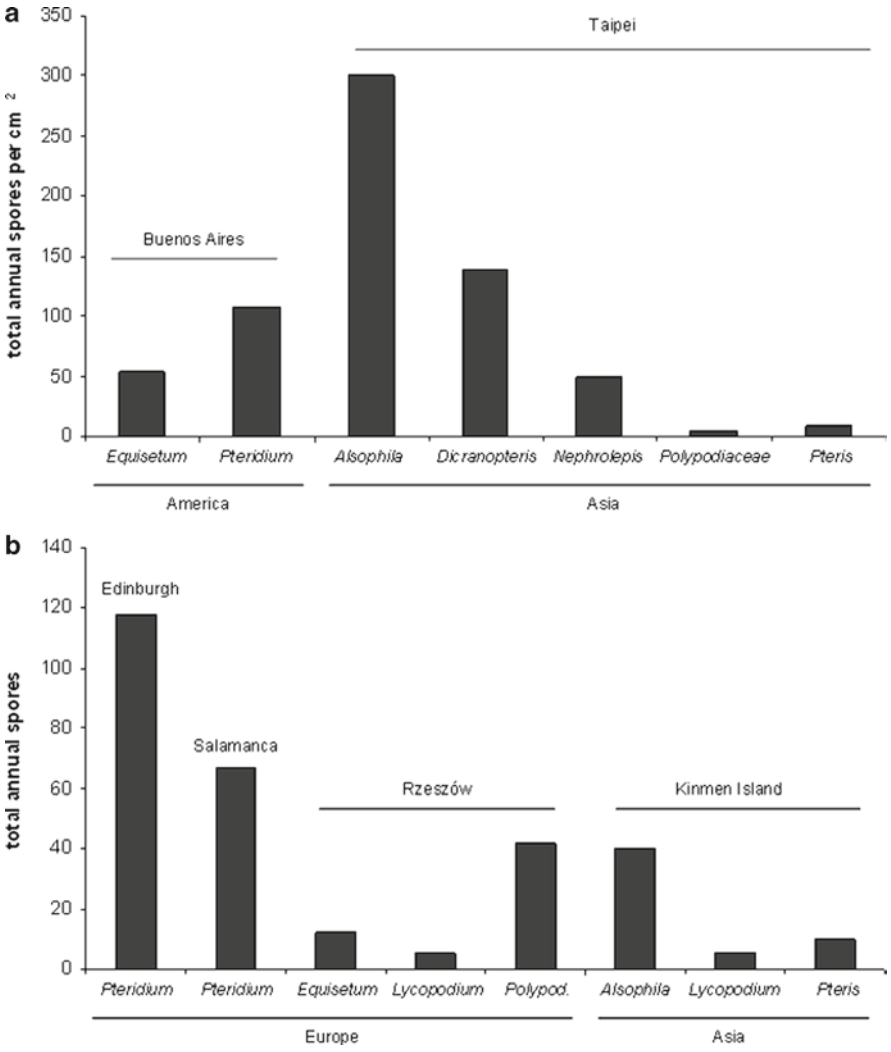

Fig. 20.1 Annual total records of some fern spores obtained by means of volumetric method (**a**), excluding *Dicranopteris*, or gravimetric method (**b**)

seasonal occurrence of fern spores between late Summer and early Autumn, specially in the case of *Pteridium* (Caulton et al. 2000), or even in early Spring in the case of *Equisetum* (Kasprzyk 2004).

Daily maximum concentrations of fern spores were reported in Asia (Chen 1984) for *Alsophila* (523 spores/cm^2, 25 July), *Dicranopteris* (148 spores/cm^2, 1 July) and *Pteridium* (82 spores/cm^2, 7 July) during the year 2003 by means of gravimetric method. The use of volumetric method registered some data about daily peaks of airborne spore levels, being 1,800 spores/m^3 of *Pteridium* during 4 September 1990, the highest daily value counted (Lacey and McCartney 1994). This value

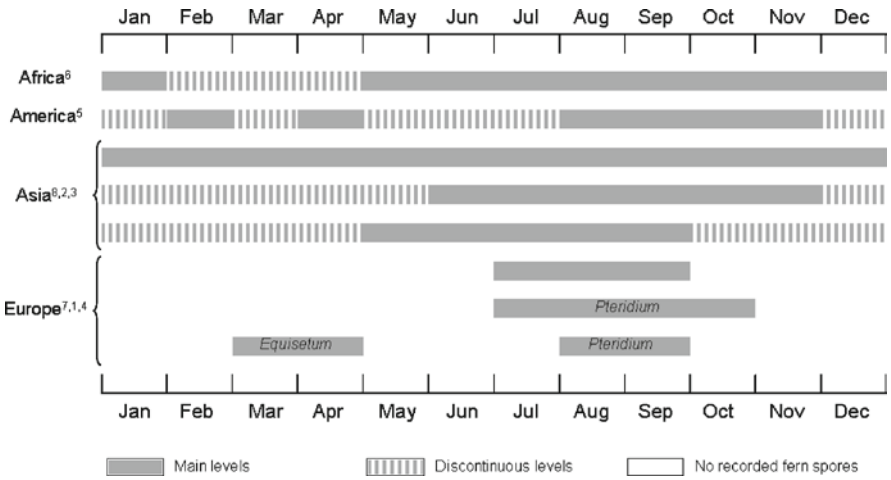

Fig. 20.2 Seasonal distribution of Pteridophyte spores (Caulton et al. 2000; Chen and Chien 1986; Chen and Huang 1980; Kasprzyk 2004; Majas and Romero 1992; Njokuocha 2006; Nilsson and Praglowski 1974; Yasmeen and Devi 1988)

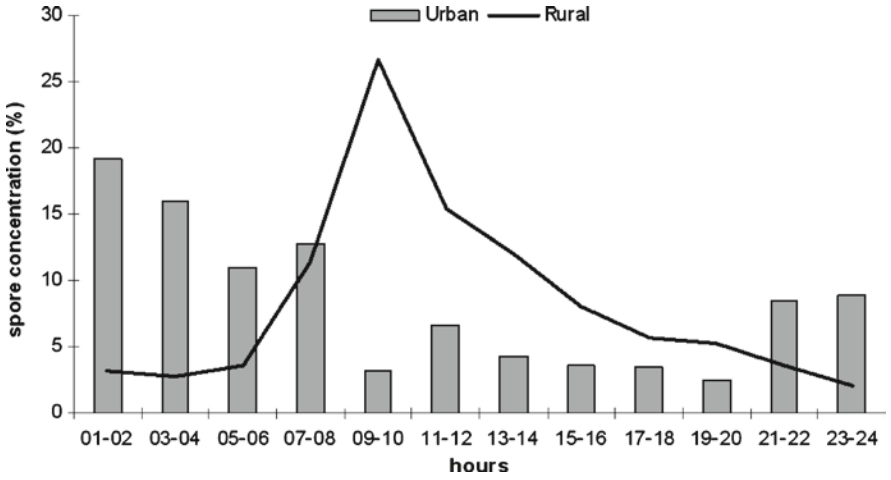

Fig. 20.3 Bi-hourly variation of bracken spore concentrations in urban and rural areas, mean daily counts (%)

could be explained with the close location of spore-trap to bracken thickets and could be also joined with intra-diurnal pattern, because in this rural area (Fig. 20.3) the levels were higher between 08:00 hours and midday, when sporing process occurs (Conway 1957). In urban areas of temperate zones, bracken and other ferns main populations are usually located far away (Favali et al. 2003), enabling higher airborne concentrations at night (Rodríguez et al. 2009), due to a probable medium-range transport according to different ways of atmospheric transport proposed by Rantio-Lehtimäki (1994). Regional transport was proved in *Castanea* pollen (Peeters and

Zooler 1988) and it was proposed for *Lycopodium* spores (Di-Giovanni et al. 1995), as well as other anemophilous pollen types.

20.4 Perspectives and Applications

Some of airborne biological particles present allergens, both included in the term "air pollution" and whose exposure represents a key factor among environmental determinants of allergy symptoms (Eder et al. 2006). The prevalence of pollen and fungal spores is increasing nowadays, especially in urban areas (Bush and Portnoy 2001; D'Amato et al. 2007). The importance of fern spores in allergic diseases was pointed out by Geller-Bernstein et al. (1987), by means of skin prick tests (SPT). Fern taxa with allergenic relevance are listed in Table 20.2, having extracts of spores in two species of *Dicranopteris* Bernh. (Chew et al. 2000), resam-fern (*Dicranopteris linearis* (Burm.f.) Underw.) and roadside fern (*Dicranopteris currani* Copel.) the highest frequency in SPT, with 34% and 31% percentages, respectively, followed by fishbone fern (*Nephrolepis auriculata* (L.) Trimen) and bird's nest fern (*Asplenium* nidus L.), with 30% and 25%, respectively, in Singapore. In addition, different works concerning the carcinogenicity of fern spores (Povey et al. 1996; Simán et al. 2000; Freitas et al. 2001) have indicated the risk for human health due to these spores which might be inhaled and ingested by humans (Wilson et al. 1998). Monitoring atmospheric fern spore levels is relevant in order to diminish exposure to fern allergens and to avoid the carcinogenic implications to human health.

In accordance with the clinical importance of atmospheric fern spore counts, climate change scenarios could be related to a greater production of airborne biological particles as pollen and fungal spores and propagules (Ziska et al. 2009) and might affect spore production in some fern spores, because in a model developed in some areas of Great Britain (Pakeman and Marrs 1996), bracken biomass (*Pteridium aquilinum* (L.) Kuhn.) would increase its presence and subsequently result in a greater spore liberation from sporangia.

Finally, some studies of modern biological particles spectra in caves can provide a reliable reflect of regional and local vegetation, directly comparable to the more

Table 20.2 Allergenic pteridophyte spores

Fern taxa	References
Acrostichum aureum L.	Yasmeen and Devi (1988), Bunnag et al. (1989)
Asplenium nidus L.	Chew et al. (2000)
Dicranopteris curranii Copel.	Chew et al. (2000)
D. linearis (Burm.f.) Underw.	Chew et al. (2000)
Lycopodium L.	Devi et al. (1989)
Nephrolepis auriculata (L.) Trimen	Chew et al. (2000)
Pteridium aquilinum (L.) Kuhn.	Chew et al. (2000)
Stenochlaena palustris (Burm.f.) Bedd.	Chew et al. (2000)

conventional spectra obtained from open sites (Coles and Gilbertson 1994; Navarro et al. 2002). In addition, fern spores have importance in a full interpretation and also in an accurate reconstruction of paleoenvironments (Barth et al. 2004; Zhu et al. 2008)

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Part IV
Therapeutical/Medicinal Applications

Chapter 21
Studies on Folk Medicinal Fern: An Example of "Gu-Sui-Bu"

Hung-Chi Chang, Sushim Kumar Gupta, and Hsin-Sheng Tsay

21.1 Introduction

Ferns and their allies are in a major division of the Plant Kingdom called Pteridophyta, and they have been around for millions of years. At one time they were a dominant part of the earth's vegetation. Ferns tend to be distributed in wetter parts of the world but it is not uncommon to find ferns in very dry or severely cold conditions. The majority inhabits wet, tropical forests and can be found from sea level to mountains over 15,000 feet in altitude. There are over 250 different genera of ferns and about 12,000 species. Ferns differ from other plants in that they do not produce flowers or seed, instead they reproduce from spores. Spores are one-celled microscopic structures that cannot be seen with the naked eye. These spores are found most often on the underside or edges of the frond, and in some cases are borne on separate stalks. Ferns and their allies are clearly unique in the modern plant world and differ in many ways from the flowering plants. Fern like plants occupy a thick slice of the fossil record; this varied group of plants includes some of the oldest species extant on the planet. They evolved from the earliest vascular plants that made their way onto land, and fossil evidence of their existence can be traced back more than 350 million years to the Devonian period.

The Ayurvedic systems of medicine referred by Sushruta (ca AD 100) and Charka (ca AD 100) recommended the medicinal uses of some ferns in their book Samhitas. Ferns are also used by the physicians in Unani system of medicine (Uddin et al. 1998). Later, modern biological and pharmaceutical studies were carried out on pteridophytes by different researchers. Benerjee and Sen (1980) conducted the only extensive survey of antibiotic activity among the ferns and reported about a hundred species having such property. Dixit and Vohra (1984) reported edible and medicinally important pteridophytic species from India. In the Chinese system of medicine, many ferns are also prescribed by local doctors (Kimura and Noro 1965).

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About 300 kinds of ferns were used as Chinese traditional medicinal herbs for the treatment of common cold, diarrhea, suffer burn, trauma bleeding, ascarid disease, and showed many bioactivities, such as antioxidant, antimicrobial, antiviral, anti-inflammatory, antitussive, antitumor, and anti-HIV. In the Chinese traditional system of medicine, "Gu-Sui-Bu" has been used for a long time in the treatment of bone injuries (Chang et al. 2003). It has been proved very effective for the treatment of inflammation, hyperlipemia, and arteriosclerosis (Anonymous 2005). Also, in several recent studies, the medicine has been claimed to have therapeutic effects on bone formation and healing (Ma et al. 1996; Sun et al. 2004; Jeong et al. 2005; Wong and Rabie 2006). However, in Chinese Material Medica, different herbs from different areas with similar common names have been mentioned for treatment of the same disease. This is evident from field records, visits to local traditional doctors, review of specimens in herbaria, and the available literature. Rhizomes of *Drynaria fortunei* (Kze.) J.Sm., *Pseudodrynaria coronans* (Wall. ex Mett.) Ching (both from Polypodiaceae), *Davallia divaricata* Bl., *Davallia mariesii* Moore ex Bak, *Davallia solida* (Forst.) Sw., and *Humata griffithiana* (Hk.) C.Chr. (from Davalliaceae) are used or called as "Gu-Sui-Bu" or "Shibu" in Taiwan. These have been claimed to cure body ache, inflammation, cancer, aging, blood stasis and bone injuries. In a publication by Pharmacopoeia Commission of People Republic of China, only *D. fortunei* has been reported as source of "Gu-Sui-Bu" (Anonymous 2005), however, no systematic investigation has been carried out so far, to evaluate comparative values of these different sources.

Over the past few years, investigations for phenolic compounds in medicinal herbs have gained importance due to their high antioxidative activity (Rice-Evans et al. 1997; Pietta et al. 1998; Zhu et al. 2004). A large numbers of reports have demonstrated that these compounds are of great value in preventing the onset and/or progression of many human diseases (Parshad et al. 1998; Lee et al. 2000). Polyphenols have many favorable effects on human health, such as the inhibition of the oxidization of low-density proteins (Frankel et al. 1993), thereby decreasing the risk of heart disease (Williams and Elliot 1997). These compounds have anti-inflammatory and anti-carcinogenic properties (Carrol et al. 1999; Maeda-Yamamoto et al. 1999). Also, flavonoids and many other phenolic compounds of plant origin have been reported as scavenger reactive oxygen species (ROS), and are viewed as promising therapeutic drugs for free radical pathologies (Parshad et al. 1998; Lee et al. 2000). Thus, measurement of the polyphenols and antioxidant activity in herbs have become important tools to understand the relative values of plant species, especially from the health point of view.

Since a finger-thick fleshy rhizome part of the fern is used in "Gu-Sui-Bu" medicine, plant is rapidly disappearing from natural habitats due to over exploitation. Currently, the plant is not under cultivation. Development of in vitro propagation procedure may help in promotion of its cultivation and compensation of its loss in the wild.

The present chapter describes different studies on "Gu-Sui-Bu," which include comparative antioxidative potencies, scavenging activities against DPPH radical, reducing power, and estimation of polyphenol contents and conservation of *D. fortune* by in vitro methodologies.

21.2 Comparative Analysis of Different Phenolic Compounds and Their Activities in Six Different "Gu-Sui-Bu"

Aqueous and organic extract were used to analyze different phenolic compounds and their quantitative analyses are listed in Table 21.1.

21.2.1 Antioxidant Activity

Antioxidants have been defined as substances that, when present at low concentrations compared with oxidizable compounds (e.g., DNA, protein, lipid, or carbohydrate), delay or prevent oxidative damage due to the presence of reactive oxygen species (ROS). Oxidative stress results from an imbalance of oxidizing species and natural antioxidants in the body, and it has been thought to have contributed to aging, cell apoptosis, and severe diseases such as cancer, Parkinson's disease, Alzheimer's disease, and even cardiovascular disorders (Giasson et al. 2002). These ROS undergo redox reactions with phenolics, resulting in inhibition of antioxidant activity in concentration dependant manner (Halliwell and Gutteridge 1990). Thus, measurement of total polyphenols and its constituents along with antioxidant activity has increasingly been used in plant samples and has become an important tool for investigation (Gordon 1990; Veglioglu et al. 1998).

21.2.2 Antioxidant Activity by ABTS Assay

Aqueous and ethanol extract (Table 21.1) were used for ABTS assay (Miller and Rice-Evans 1997). Antioxidant properties of "Gu-Sui-Bu" extracts were expressed as Trolox Equivalent Antioxidant Capacity (TEAC), calculated from at least three different concentrations of extract tested in the assay giving a linear response. TEAC values determined from the calibration curve for six sources of "Gu-Sui-Bu" are shown in Fig. 21.1. Antioxidant activities of both aqueous and ethanol extracts of the six sources were in the following decreasing order: DMW (1.27 mM) > DDW (0.96 mM) > DSW (O.91 mM) > PCW (0.77 mM) > DSE (O.32 mM) > DFW (0.26 mM) > HGE (0.18 mM) > DME and DDE (0.17 mM) > PCE (0.11 mM) > DFE (0.07 mM). Thus, it was observed that most samples in aqueous extracts had higher antioxidant potencies than ethanol extracts (Chang et al. 2007b).

21.2.3 Antioxidant Activity by Dot-Blot and DPPH Staining

The free radical scavenging activity of the extract was screened by the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The reduction of the DPPH was determined on TLC plate spotted with equal volume of samples loaded in order of

Table 21.1 Particulars of six sources of medicinal fern used as "Gu-Sui-Bu," their comparative yields in ethanol and aqueous extracts

		Ethanol extract		Aqueous extract		Medical use/disease treated
Species	Common name	Code	Yield (%)[a]	Code	Yield (%)	
Drynaria fortunei (Polypodiaceae)	Gusuibu	DFE	11.2	DFW	12.8	Inflammation, hyperlipemia and arteriosclerosis (Anonymous 2005); Cancer (Cai et al. 2004)
Pseudodrynaria coronans (Polypodiaceae)	Gusuibu	PCE	15.0	PCW	18.6	Bone injuries, tinnitus, and lumbago (Anonymous 1999)
Davallia divaricata (Davalliaceae)	Dayegusuibu	DDE	14.6	DDW	20.4	Bone injuries, tinnitus, and lumbago (Anonymous 1999); Joint pain (Hwang et al. 1989)
D. mariesii (Davalliaceae)	Haizhougusuibu	DME	8.5	DMW	29.0	Common cold, neuralgia, stomach cancer, lumbago, rheumatalgia, odontalgia, and tinnitus (Cui et al. 1990)
D. solida (Davalliaceae)	Koyegusuibu	DSE	12.7	DSW	14.5	Body ache, inflammation, cancer, and bone injuries
Humata griffithiana (Davalliaceae)	Begaigusuibu	HGE	6.7	HGW	16.1	Body ache, inflammation, cancer, and bone injuries

[a]Dry weight basis

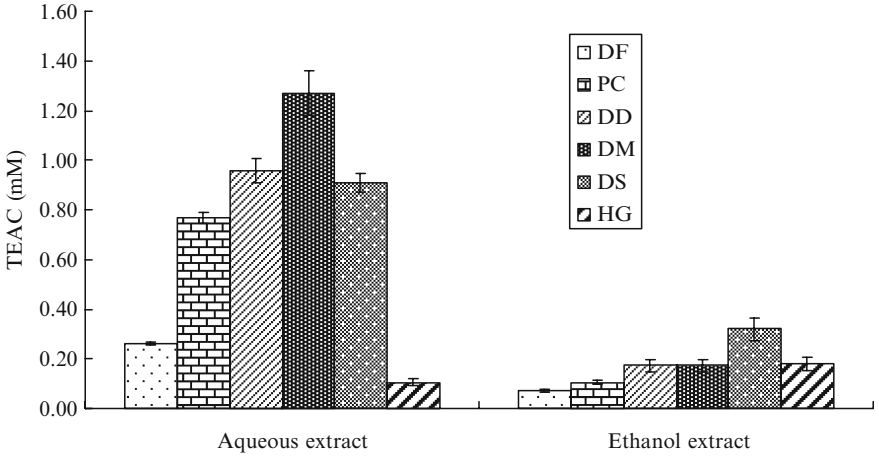

Fig. 21.1 Trolox equivalent antioxidant capacity (*TEAC*) values of aqueous and ethanol extracts of six sources of Gusuibu. *DF Drynaria fortunei*, *PC P. coronans*, *DD D. divaricata*, *DM D. mariesii*, *DS D. solida*, *HG H. griffithiana*. In text, all ethanol extracts have been designated as E and aqueous extracts as W after the species code

decreasing concentration along the row. The staining of the silica plate was based on the procedure of Soler-Rivas et al. (2000). It was observed that, white spots with strong intensity appeared fast up to the dilution of 25 μg mL^{-1} for DSE (the final amount in the spot: 0.075 μg dry matter), 50–100 μg mL^{-1} for PCE, PCW, DDE, DDW, DSW, and HGE (final amount: 0.15–0.3 μg dry matter); and the lowest intensities of DFE, DFW, and HGW. For antioxidant analysis, fast-reacted and strong intensities of white spots appeared were also up to dilutions of 50–100 μg mL^{-1} of BHT and GSH (final amount: 0.15–0.3 μg dry matter). Appropriate dilution reacts positively with DPPH depending upon their free radical scavenging capacity (RSC), its nature (Chang et al. 2002) and results in a dot of certain diameter and color intensity, which is an indication of radical scavenging capacity. Darker dot indicates higher RSC value. According to the color intensities, RSC values of ethanol extracts in decreasing order were as follows: DSE (E_5) > DDE (E_3) > HGE (E_6) > DME (E_4) > PCE (E_2) > DFE (E_1), while RSC values of aqueous extracts in the decreasing order were as follows: DMW (W_4) > DSW (W_5) > DDW (W_3) > PCW (W_2) > DFW (W_1) > HGW (W_6) (Fig. 21.2) (Chang et al. 2007b).

21.2.4 Determination of "Reducing Power"

The reducing power of the extracted samples (dissolved in ethanol or distilled water), glutathione (GSH, dissolved in distilled water), or butylated hydroxytoluene (BHT, dissolved in ethanol) was determined according to the method of Jayaprakasha et al. (2002). Increase in absorbance of the reaction indicated the reducing power of the

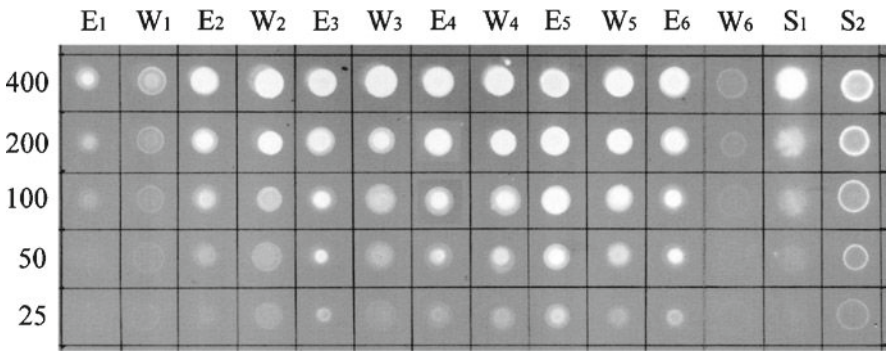

Fig. 21.2 Dot-blot assay of free radical scavenging capacity (*RSC*) on a silica sheet stained with a DPPH solution in methanol. Each 3 μL of plant extract (400, 200, 100, 50, and 25 μg mL^{-1}) was applied from top to down. Dots from left to right are: *Drynaria fortunei* (E_1, W_1), *P. coronans* (E_2, W_2), *D. divaricata* (E_3, W_3), *D. mariesii* (E_4, W_4), *D. solida* (E_5, W_5), and *H. griffithiana* (E_6, W_6). Antioxidants; butylated hydroxytoluene (*BHT*) (S_1), glutathione (GSH) (S_2) were used as positive controls (E_1–E_6: Ethanol extracts; W_1–W_6: Aqueous extracts)

samples. EC_{50} value (μg extract mL^{-1}) was the effective concentration at which the absorbance was 0.5 for reducing capacity and was obtained by interpolation from linear regression analysis. Each sample extract exhibited the scavenging activity and was found to be dose-dependent (data not shown). The EC_{50} values are given in Table 21.2. It was found that ethanol extract of fern species *Davallia solida* (DSE) had the highest radical scavenging activity (expressed as μg extract/mL) (26.89), followed by DDW (52.81), DDE (87.95), HGE (93.50), PCW (98.23), DSW (166.72), DMW (173.03), and PCE (190.81). Lower radical scavenging activities (>400) were observed in case of DFE, DFW, and HGW. The EC_{50} scavenging ability on DPPH radicals of standard sample glutathione (GSH) was lower (89.64) compared to BHT (42.28).

21.2.5 Scavenging Activity Against DPPH Radical

This is the most widely reported method for screening of antioxidant activity of many medicinal herbs (Blois, 1958; Duh, 1998). DPPH assay method is based on the reduction of methanolic solution of colored free radical DPPH by free radical scavenger. The percentage of DPPH discoloration of the sample was calculated according to the equation: % discoloration = (Abs control − Abs sample/Abs control) × 100. EC_{50} value (μg extract/mL) is the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis. GSH and BHT were used as positive controls for comparison. Extracts of all six sources of "Gu-Sui-Bu" exhibited the reducing powers and were concentration dependent. The reducing powers (expressed as μg extract/mL) of the samples studied have been given in Table 21.2. Lower the EC_{50} values means higher the reducing capacity. It was found that the aqueous extract of fern species *Davallia mariesii*

Table 21.2 EC_{50} values of ethanol and aqueous extracts in antioxidant properties of six sources of "Gu-Sui-Bu"

	EC_{50} value (μg extract mL^{-1})[a]			
	Reducing capacity		Scavenging ability on DPPH radicals	
Sources of Gusuibu[b]	Ethanol extract	Aqueous extract	Ethanol extract	Aqueous extract
Drynaria fortunei (DF)	>400	193.55 ± 1.31 A	>400	>400
Pseudodrynaria coronans (PC)	>400	38.96 ± 0.14 CD	190.81 ± 4.15 A	98.23 ± 14.36 B
Davallia divaricata (DD)	349.46 ±2.53 A[c]	43.36 ± 1.53 C	87.95 ± 0.88 B	52.81 ± 1.01 C
D. mariesii (DM)	364.55 ± 15.83 A	26.58 ± 0.26 E	95.87 ± 8.42 B	173.03 ± 10.68 A
D. solida (DS)	185.10 ± 15.25 D	32.16 ± 0.04 DE	26.89 ± 0.53 D	166.72 ± 7.24 A
Humata griffithiana (HG)	317.80 ± 19.90 B	>400	93.50 ± 8.60 B	>400
Glutathione (GSH)	ND[d]	135.11 ± 12.25 B	ND	89.64 ± 2.88 B
2,6-Di-tert-butyl-4-methylphenol (BHT)	229.01 ± 0.60 C	ND	42.28 ± 0.87 C	ND

[a]EC50 value: the effective concentration at which the absorbance was 0.5 for reducing capacity; 1,1′-diphenyl2-picrylhydrazyl (DPPH) radicals were scavenged by 50%. EC_{50} value was obtained by interpolation from linear regression analysis

[b]In text, all ethanol extracts have been designated as E and aqueous extracts as W after the species code

[c]Each value is expressed as mean ± standard deviation ($n = 3$). Means with different letters within a column are significantly different ($p < 0.05$)

[d]ND: not detected

(DMW) had higher degree of reducing power (26.58), followed by DSW (32.81), PCW (38.96), DDW (43.36), DSE (185.10), DFW (193,55), HGE (317.8), DDE (349.46), and DME (364.55). DFE, PCE, and HGW showed lower reducing power (>400). Between the two standards, aqueous extract of glutathione (GSH) had more reducing power (135.11) compared to BHT (229.01).

21.2.6 Total Polyphenols Content (Flavonoids, Flavonols, Condensed Tannin, and Proanthocyanidin) Determination in "Gu-Sui-Bu"

Total phenolic compounds were estimated using the Folin- Ciocalteu method (Ragazzi and Veronese 1973). The total polyphenols, flavonoids, flavonols, condensed tannin, and proanthocyanidin in ethanol and aqueous extracts of six sources of "Gu-Sui-Bu" are given in Table 21.3. Total polyphenols content (expressed as μg catechin equivalent/mg dry weight) varied significantly among the ethanol or aqueous extracts of six sources of "Gu-Sui-Bu." The quantities of total polyphenols ranged from a minimum 53.44 μg in ethanol extract of *D. fortunei* (DFE) to the

Table 21.3 Phenolic contents in ethanol and aqueous extracts of six sources of "Gu-Sui-Bu"

Sources of Gusuibu[a]	Total polyphenols[b]	Total flavonoids[c]	Total flavonols[b]	Condensed tannins[b]	Proanthocyanidins[d]
Ethanol extract					
Drynaria fortunei (DF)	53.44 ± 2.78	2.36 ± 1.54	0.75 ± 0.00	3.17 ± 5.63	89.17 ± 0.00
Pseudodrynaria coronans (PC)	108.18 ± 3.21	0	12.91 ± 0.21	32.73 ± 11.26	103.62 ± 1.28
Davallia divaricata (DD)	134.15 ± 12.13	0	49.39 ± 2.24	108.76 ± 14.07	128.63 ± 1.11
Davallia mariesii (DM)	140.64 ± 8.50	0	30.41 ± 0.86	53.85 ± 11.26	128.63 ± 1.11
Davallia solida (DS)	291.86 ± 7.00	122.44 ± 1.78	66.14 ± 2.61	260.82 ± 22.52	180.88 ± 2.12
Humata griffithiana (HG)	166.62 ± 5.79	11.64 ± 0.00	31.03 ± 0.86	79.19 ± 11.26	168.09 ± 3.85
Aqueous extract					
Drynaria fortunei (DF)	326.18 ± 12.13	16.28 ± 0.00	16.51 ± 0.56	108.76 ± 5.63	132.15 ± 3.39
Pseudodrynaria coronans (PC)	1120.29 ± 20.51	41.01 ± 1.54	145.31 ± 2.61	636.73 ± 14.07	341.13 ± 2.56
Davallia divaricata (DD)	1203.78 ± 12.55	3.91 ± 1.54	263.81 ± 4.85	1029.54 ± 30.97	258.13 ± 3.85
Davallia mariesii (DM)	1635.16 ± 48.39	3.91 ± 0.00	295.70 ± 3.33	1422.35 ± 14.07	467.85 ± 2.56
Davallia solida (DS)	1400.45 ± 34.87	123.98 ± 5.42	246.93 ± 5.02	1164.70 ± 25.34	429.32 ± 3.85
Humata griffithiana (HG)	183.32 ± 49.88	14.22 ± 1.78	12.041 ± 1.07	32.73 ± 14.07	106.21 ± 1.28

[a]In text, all ethanol extracts have been designated as E and aqueous extracts as W after the species code
[b]Expressed as μg catechin equivalent/mg dry weight
[c]Expressed as μg rutin equivalent/mg dry weight
[d]Expressed as μg cyanidin chloride equivalent/mg dry weight equivalent

maximum 1635.16 μg in aqueous extract of *Davallia mariesii* (DMW) (Table 21.3). Depending upon species (source of "Gu-Sui-Bu"), the quantities of total polyphenols in aqueous extracts were 5–12 times higher compared to ethanol extracts. The only exception was fern species *Humata griffithiana* (HG), where margin of difference in total polyphenols in both the solvents was narrow.

21.2.6.1 Flavonoids Content

The $AlCl_3$ method (Lamaison and Carnet 1990) was used for estimation of the total flavonoids content of the extracted samples. Flavonoids contents were calculated from the calibration curve of rutin standard solutions, and expressed as μg rutin equivalent/mg dry weight. Total flavonoids contents (expressed as μg rutin equivalent/mg dry weight) ranged from 0.0 to 122.44 μg in ethanol extracts and from 3.91 μg to 123.98 μg in aqueous extracts of the six sources. There were no flavonoids contents in ethanol extracts of fern species *Pseudodrynaria coronans* (PC), *Davallia mariesii* (DM), and *Davallia solida* (DS) (Table 21.3).

21.2.6.2 Flavonols Content

The total flavanols content was estimated using the p-dimethylaminocinnamaldehyde (DMACA) method (Arnous et al. 2001). This method has a great advantage over the widely used vanillin method, since there is no interference by anthocyanins. Furthermore, it provides higher sensitivity and specificity (Li et al. 1996). The concentration of total flavanols was estimated from a calibration curve. Results are expressed as μg catechin equivalent/mg dry weight. Quantities of total flavonols in ethanol and aqueous extracts of six sources of "Gu-Sui-Bu" varied significantly (Table 21.3). With the exception of fern species *Humata griffithiana* (HG), aqueous extracts had 3–22 times higher total flavonol contents compared to ethanol extracts. However, ethanol extract of *Humata griffithiana* showed 2.5 time higher quantity than aqueous extract. The minimum quantity of total flavonols (0.75 μg) was found in *D. fortunei* (DFE) and the maximum (295.70 μg) in *Davallia mariesii* (DMW) (Table 21.3).

21.2.6.3 Condensed Tannins/Proanthocyanidin Content

Condensed tannins content was estimated using the vanillin assay method (Julkunen-Titto 1985). Concentration of tannins was calculated as μg catechin equivalent/mg dry weight from calibration curve. The proanthocyanidin content was estimated using HCl/butanol assay method (Porter et al. 1986). Results were expressed in mg cyanidin chloride g^{-1} fresh weight. In case of condensed tannins and proanthocyanidin, more or less similar trends like those for total flavonols were observed among the six sources. Aqueous extracts had several times higher values compared to ethanol extracts with the exception of *Humata griffithiana* (Table 21.3).

21.3 In Vitro Studies on *D. fortunei*

A typical fern completes its life cycle in two forms, a diploid sporophyte (most often) and a haploid gametophyte. These forms alternate each other. Gametophyte begins with the germination of spore and further gradual development of prothallus (gametophyte). The phenomenon of spore germination and subsequent growth of gametophyte has been reported to be influenced by several physicochemical factors including light. Numerous studies have been carried out to understand basic regulatory mechanisms in spore germination and development of gametophyte and sporophyte (Raghavan 1989). In addition to light and GA-like compounds, other factors including the pH of the culture medium (Conway 1949), ethylene (Edwards and Miller 1972a, b), CO_2 (Edwards 1977), and calcium availability (Wayne and Hepler 1984; Wayne and Hepler 1985) have been shown to influence the ability of spores to germinate and the subsequent growth of gametophytes. Thus, in a large number of reports on ferns available so far, it has been seen that optimum conditions for spore germination vary among fern species. Spores of *D. fortunei* were studied by Chang et al. (2007a), to investigate the effects of different factors like strength of MS basal medium, sucrose concentrations, pH, sugars, and spectra of light on in vitro germination, development of gametophytes, and reproductive organs.

21.3.1 Effects of MS Basal Medium, Sucrose Concentration, and Sugars on Germination of Spores and Development of Gametophytes

Germination of *D. fortunei* spores commenced by the rupturing of the spore coat and division of the spore cell. However, spores underwent only one asymmetric cell division giving rise to rhizoid and protonemal cell which continued to divide to form the gametophyte. Although spores of several ferns germinate in water, the gametophyte requires a variety of mineral elements found in a balanced nutrient solution for continued growth, in absence of which deficiency symptoms appear (Raghavan 1989). Among different concentrations of MS basal medium at pH 5.7, 1/4X strength of MS, resulted in highest spore germination (32.5%) as compared to half strength MS (27.6%); however, further growth of gametophytes in the former medium was much restricted (Murashige and Skoog 1962). MS basal medium at full strength (1X) and double strength (2X) resulted in 15.3 and 7.8% spore germination, respectively. As evident from the present study, strength of salts in MS basal medium influenced not only spore germination but also growth of the gametophytes. Higher percentage of spore germination but restricted growth of prothalli in 1/4 strength of MS salts could be due to deficiency (sub-optimal level) of nutrients, whereas decreased germination and restricted growth on 1X and 2X strengths of the basal medium could be due to supra-optimal levels of mineral salts. Thus, it appears that gametophytes require mineral salts in a balanced manner for their normal growth.

Sucrose in culture medium functions both as a carbon source and osmotic regulator. Sucrose is rapidly hydrolyzed to glucose and fructose, nearly doubling the osmolarity of the medium. Sucrose has been used as a major carbohydrate source in the induction medium. There are contrasting reports on the effect of sucrose on spore germination and gametophyte development in several fern species. In a study carried out with four fern species, percentage of spore germination significantly increased by inclusion of sucrose in the culture medium (Sheffield et al. 2001). However, in two other separate studies, sucrose had stimulatory effect (in terms of cell number) in development of gametophyte of *Platycerium* (Camloh 1993), whereas inhibited 2-D growth of gametophytes of *Anemia* and *Pteridium* (Douglas 1994). In several other ferns, beneficial effects of sugars like fructose, maltose, ribose, and xylose in development of gametophytes have been documented (Raghavan 1989). Different concentration of sucrose in the range of 0–7%, the maximum germination (72.3%) was recorded with 2% sucrose. Sucrose levels higher than 2% decreased spore germination drastically. The lowest germination rate (20.0%) was recorded in a medium with 7% sucrose. Also, we optimized sources of carbohydrate in the medium. Different sugars influence differently on spore germination in *D. fortunei*; of the four sugars tested, sucrose affected the highest spore germination (74.7%) compared to maltose (59.7%), glucose (23.3%), and no germination was reported with fructose (0%).

21.3.2 Effects of pH on Spore Germination, Development of Gametophytes, and Reproductive Organs

The study of pH on spore germination under controlled conditions is an important aspect of germination of fern spores, since this group of plants survive and grow under different soil types and ecological niches. In previous reports, it was observed that spores of most ferns germinate at slightly acidic or neutral pH (Raghavan 1989). Tolerance of spores of some fern species including *Notholaena cochisensis* and *Pellaea limitanea* to pH in the range of 9–10 had correlation with high alkalinity of the soil in which they grew (Hevly 1963). It is to be expected that germination of spores is limited in its ability to occur within a narrow range of pH on either side of neutrality (Raghavan 1989). However, to our surprise, germination of *D. fortunei* spores occurred in varying percentages on a wide range of pH from 3.7 to 9.7, though the highest germination percentage was achieved on a medium with pH towards alkalinity (pH 7.7).

A range of pH 3.7–9.7 had significant effects on spore germination in *D. fortunei* (Fig. 21.3). The maximum spore germination (63.3%) was observed on the medium at pH 7.7 and the minimum (0.8%) at pH 9.7. Increasing order of pH, i.e., 3.7, 4.7, 5.7, 6.7, and 7.7 exhibited linear increase in germination percentage, i.e., 2.1, 7.9, 27.1, 46.8, and 63.3, respectively. pH levels higher than 7.7 resulted in decreased germination. At pH 8.2, 8.7, 9.2, and 9.7, 58.4, 51.9, 24.6, and 0.8% germination occurred, respectively (Fig. 21.3). Besides, spore germination, pH affected further

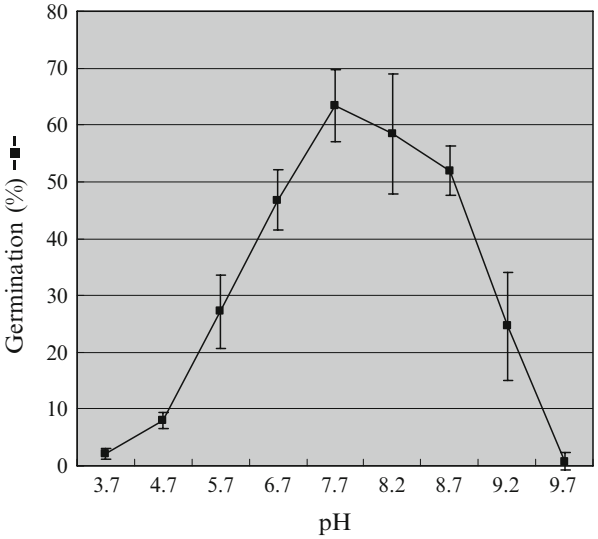

Fig. 21.3 Influence of pH on spore germination in *Drynaria fortunei*. Culture medium and conditions: MS basal 1/2 strength, sucrose 2%, white light

growth patterns of gametophytes. Growth of gametophytes was much restricted at pH levels lower or higher than 7.7 (Fig. 21.6a–c). The lowest and the highest pH i.e., 3.7 and 9.7 inhibited cell divisions in protonema. Also, development of biplanar gametophytes was observed only at pH 7.7 (Fig. 21.6b). Media with different pH had influence on number of archegonia formation in the gametophytes after 14 weeks of inoculation of spores. The trend was more or less identical to the spore germination results (Fig. 21.7a). The maximum archegonia (an average of 27.6 ± 2.7/per gametophyte) were observed at pH 7.7 followed by pH 6.7 (20.7 ± 3.8/per gametophyte). At pH 4.7, the least number of archegonia (0.7 ± 0.5/per gametophyte) was formed where as gametophytes in media at pH 3.7 and 9.7 did not form any archegonia.

21.3.3 Effects of Light Spectra on Spore Germination, Development of Gametophytes, and Reproductive Organs

Light is one of the most important factors which have drastic influences on events in life cycle of a fern. Light is one signal known to awaken dormant fern spore. With varying degrees of complexity, involvement of a light sensitive pigment phytochrome in the germination of spores has been reported. Phytochrome acts like a germination switch, and the wavelength of light flips the switch back and forth between off (the Pr form: P for phytochrome and r for absorbing red light) and on

(the Pfr form: fr for absorbing far-red light) in a photo reversible manner (Raghavan 1992). Studies on the sensitive fern *Onoclea sensibilis* revealed that these effects by phytochrome have been brought about by altering the intracellular calcium levels in the spore cell (Wayne and Hepler 1984, 1985). Thus, light triggers a cascade of biochemical activity in a dormant fern spore and spores incubated under different light spectra show varying germination response due to sequential changes in these pigments making germination a complex event.

Like spores, sensitivity of protonemata (five- to six-celled stage gametophyte) of ferns to blue, red, and far-red lights vary greatly since, contrasting results have been observed in several reports (Raghavan 1989). As shown in present study, gametophytes of *Lygodium japonicum* assumed planar morphology in both red and blue light, but remained filamentous in far-red light (Swami and Raghavan 1980). These light affected growth forms are results of changed patterns of cell divisions in the protonemata (Ito, 1970). The pronounced inhibition of elongation growth accompanied by growth in width of the filamentous form provides a firm basis to quantify growth data in terms of length/width ratio, designated as morphogenetic index (Mohr 1956). The morphogenetic index of gametophytes cultured under far-red is high indicating the occurrence of increased cell elongation coupled with low incidence of cell division. Under white, blue, and red light, the index is very low and there is significant increase in number of cells formed. Development of gametophytes with normal, planar growth under white light is a result of increased mitotic activity and increase in surface area of the gametophytes.

Transfer of gametophytes from one light to another also introduces corresponding changes in their morphology. Morphogenetic effects of different light spectra on the development of protonemata in ferns have often been interpreted as a result of differential mitotic activity. Thus, light regimens could act antagonistically by modulating the elongation potential or division potential of cells as evident from the present study (Fig. 21.6g, h).

In contrast to the present results on *D. fortunei*, it was observed that spores of *Stromatopteris moniliformis* (Gleicheniaceae) did not germinate under light and took 3 months to germinate in dark on a nutrient medium containing minerals and 0.5% glucose (Whittier 1999). In *Diphasiastrum sitchense*, only 0.5% spores could germinate after 5 months of culture in dark, and light did not affect germination even after 11 months of culture (Whittier 2003). After attaining maturity, gametophytes of fern species in general, behave as centers of growth and morphogenesis and develop reproductive organs e.g., archegonia (female) and antheridia (male). In majority of the homosporous ferns, development of antheridia precedes archegonia (Raghavan 1989). However, in case of *D. fortunei*, development of archegonia preceded antheridia. What triggers this differentiation in different ferns still remains unknown. Though various reports have demonstrated involvement of endogenous hormones in the induction of sexuality in fern gametophytes, the issue is quite complex and needs separate investigation.

It was observed that spores of *D. fortunei* required light to germinate, since the same did not germinate in complete darkness. Further experiments with dif-

ferent light spectra on spore germination i.e., red, far-red, blue, and white light resulted in 71.3, 42.3, 52.7, and 71.0% spore germination, respectively (Fig. 21.4). However, striking differences were observed in the development of gametophytes in different light spectra (Fig. 21.6d–h). After 4 weeks of culture, there was no morphological difference among gametophytes grown under white or blue light. Cordate type gametophytes developed in both the lights. In red light, elongated or filamentous but broad gametophytes developed (Fig. 21.6d). In far-red light, gametophytes grew as uniseriate filaments and consisted of mostly elongated cells (Fig. 21.6f). Morphogenetic index (length/width ratio) of gametophytes under different lights was 0.9 ± 0.1, 2.6 ± 1.2, 37.4 ± 2.0, 0.8 ± 0.1 under blue, red, far-red, and white lights, respectively (Fig. 21.5). Experiments were carried out to study the reversible effects of white and

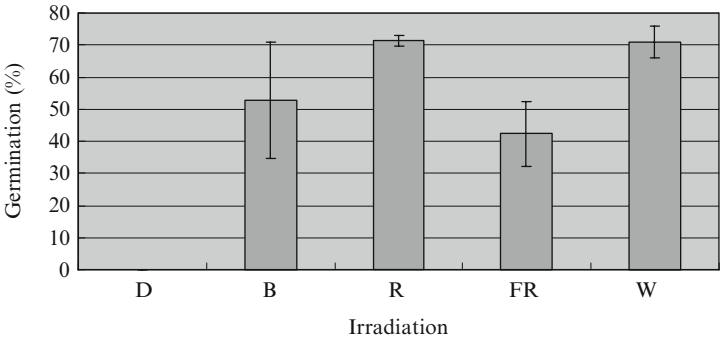

Fig. 21.4 Influence of different light spectra on spore germination in *Drynaria fortunei* (*D*=complete darkness; *B*=blue light (450 nm); *R*=red light (660 nm); *FR*=far-red light (735 nm) and *W*=white light (380–780 nm)). Culture medium and conditions: MS basal 1/2 strength, sucrose 2%, pH 7.7

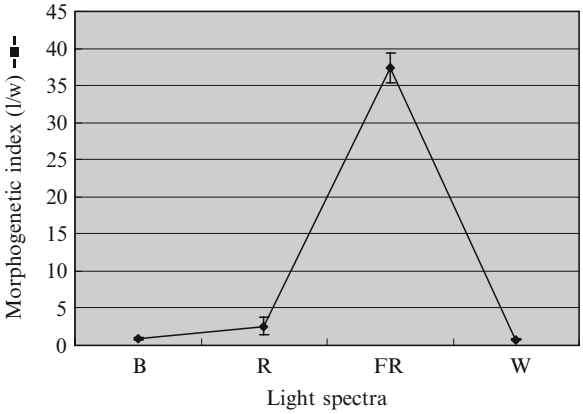

Fig. 21.5 Influence of different light spectra on morphogenetic index (*l*=length/*w*=width) of gametophytes of *Drynaria fortunei* (*B*=blue light (450 nm); *R*=red light (660 nm); *FR*=far-red light (735 nm) and *W*=white light (380–780 nm)). Culture medium and conditions: MS basal 1/2 strength, sucrose 2%, pH 7.7

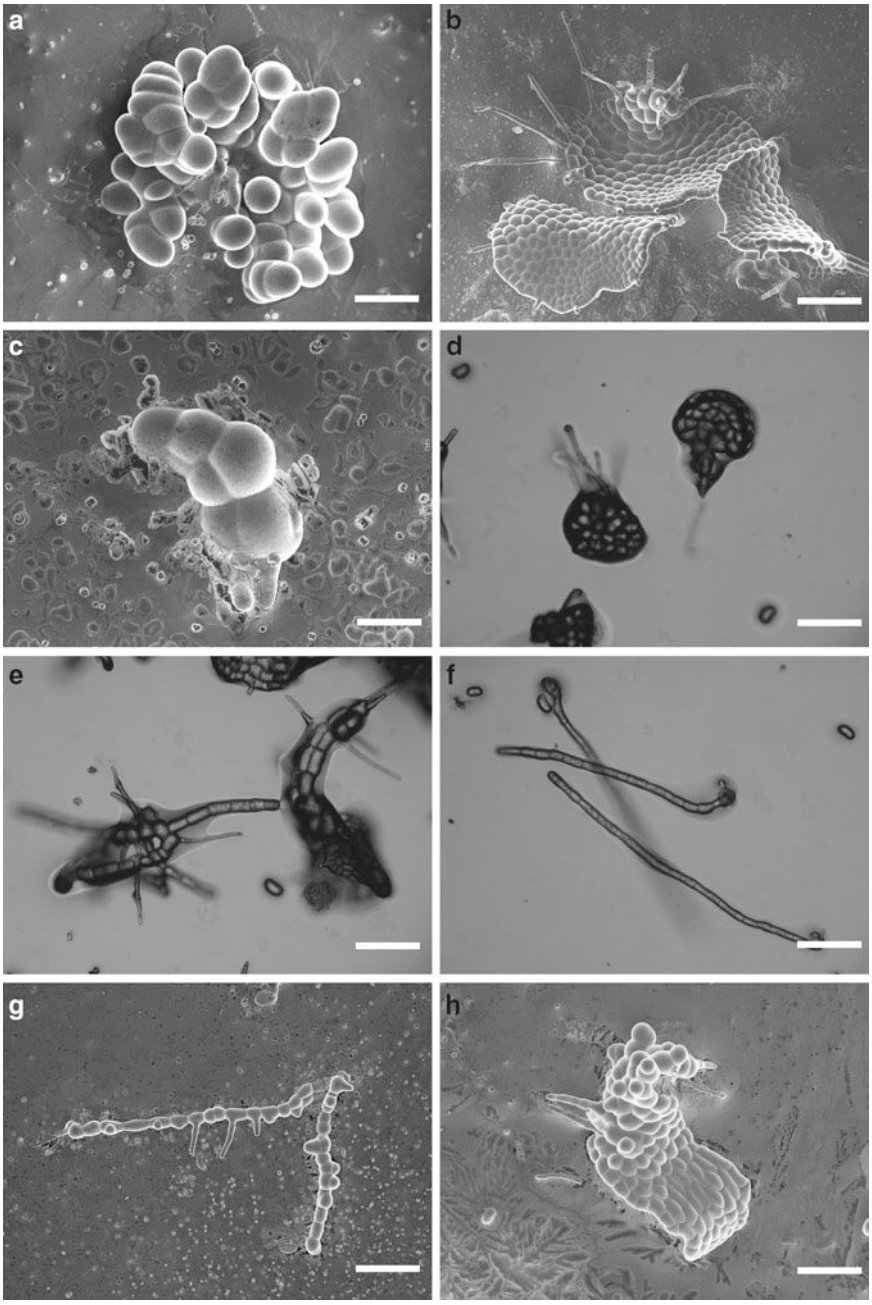

Fig. 21.6 A-H, Development of gametophytes under different pH and light spectra. Gametophytes grown on the medium at pH 3.7 (bar = 34 μm) (**a**); at pH 7.7 and under white light (Control) (bar = 146 μm) (**b**); at pH 9.7 (bar = 34 μm) (**c**); blue light (bar = 230 μm) (**d**); red (R) light (bar = 230 μm) (**e**); far-red (FR) light (bar = 230 μm) (**f**); Gametophytes grown under white light for 1 week then shifted to FR for 2 weeks and then to white light for 1 wk (bar = 146 μm) (**g**); Grown under FR for 1 week then shifetd to white light for 2 weeks and then to FR for 1 week (bar = 146 μm) (**h**)

infra-red lights on development of gametophytes. Spores germinated in white light for 1 week when shifted to FR for 2 weeks, thereafter to white light for further 2 weeks showed changed pattern of cell divisions in protonema according the light regime during incubation period (Fig. 21.6g). Similarly, spores germinated under FR for 1 week when shifted to white light for 2 weeks, thereafter to FR for further 2 weeks showed a multi-seriate, filamentous gametophyte, which reflected the cell divisions under both IR and white light regimen (Fig. 21.6h).

Light spectra had influence on development of reproductive organs in gametophytes of *D. fortunei*. But Chang et al. (2007a) reported that 5 months of inoculation of spores revealed no antheridia and archegonia under far-red light (Table 21.3). Under blue light, archegonia alone or both archegonia and antheridia were observed in 60 and 6.7% gametophytes, respectively, while 33.3% gametophytes did not show any sex organs. Red light favored antheridia formation, since these male organs were observed in 33.3% gametophytes, while 53.3% did not develop any reproductive organs. Very small percentage of gametophytes (6.7) developed either archegonia alone or both the sex organs (bisexual) under both red and blue light (Table 21.4). Under white light, percentages of gametophytes developed only archegonia (Fig. 21.7a) or antheridia (Fig. 21.7b) were 50.0, and 3.3, respectively, while 23.3% had both antheridia and archegonia and 23.3% were asexual (showed no sex organs) (Table 21.4). In far-red light, elongated and uniseriate gametophytes in *D. fortunei* were observed. These gametophytes did not have normal growth and maturity like gametophytes of *Cryptogramma crispa* (Pajaron et al. 1999). The in vitro growth of sporophytes is a very slow process; it takes 20–22 weeks to form compact mass of gametophytes (Fig. 21.7c) and juvenile sporophytes appears after 12–16 weeks (Fig. 21.7d, e). The sporophytes grow further to develop rhizome (Fig. 21.7f). Hence we can say that the physicochemical factors play important roles in in vitro spore germination, and gametophyte development in *D. fortunei*.

Table 21.4 Influence of light spectra on development of antheridia and/or archegonia in *Drynaria fortune*

	% of gametophytes showing development of sex organs			
	Male	Female	Bisexual	Asexual
Light spectra	(Antheridia)	(Archegonia)	(Both Antheridia and Archegonia)	(No sex organs)
Blue (450 nm)	0	60 (18)	6.7 (2)	33.3 (10)
Red (660 nm)	33.3 (10)	6.7 (2)	6.7 (2)	53.3 (16)
Far-red (735 nm)	0	0	0	100 (30)
White (380–780 nm)	3.3 (1)	50 (15)	23.3 (7)	23.3 (7)

Percentages of sexual expression in isolated gametophytes observed after 5 months of inoculation of spores. Absolute numbers of gametophytes are in parentheses

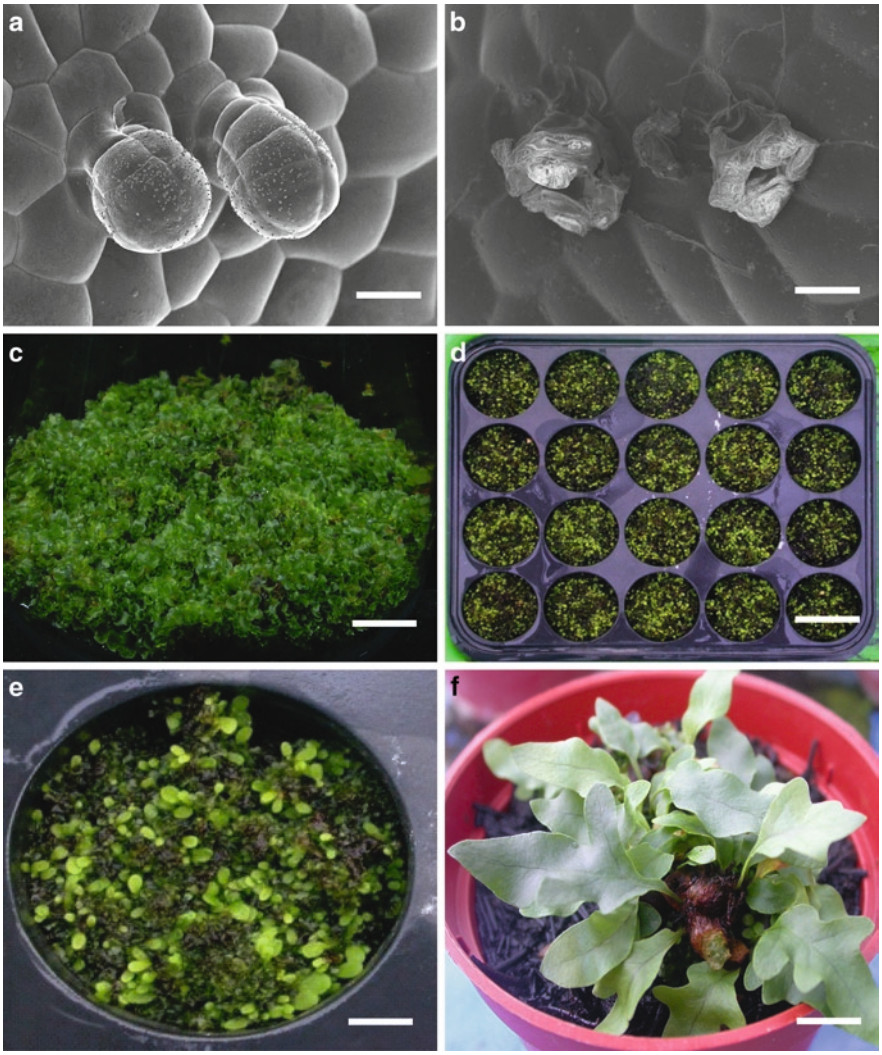

Fig. 21.7 Reproductive organs (female-archegonium, and male-antheridium) in *Drynaria fortunei.* Archegonia in *D. fortunei* in 14 wks old gametophyte (**a**); antheridia in 5-month old gametophyte (**b**). Mass of old and new gametophytes in culture flask, 3 months after transfer of gametophytes from Petri dishes (**c**); Sporophytes developed after 14 weeks of transfer of gametophytes to plastic-cell tray kept in green house (**d**); Sporophytes in a single plastic tray cell (**e**); Sporophytes transferred to a pot kept in green house showing rhizome formation after 5 months of potting (arrow indicates rhizome) (**f**)

21.4 Conclusion

Thus, the present chapter describes the antioxidant properties of "Gu-Sui-Bu" and optimum requirement of some of the physicochemical factors for in vitro spore germination, gametophyte development in *D. fortunei*. The presented investigation may

be of help in promotion of cultivation of not only *D. fortunei* but other genera of "Gu-Sui-Bu" at large scale and compensation of its depletion in the wild.

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Chapter 22
Ecdysteroids in Ferns: Distribution, Diversity, Biosynthesis, and Functions

René Lafont, Raimana Ho, Phila Raharivelomanana, and Laurie Dinan

22.1 Introduction

Phytoecdysteroids are plant analogs of insect molting hormones. They were first discovered in a gymnosperm in 1966 and almost simultaneously in the fern *Polypodium vulgare* by Jizba et al. (1967a, b). These steroids are characterized by a *cis*-fused A/B-ring junction, a 7-en-6-one chromophore, a 14α-hydroxyl group, and they usually retain the entire carbon skeleton of their parent sterols (Bergamasco and Horn 1983).

22.2 Distribution and Diversity

After the pioneering investigations of Jizba et al. (1967a, b) and Heinrich and Hoffmeister (1967, 1968), two types of investigations have been performed. Some groups, mainly in Japan and New Zealand, analyzed the distribution of phytoecdysteroid-containing ferns, and they investigated many fern species using insect-based bioassays to check for the presence of these molecules (Imai et al. 1969a; Matsuoka et al. 1969; Russell and Fenemore 1971; Hikino et al. 1973; Takemoto et al. 1973; Yen et al. 1974). More recently, additional systematic studies were performed using immunoassays (Volodin et al. 2007). To date, more than 500 fern species have been investigated for ecdysteroid presence, and ca. 50% were positive (Dinan and Lafont 2007), a value that has to be compared to the 5–6% of positive species when considering all tested plants (Dinan 2001; Dinan et al. 2001). Ecdysteroids have not been detected in all fern families (Fig. 22.1); in some families, they are absent, or present in a few species only, whereas in

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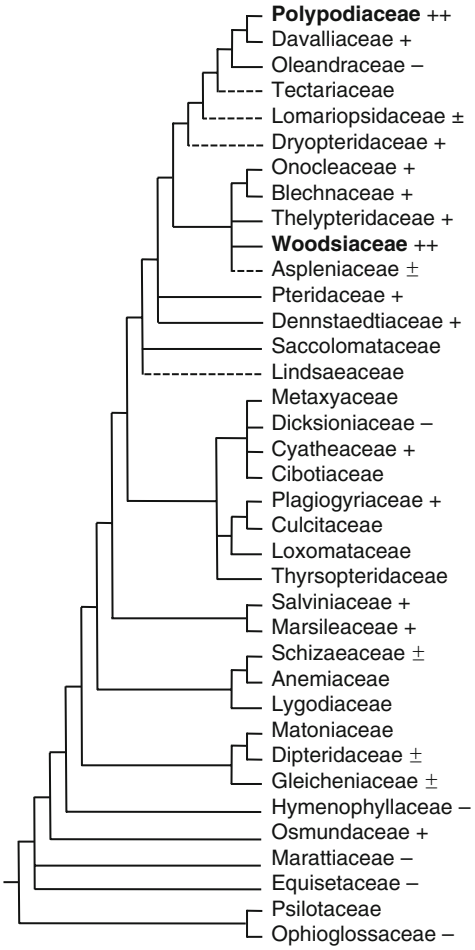


Fig. 22.1 Distribution of ecdysteroid-containing species among fern families (++ very frequent; +: frequent; ±: present in a few species only; -: none detected in species tested so far). Fern classification is after Smith et al. (2006)

others (e.g., Polypodiaceae) almost all investigated species contain ecdysteroids, sometimes at very high concentrations. Other investigations were performed with the aim of isolating ecdysteroids, and presently as many as ca. 40 different ecdysteroids have been isolated from various fern species, including several unique molecular structures (Table 22.1, Fig. 22.2).

Ecdysteroids may be very abundant (total concentration up to $\geq 2\%$ of the dry weight in some fern species). When considering their chemical nature, it is noticeable that (1) most of them are 27C molecules, (2) ecdysone is found in many fern species (although it is rarely found in ecdysteroid-positive higher plants), and is even a major component in some of them, e.g., in the *Microsorum* genus

Table 22.1 Ecdysteroids isolated from ferns (see also Dinan and Lafont (2007) for a more detailed review of ecdysteroid-containing species)

Species	Ecdysteroids	References
Acrostrichum aureum	PonA, pterosterone	Bergamasco and Horn (1983)
A. speciosum	E, 20E	Bergamasco and Horn (1983)
Anemia phyllitidis	20E, PonA (tentative)	Bürcky (1977)
Athyrium niponicum	20E, PonA, pterosterone	Hikino and Hikino (1970)
A. yokoscense	20E	Imai et al. (1969b)
	24-*epi*-pterosterone	Ohta et al. (1996)
Blechnum amabile	20E, PonA	Takemoto et al. (1969)
B. minus	2dE, 2d20E, E	Chong et al. (1970)
	PonA	Bergamasco and Horn (1983)
	Blechnosides A, B	Suksamrarn et al. (1986)
B. niponicum	Shidasterone (= stachysterone D)	Takemoto et al. (1968f), Hikino et al. (1975)
	20E, PonA	Takemoto et al. (1969)
B. spicant	PonA	Jizba and Herout (1974)
B. vulcanicum	2dE, 3-*epi*-2dE	Russell et al. (1981)
Brainea insignis	PonA, 20E, Ponasteroside A, Brainesterosides A-E	Fang et al. (2008), Wu et al. (2010)
Cheilanthes mysurensis	Cheilanthone B	Iyer et al. (1973)
C. seiberi	E, 20E, PonA, 2dE, 2d20E	Bergamasco and Horn (1983)
C. tenuifolia	E, Cheilanthones A, B	Faux et al. (1970)
Cryosinus hastatus	20E	Hikino and Hikino (1970)
Cyathea cooperi	20E	Bergamasco and Horn (1983)
Cyclosorus acuminatus	20E	Imai et al. (1969b)
Diplazium donianum (= *Athyrium aphanoneuron*)	Makisterone A, Makisterone D	Hikino et al. (1976)
Doodia aspera	20E	Bergamasco and Horn (1983)
Gleichenia glauca	PonA	Hikino and Hikino (1970)
Lastrea japonica	20E	Hikino and Hikino (1970)
L. thelypteris	20E, PonA	Hikino and Hikino (1970)
	Pterosterone	Takemoto et al. (1968b)
Lemmaphyllum microphyllum	E, Pterosterone	Takemoto et al. (1968a)
	20E, Makisterone C	Takemoto et al. (1968e)
Lepisorus ussuriensis	2α,3β-(22*R*)-trihydroxycholestan-6-1-22-O-β-D-glucopyranosyl-(1 → 2)-α-L-arabinopyranoside	Choi et al. (1999)
Lygodium japonicum	Ponasteroside A, Lygodiumsteroside A	Zhu et al. (2009)
Matteucia struthiopteris	20E, Pterosterone	Takemoto et al. (1967)
	PonA	Hikino and Hikino (1970)
Microsorum commutatum	E	Ho et al. (2007)

(continued)

Table 22.1 (continued)

Species	Ecdysteroids	References
M. maximum	E, 20E, 2d20E, Inokosterone, Makisterone A	Ho et al. (2007)
M. membranifolium	E, 20E, 2dE, 2d20E, 2dE 3-glucosylferulate, 2d20E 3-glucosylferulate, 2dE 25-rhamnoside	Ho et al. (2007, 2008)
M. punctatum	E, 20E, Makisterone A	Ho et al. (2007)
M. scolopendria	E, 20E, Makisterone A, Makisterone C, Inokosterone, Amarasterone A, Poststerone, 25dE 22-glucoside, 24-methyl-ecdysone, 24,28-*diepi*-cyasterone	Snogan et al. (2007)
Neocheiropteris ensata	20E	Takemoto et al. (1968e)
	E	Hikino and Hikino (1970)
Onoclea sensibilis	20E	Hikino and Hikino (1970)
	Pterosterone	Takemoto et al. (1968b)
Osmunda asiatica	E, 20E, PonA	Takemoto et al. (1968c)
O. japonica	20E	Imai et al. (1969b)
	E, 20E, PonA	Takemoto et al. (1968c)
Pellaea falcata	20E	Bergamasco and Horn (1983)
Phymatodes novae-zelandae	E, 20E, PolB	Russell (1972)
Plenasium banksiifolium	E, 20E	Murakami et al. (1980)
Pleopeltis thunbergiana	20E	Takemoto et al. (1968e)
Polypodium aureum	20E	Jizba et al. (1974a)
	Polypodaurein	Jizba et al. (1974b)
P. glycyrrhiza	Polypodoside A	Kim et al. (1988)
	Polypodosides A, B, C	Kim and Kinghorn (1989)
P. japonicum	20E	Imai et al. (1969b)
P. virginianum	E, 20E	Hikino (1976)
P. vulgare	E, 20E	Heinrich and Hoffmeister (1967)
	20E	Jizba and Herout (1967), Jizba et al. (1967a)
	PolB	Jizba and Herout (1967); Jizba et al. (1967b)
	Polypodosaponin	Jizba et al. (1971a)
	Osladin	Jizba et al. (1971b)
	20,26-Dihydroxyecdysone	Marco et al. (1993)
	E, 20E, PolB, abutasterone, inokosterone, 5-hydroxy-abutasterone, pterosterone, 24-hydroxyecdysone	Coll et al. (1994)

(continued)

Table 22.1 (continued)

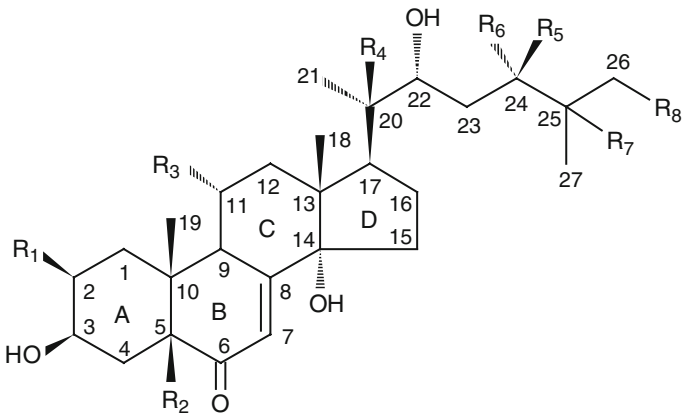
Species	Ecdysteroids	References
Pteridium aquilinum	E, 20E	Kaplanis et al. (1967), Selvaraj et al. (2005)
	20E, PonA	McMorris and Voeller (1971)
	PonA, inokosterone	Zhang et al. (2004)
P. aquilinum var. *latiusculum*	Ponasteroside A	Takemoto et al. (1968g), Hikino et al. (1969)
	PonA, pterosterone	Takemoto et al. (1968d)
Pteris cretica	20E	Imai et al. (1969b)
Schizaea dichotoma	20E, PonA, Pterosterone, Ajugasterone C	Bergamasco and Horn (1983)
	Schizaeasterones A, B	Fuchino et al. (1997)
Stenochlaena palustris	20E	Bergamasco and Horn (1983)
Tectaria spp.	E, 20E	Bergamasco and Horn (1983)

E ecdysone, *20E* 20-hydroxyecdysone, *2dE* 2-deoxyecdysone, *2d20E* 2-deoxy-20-hydroxyecdysone, *PonA* ponasterone A, *PolB* polypodine B

(Ho et al. 2007, 2008; Snogan et al. 2007), (3) 2-deoxy-ecdysteroids are also quite common, and (4) glycosides have been found in several species. Unique ecdysteroid glycosyl-ferulate conjugates have been recently isolated from *Microsorum membranifolium* (Polypodiaceae: Ho et al. 2008); these conjugates are present together with free ecdysteroids and constitute a complex cocktail, of which only the major components have been identified as yet (Fig. 22.3).

Most of the available data about ecdysteroid distribution within the plants have been obtained with *P. vulgare*, i.e., the first fern species from which ecdysteroids were isolated. Ecdysteroids are not distributed equally within plants of this species: their total concentration is high in the rhizomes, somewhat lower in the roots and lowest in the fronds, and the (complex) ecdysteroid patterns also differ between organs (Reixach et al. 1996). However, there is no general distribution pattern: thus, in the two related species, *Microsorum membranifolium* and *M. scolopendria*, the former had higher concentrations in the fronds, whereas the latter had higher ones in the rhizomes (Ho et al. 2007).

The ecdysteroid content fluctuates heavily with season: in *P. vulgare* rhizomes, the 20-hydroxyecdysone concentration was 0.4% (relative to dry weight) in January and only 0.04% in October (Messeguer et al. 1998), and values above 0.25% of the fresh weight have been reported by Jizba et al. (1967a), but the period of sample collection was not indicated. In *Pteridium aquilinum* fronds, the 20-hydroxyecdysone changed from undetectable levels in July–August to 53 μg/kg fresh weight in October (Jones and Firn 1978). In order to better understand the dynamics of these systems it would be of interest to analyze ecdysteroid distribution more precisely over a 1-year period in both annual fronds and perennial rhizomes.



Name	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
Ecdysone (E)	OH	H	H	H	H	H	OH	H
20E	OH	H	H	OH	H	H	OH	H
2-deoxyE (2dE)	H	H	H	H	H	H	OH	H
2-deoxy20E (2d20E)	H	H	H	OH	H	H	OH	H
Ponasterone A	OH	H	H	OH	H	H	H	H
25-deoxyE (25dE)	OH	H	H	H	H	H	H	H
Inokosterone	OH	H	H	OH	H	H	H	OH
Pterosterone	OH	H	H	OH	H	OH	H	H
Abutasterone	OH	H	H	OH	H	OH	OH	H
24-Hydroxyecdysone	OH	H	H	H	H	OH	OH	H
Polypodine B	OH	OH	H	OH	H	H	OH	H
Ajugasterone C	OH	H	OH	OH	H	H	H	H
Makisterone A	OH	H	H	OH	CH_3	H	OH	H
Makisterone C	OH	H	H	OH	C_2H_5	H	OH	H

Fig. 22.2 Structures of the most common fern ecdysteroids. For other structures, see the Ecdybase (Lafont et al. 2002)

22.3 Biosynthesis

In *P. vulgare*, the highest concentrations of phytoecdysteroids were found in prothalli, i.e., the haploid gametophytes formed from spores. Owing to their small size, easy in vitro cultivation, high ecdysteroid content, and efficient uptake of exogenous (radioactive) precursor molecules, prothalli represent a particularly favorable biological material for metabolic studies (Reixach et al. 1996, 1997, 1999; Messeguer et al. 1998). There are only a few studies on the ecdysteroid biosynthetic pathway in ferns; three kinds of radioactive molecules were tested for that purpose: acetate or mevalonate (i.e., general sterol precursors), cholesterol (or 25-hydroxycholesterol), and putative ecdysteroidal intermediates already used with insects or other arthropods and lacking one (ecdysone) or several hydroxyl groups.

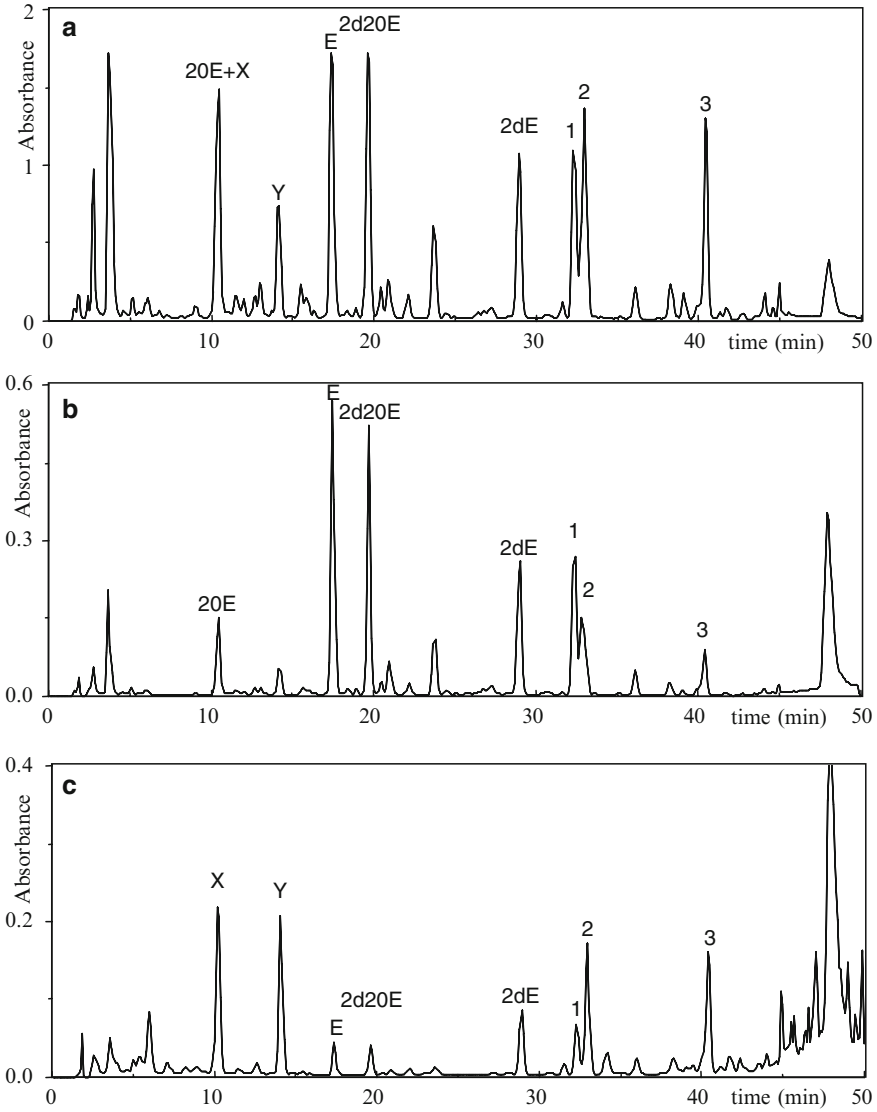


Fig. 22.3 RP-HPLC analysis of an extract from fronds of the fern *Microsorum membranifolium*. HPLC conditions: column ACE C_{18} (150 mm, 4.6 mm i.d.); flow-rate: 1 mL.min^{-1}; linear gradient 15–35% (in 40 min), then 35–100% (in 10 min) of ACN-isopropanol [5:2, v/v] in water containing 0.1% TFA. (**a**) Crude butanolic fraction; (**b**) fraction not retained on a polyamide column. (**c**) fraction retained on the polyamide column and eluted with ethanol. Compounds X and Y are unidentified phenolic compounds; *1* 2dE 25-α-L-rhamnopyranoside; *2* 2d20E 3-[4-(1-β-D-glucopyranosyl)]-ferulate; *3* 2dE 3-[4-(1-β-D-glucopyranosyl)]-ferulate (for additional information, see Ho et al. 2008)

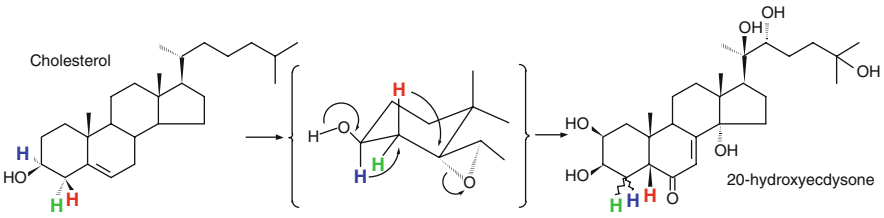


Fig. 22.4 Origin of the 5β-H of ecdysteroids in *Polypodium vulgare* (Modified after Dinan et al. 2009)

The first metabolic studies (Cook et al. 1973; Lockley et al. 1975; Davies et al. 1980) were aimed at understanding the mechanism of B-ring (5βH-7-en-6-one) functionalization using precursors which were labeled with tritium atoms introduced at specific positions, and the same group also analyzed the conversion of the same molecules in an insect system (locust molting glands). The conclusions of these studies indicate that these early steps do not proceed in the same way in ferns and insects: in *P. vulgare*, the 4β-H of cholesterol moved to the 5β-H position (Fig. 22.4), whereas in locust the 4β-H was lost during this process. A third situation was observed in the angiosperm, *Ajuga reptans*, where the 5β-H originates from the 6-H of cholesterol (see review by Dinan et al. 2009). Differences between insects and plants also concern the further biosynthetic steps. Conversion studies with 2,22,25-trideoxyecdysone or 2,22-dideoxyecdysone failed to generate labeled 20-hydroxyecdysone (although the same biological system efficiently converted cholesterol, 22*R*-hydroxycholesterol, and 25*S*-hydroxycholesterol into ecdysone and 20-hydroxyecdysone), which means that the sequence of hydroxylation differs from that of insects, and in particular that 22-hydroxylation must take place at an early stage (Reixach et al. 1999). On the other hand, 25-hydroxylation may occur at any stage, as both 25-hydroxycholesterol and ponasterone A (25-deoxy-20-hydroxyecdysone) are converted into 20-hydroxyecdysone. All the available results suggest an independent evolutionary origin of ecdysteroids in plants and insects, as well as a probably polyphyletic origin in plants.

P. vulgare prothalli contain also minor amounts of a 5β-OH ecdysteroid, polypodine B, which does not exist in animals. Labeling studies performed with tritiated ecdysone (Reixach et al. 1996) resulted in its significant conversion into polypodine B, which means at least that 5β-hydroxylation may proceed at a late stage of the biosynthetic pathway, as suggested by early experiments (De Souza et al. 1970). The same holds true for hydroxylation on carbon-24 (Reixach et al. 1996).

Almost no data exist about the ecdysteroid biosynthetic enzymes in plants in general or in ferns in particular. Classically, steroid hydroxylations are performed by cytochrome-P450 enzymes. The only attempt to purify an ecdysteroid biosynthetic enzyme in ferns was performed on the 20-hydroxylase, an enzyme which represents the last step of 20-hydroxyecdysone biosynthesis, at least in insects: this may not hold true for plants. The enzyme was characterized in *P. vulgare* callus cultures

(Canals et al. 2005). The enzyme is inhibited by classical P450 inhibitors (which bind to the haem group; e.g., ketoconazole), it is rapidly (within 24 h) induced by its substrate (exogenously applied ecdysone) and it was purified 400-fold from callus microsomes. Interestingly, the enzyme showed a dose-dependent inhibition by its reaction product (20-hydroxyecdysone) even at very low concentrations (0.01 μM).

22.4 Regulation of Ecdysteroid Biosynthesis and Accumulation

The analysis of ecdysteroid production in whole plants is not convenient, and the analysis of their distribution does not provide information about the sites of biosynthesis, as they may be different from the sites of accumulation. In the case of ferns, studies were either made on prothalli (or calli) as discussed above, or with cell suspensions obtained from callus cultures after repeated selection procedures using mechanical sieving of calli through a metal sieve, a process used with *P. aquilinum* (Vaněk et al. 1990; Macek and Vaněk 1994; Svatoš and Maceˇk 1994). In every case, the ecdysteroid pattern may differ more or less from that of the intact plant.

Cell suspensions of *P. aquilinum* produced a complex ecdysteroid mixture, with ponasterone A being the major component (Vaněk et al. 1990). The production was analyzed over 55 days of culture with cells and medium being analyzed separately by HPLC and this showed a progressive accumulation of ecdysteroids in the medium, accounting for one half of total ecdysteroids by the end of the experiment (Fig. 22.5).

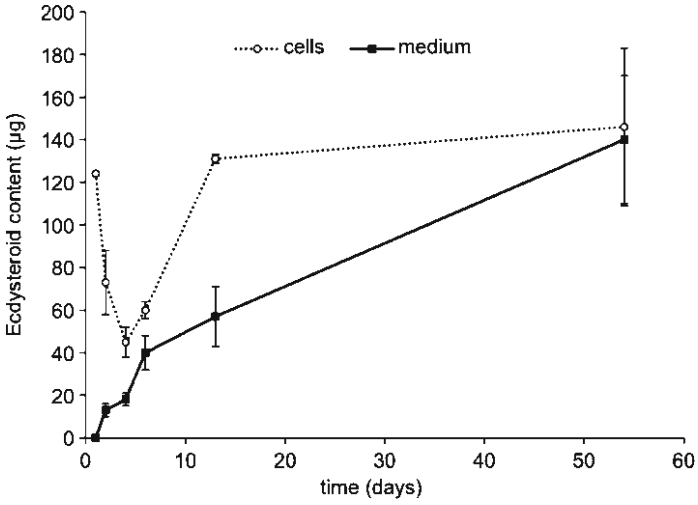


Fig. 22.5 Kinetics of total ecdysteroids in a cell suspension culture of *Pteridium aquilinum*. Cells and medium were separately analyzed (original drawing based on the data of Svatoš and Maceˇk 1994)

In the case of *P. vulgare*, prothalli produced large amounts of ecdysteroids (Reixach et al. 1996). Callus cultures produced almost no ecdysteroids, although they were able to convert 25-hydroxycholesterol to 20-hydroxyecdysone at a low rate and also to efficiently metabolize exogenously applied putative intermediates like 2-deoxyecdysone (Reixach et al. 1999). Growing prothalli gradually accumulate ecdysteroids over time until this amount reaches a plateau after ca. 60 days (Camps et al. 1990). In fact, during this period, the concentration of ecdysteroids remains almost constant (0.6–0.8% of dry weight for 20-hydroxyecdysone, 0.07–0.1% for polypodine B, and 0.03–0.06% for ecdysone). This is indicative that the accumulation of ecdysteroids is precisely regulated in this system, by some kind of negative feedback mechanism. This conclusion was established by heat-shock experiments; when prothalli were incubated in warm water (45 °C for 60 min), they released most (97%) of their ecdysteroids into the water. Subsequently, when cultivated again under normal conditions, ecdysteroid production was greatly stimulated and their concentration rapidly returned to initial levels (Reixach et al. 1997). After this treatment, the incorporation of exogenous precursors was strongly enhanced, so that, for instance, >30% of [^{3}H]25-hydroxycholesterol was converted into the ecdysteroids ecdysone and 20-hydroxyecdysone within as little as 20 h (Reixach et al. 1999). By maintaining prothalli in water at 45°C with regular renewal, it was possible to increase ecdysteroid production by up to 20-fold, as ecdysteroids were continuously released.

External conditions may modifiy ecdysteroid content: when cultured under controlled light, the ecdysteroid content of both fronds and rhizomes of *P. vulgare* decreased with increasing light intensity (Chae et al. 2001). The same authors also showed that treatment of this fern with plant hormones (methyl jasmonate, 6-benzylaminopurine, and thidiazuron) increases ecdysteroid content.

22.5 Function(s)

Ferns are thought to be under-utilized by insects when compared with Angiosperms (Cooper-Driver 1978), which may be connected with the presence of various chemical defenses. They nevertheless host various insects including specialist feeders.

Ecdysteroids may contribute to phytophagous insect control, as shown with the same molecules isolated from various plant species (Dinan 2001). In the case of ferns, this question was addressed by Jones and Firn (1978) for the ecdysteroids of *P. aquilinum*. They observed two types of effects, i.e., feeding deterrency and disturbance of development and/or reproduction. The former usually only required low concentrations, at least with some species like *Pieris brassicae*, but in other cases some insect species were unaffected even by concentrations well above those found in the fern.

Selvaraj et al. (2005) tested the toxic properties of *P. aquilinum* ecdysteroid-containing extracts on two insect pests, *Helicoverpa armigera* and *Spodoptera*

litura, but, as they did not use pure ecdysteroids, it is difficult to assess whether the observed effects are due only to ecdysteroids, as ferns also contain many other secondary metabolites potentially harmful for insects (Jones and Firn 1979; Markham et al. 2006.

In addition, ecdysteroids exert various pharmacological effects on mammals/humans (e.g., Dinan and Lafont 2006), and extracts from several fern species containing large amounts of ecdysteroids are used in traditional medicine (see Chap. 23 by Ho et al.).

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Chapter 23
Ferns: From Traditional Uses to Pharmaceutical Development, Chemical Identification of Active Principles

Raimana Ho, Taivini Teai, Jean-Pierre Bianchini, René Lafont, and Phila Raharivelomanana

23.1 Introduction

Pteridophytes (Fern and fern allies) have a long geological history on earth as pioneer plants which colonized our planet for millions of years. They form one of the oldest land plant groups on earth constituted by a vast group of vascular cryptograms including more than 10,000 species grown in varied climatic regions and geographically distributed. The medicinal use of ferns as a source of relief and treatment of various ailments is as old as humankind itself. Some of these uses of ferns have been transmitted through centuries. Later on, pharmacological and phytochemical investigations have tended to demystify and bring better knowledge about the contained plant metabolites and their biological properties.

23.2 Traditional Medicine Uses of Ferns

Folk medicinal uses of ferns have been transmitted through centuries orally from generation to generation or recorded in ancient literature of traditional pharmacopoeia. Collected data from different countries report ethnopharmacological investigations on medicinal plant uses including ferns and allies, which have been used since ancient times. Different fern species throughout the world are presently utilized to treat various ailments, mostly in developing countries, where herbal products still occupy a significant place in primary healthcare for cultural and economic reasons. The non exhaustive list given in Table 23.1 shows that most of the plants are used to cure common diseases because of their purgative and antibacterial properties or to treat gastric and renal infections; they were also used as diuretics, pain killers (to treat headache, stomachaches, gastrointestinal aches) and anti-inflammatory agents. Related literature

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Table 23.1 Ferns used in traditional medicine

Family	Genus	Species	Geographical origin	Traditional uses	Literature
Adiantaceae	*Acrostichum*	*aureum*	Polynesia and Fiji	To promote healthy pregnancy, as a purgative, in wound healing, in sore throat, and for the treatment of elephantiasis	Grepin and Grepin (1984), Pétard (1986), Morrison et al. (1994), and Cambie and Ash (1994)
	Adiantum	*ceneatum*	Brazil	Pain killer	Bresciani et al. (2003)
		capillus-veneris	Callejón de Huaylas (Perú)	In bronchitis and cough treatment, as an emmenagogue	Hammond et al. (1998)
		capillus-veneris	Kumaun Himalaya	Febrifuge, diuretic, expectorant, emmenagogue, general tonic, in bronchitis and in cold and cough treatments	Upreti et al. (2009)
		capillus-veneris	Yunnan (China)	In dermatitis and cystitis treatment	Lee et al. (2008)
		flabellulatum	Hainan island (China)	Nail cicatrization and snake bite treatment	Zheng and Xing (2009)
		incisium	Kumaun Himalaya	Control of internal burning sensation of the body, as antitussive, antidiabetic, febrifuge, in the treatment of skin diseases	Upreti et al. (2009)
		lunulatum	India	contraceptive	Dhiman (1998)
		philippense	Kumaun Himalaya	Antitussive, febrifuge, leprosy, asthma, and as a treatment for hair fall	Upreti et al. (2009)
		soboliferum	Hainan island (China)	Nail cicatrization	Zheng and Xing (2009)
		venustum	Kumaun Himalaya	Tonic, expectorant, astringent, emetic, diuretic, febrifuge, in scorpion bite treatment	Upreti et al. (2009)

	Cheilanthes	*farinosa*	Ethiopia	In the treatment of inflammatory skin disorders	Yonathan et al. (2006)
Aspleniaceae	*Asplenium*	*indicum*	India	Gonorrhea	Rout et al. (2009)
		laciniatum	India	Leukorrhea	Rout et al. (2009)
		nidus	India	Antipyretic, in the treatment of elephantiasis, emollient in cough and chest diseases, leaves are smoked to cure cold	Benjamin and Manickam (2007)
		nidus	Kumaun Himalaya	In the treatment of jaundice, malaria, for the removal of calculi	Upreti et al. (2009)
		nidus	Polynesia	Contraceptive	Pétard (1986), Bourdy (1996), Morrison et al. (1994)
		polydon	India	To promote parturition, as anticancer agent	Benjamin and Manickam (2007)
		pseudolaserpitiifolium	Hainan island (China)	In rheumatoid arthritis treatment	Zheng and Xing (2009)
		trichomanes	Italy	Emmenagogue, expectorant, antitussive, laxative	Negri (1979), Pomini (1990)
			North America	As an abortifacient and in amenorrhea	Moerman (1998)
			Kumaun Himalaya	Laxative, expectorant, in the treatment of cold	Upreti et al. (2009)
	Neottopteris	*nidus*	Hainan island (China)	In the treatment of rheumatoid arthritis	Zheng and Xing (2009)
Blechnaceae	*Blechnum*	*occidentale*	Brazil	Anti-inflammatory, in urinary infections, in pulmonary and liver disease treatments	Nonato et al. (2009)
		orientale	India	Impotence	Dhiman (1998)

(continued)

Table 23.1 (continued)

Family	Genus	Species	Geographical origin	Traditional uses	Literature
		orientale	Tahiti, Cook Islands	In cicatrization, general tonic,	Pétard (1986), Defilipps et al. (1998)
		orientale	Kumaun Himalaya	Anthelmintic, in typhoid treatment	Upreti et al. (2009)
Cyatheaceae	*Alsophila*	*costularis*	Yunnan (China)	Hepatitis treatment	Lee et al. (2008)
	Cyathea	*affinis*	Polynesia	Haemostatic	Pétard (1986)
		medullaris	Polynesia	Haemostatic	Pétard (1986)
		phalerata	Brazil	Varicose veins and hemorrhoid treatment, anti-inflammatory	Appel Hort et al. (2008)
	Hemitelia	*stokesii*	Rapa Nui (Chile)	Haemostatic	Pétard (1986)
Davalliaceae	*Davallia*	*fijiensis*	Fiji	Healing of fractured bones	WHO (1998)
		mariesii	China	Healing of fractured bones, pain killer, anti-inflammatory, anticancer, antiaging, hemostasis	Chang et al. (2007)
		solida	Polynesia	Cleansing bath for newborn, in dysmenorrhea, gonorrhea, leukorrhea, sore throat, asthma, as a purgative, for wound healing, as antiemetic, in sprain, and for the healing of fractured bones	Grepin and Grepin (1984), Pétard (1986), Whistler (1992a)
Dicksoniaceae	*Cibotium*	*barometz*	Yunnan (China)	Antihemorrhagic	Lee et al. (2008)
Dryopteridaceae	*Nephrolepis*	*biserrata*	Polynesia	Newborn umbilical cicatrization	Pétard (1986)
		exaltata	Polynesia	Newborn umbilical cicatrization	Pétard (1986)
		hirsulata	Tonga	Postpartum care, bellyache	Bloomfield (2002)
		hirsutula	Polynesia	Newborn umbilical cicatrization	Pétard (1986)
	Polystichum	*pungens*	Eastern Cape (South Africa)	Wound healing	Grierson and Afolayan (1999)

Equisetaceae	*Equisetum*	*ramosissimum*	South Africa	Improve the fertility of women diuretic, Haemostatic, antirheumatic, antifungal, antiviral, in bone injury	Benjamin and Manickam (2007)
Gleicheniaceae	*Dicranopteris*	*linearis*	India	Treatment of sterility	Vasudeva (1999)
		linearis	Kumaun Himalaya	Laxative, anthelmintic, antibacterial, in asthma and treatment of infertility in women	Upreti et al. (2009)
	Gleichenia	*linearis*	Polynesia	Gonorrhea treatment, hernia	Grepin and Grepin (1984) and Pétard (1986)
Helminthostachyaceae	*Helminthostachys*	*zeylanica*	Kumaun Himalaya	Intoxicant, in sciatica, aphrodisiac	Upreti et al. (2009)
Hemionitidaceae	*Hemionitis*	*arifolia*	India	Antidiabetic, aches and burns	Ajikumaran et al. (2006)
Isoetaceae	*Isoetes*	*coramandeliana*	Europa	Treatment of spleen and liver diseases	Benjamin and Manickam (2007)
Lindsaeaceae	*Odontosoria*	*chinensis*	Mauritius	In chronic enteritis	Benjamin and Manickam (2007)
	Sphenomeris	*chinensis*	Kumaun Himalaya	Diuretic, in chronic enteritis, and sprain treatment	Upreti et al. (2009)
Lycopodiaceae	*Huperzia*	*serrata*	China	Febrifuge, blood disorder treatment, contusions, strains, swelling, myasthenia gravis, schizophrenia	Ma et al. (2007) Zhang et al. (2008)
	Lycopodiella	*cernua*	Kumaun Himalaya	Antitussive, beriberi and skin eruption treatment	Upreti et al. (2009)
		cernua	Fiji	Rheumatism treatment	Cambie and Ash (1994)
Lygodiaceae	*Lygodium*	*conforme*	Hainan island (China)	Hepatitis treatment	Zheng and Xing (2009)
		flexuosum	Yunnan (China)	Rheumatoid lumbago treatment, gallstone	Lee et al. (2008)
		flexuosum	Kumaun Himalaya	Expectorant, rheumatism, sprains, scabies, eczema and for treating cut and also liver disorders	Upreti et al. (2009)

(continued)

Table 23.1 (continued)

Family	Genus	Species	Geographical origin	Traditional uses	Literature
		japonicum	Kumaun Himalaya	Diuretic, antispasmodic, in rheumatism, and in treatment of pulmonary, and renal diseases	Upreti et al. (2009)
		japonicum	Hainan island (China)	Diuretic, purgative, in colds, headaches, gastrointestinal disorders, hepatitis	Zheng and Xing (2009)
		reticulatum	Fiji	Dysmenorrhea, digestive, contraceptive, healing of fractured bones and for the treatment of hemorrhoids and impotence	Cambie and Ash (1994)
Marattiaceae	*Angiopteris*	*evecta*	Polynesia & Fiji	Wound healing	Pétard (1986), Cambie and Ash (1994)
	Marattia	*fraxinea*	South Africa	Remedy for ancylostomiasis	Benjamin and Manickam (2007)
		salicina	Polynesia	Wound healing, antibacterial	Pétard (1986)
Nephrolepidaceae	*Nephrolepis*	*cordifolia*	Kumaun Himalaya	Wound healing, antitussive, intestinal disorders, stomach ulcer and treatment of stomach ulcers or excess of acidity	Upreti et al. (2009)
Ophioglossaceae	*Ophioglossum*	reticulatum	Kumaun Himalaya	To treat burns, as a tonic, in wound healing	Upreti et al. (2009)
		reticulata	Polynesia	Newborn umbilical cicatrization, purgative, treatment of lip and mouth infections	Grepin and Grepin (1984), Pétard (1986), Whistler (1992a), WHO (1998)
		vulgatum	Yunnan (China)	Jaundice and hepatitis treatment	Lee, Xiao and Pei (2008)
	Osmundopteris	*lanuginosa*	Yunnan (China)	Hypertension treatment	Lee et al. (2008)
Polygonaceae	*Polygonum*	*chinensis*	Hainan island (China)	Hematochezia, diarrhea, febrifuge, diuretic	Zheng and Xing (2009)

Polypodiaceae	*Drynaria*	*fortunei*	Yunnan (China)	Lumbago treatment	Lee, Xiao and Pei (2008)
		rosii	Hainan island (China)	Hematochezia, diarrhea, febrifuge, diuretic	Zheng and Xing (2009)
	Lemmaphyllum	*microphyllum*	Hainan island (China)	In rheumatoid arthritis, sore throat and cough treatment	Zheng and Xing (2009)
	Microgramma	*squamulosa*	São Paulo (Brazil)	Antiulcer	Suffredini et al. (1999)
	Microsorum	*commutatum*	Polynesia	Renal and urological disorders	Grepin and Grepin (1984), Pétard (1986)
		grossum	Fiji	Healing of fractured bones, as a painkiller, in influenza, diarrhea and in stomachache treatment, as a muscle relaxant	Cambie and Ash (1994)
		membranifolium	Polynesia	Cleansing bath for newborn, in dysmenorrhea, gonorrhea and leukorrhea, sore throat, asthma, as a purgative, in wound healing, antiemetic, in sprains, and healing of fractured bones	Grepin and Grepin (1984), Pétard (1986)
		scolopendria	Polynesia	Skin inflammation treatment, wound healing, purgative, anti-inflammatory	Whistler (1992a), Whistler (1992b), Bloomfield (2002)
	Phyllitis	*scolopendrium*	Romania	Diuretic, astringent, expectorant, wound healing	Oniga et al. (2004)
		scolopendrium	Catalonia (Spain)	Anti-inflammatory, pulmonary and liver disease treatment	Bonet and Vallès (2007)
	Phymatodes[a]	*scolopendria*	Madagascar	Anti-inflammatory, antitussive and asthma treatment	Beaujard (1988) Ramanitrahasimbola et al. (2005)

(continued)

Table 23.1 (continued)

Family	Genus	Species	Geographical origin	Traditional uses	Literature
	Phymatosorus[b]	*scolopendria*	Polynesia	Cleansing bath for newborn, dysmenorrhea, gonorrhea and leukorrhea, sore throat, asthma, purgative, wound healing, antiemetic, sprain, healing of fractured bones	Pétard (1986), Whistler (1992a), WHO (1998)
	Polypodium	*angustifolium*	South America	Anti-inflammatory	Pareja (1988)
		decumanum	South America	Skin disorders and psoriasis treatment	Bustillos et al. (2002)
		leucotomos	South America, India	Antiphlogistic, antitumoral, anti-inflammatory for skin disease, psoriasis treatment, general tonic	Lucca (1992) and House et al. (1994)
		subpetiolatum	Guatemala	Anti-inflammatory	Anderson et al. (1979)
		vulgare	Spain	Jaundice	Bonet and Vallès (2007)
	Pyrrosia	*adnascens*	Hainan island (China)	Rheumatoid arthritis treatment	Zheng and Xing (2009)
		lanceolata	Mexico	Skin disorders with intense irritation	Benjamin and Manickam (2007)
		lanceolata	South Africa	Curing colds and sore throats	Benjamin and Manickam (2007)
		lingua	Yunnan (China)	Cystitis treatment	Lee et al. (2008)
		nuda	Yunnan (China)	Cystitis treatment	Lee et al. (2008)
Psilotaceae	*Psilotum*	*nudum*	Hawaï	Laxative, general tonic	Whistler (1992a)
Pteridaceae	*Pityrogramma*	*calomelanos*	Ayabaca (Peru)	Wound healing	De Feo (2003)
		calemelanos	Trinidad	In hypertension, fever, and cough	Benjamin and Manickam (2007)
		calemelanos	Philippines	Renal disorders	Benjamin and Manickam (2007)
		calomelanos	Ayabaca (Peru)	Wound healing	De Feo (2003)

	Pteridium	*aquilinium*	India	Astringent, anthelmintic, useful in diarrhoea.	Benjamin and Manickam (2007)
		aquilinium	Brazil	Astringent, anthelmintic, useful in diarrhoea	Santos et al. (1987), Alonso-Amelot (2002)
	Pteris	*biaurita*	Kumaun Himalaya	Chronic disorders	Upreti et al. (2009)
		ensiformis	Vanuatu	Dysmenorrhea treatment	Morrison et al. (1994)
		ensiformis	Hainan island (China)	Rheumatoid arthritis treatment, digestive, purgative	Zheng and Xing (2009)
		multifida	Hainan island (China)	Haemostatic, to treat colds	Zheng and Xing (2009)
		semipinnata	Hainan island (China)	Snake bite treatment	Zheng and Xing (2009)
		wallichiana	Kumaun Himalaya	Astringent, dysentery, and skin infection treatment	Upreti et al. (2009)
Selaginellalaceae	*Selaginella*	*bryopteris*	Kumaun Himalaya	Diuretic and gonorrhea treatment	Upreti et al. (2009)
Tectariaceae	*Tectaria*	*coadunate*	Kumaun Himalaya	Colitis and stomachache treatment	Upreti et al. (2009)
Thelypteridaceae	*Pronephrium*	*gymnopteridifrans*	Hainan island (China)	Cold	Zheng and Xing (2009)
	Thelypteris	*arida*	Kumaun Himalaya	Wound healing	Upreti et al. (2009)

[a]*Phymatodes scolopendria* is a synonym of *Microsorum scolopendria* (Nooteboom 1997)
[b]*Phymatosaurus scolopendria* is a synonym of *Microsorum scolopendria* (Nooteboom 1997)

described that different parts of the plants (rhizomes, stems, fronds, pinnae, and spores) were utilized in various ways using recipes specific to each region. Plant-based remedies are delivered either by external application (topical applications such as lotions, frictions, poultices, eye drops, fumigations, baths, and gargles) or by internal intake formulated in different ways (potions) (Grepin and Grepin 1984; Pétard 1986; Whistler 1992a, b; Cambie and Ash 1994; Whistler 1996; Bloomfield 2002; Lee et al. 2008; Upreti et al. 2009; Zheng and Xing 2009).

Poor specificity of the remedy is also noticed, as the same plant may be used to treat different ailments, for example, the uses of *Microsorum scolopendria* in Polynesia (cleansing baths for newborns, dysmenorrheas, gonorrheas, leukorrheas, sore throats, asthma, purges, wound healing, antiemetic, sprain and bone injury treatments) or the uses of *Adiantum capillus-veneris* in Kumaun Himalaya (febrifuges, diuretics, expectorant, emmenagogue, bronchitis, general tonic, and cold and cough treatments) (Upreti et al. 2009). On the other hand, it is often reported that one specific remedy may include the use of different plants as ingredients for the same medicinal preparation, which is the case of the traditional Chinese remedy called "Gusuibu" containing six different ferns (*Drynaria fortunei*, *Pseudodrynaria coronans*, *Davallia divaricata*, *Davallia mariesii*, *Davallia solida*, and *Humata griffithiana*) (Chang et al. 2007).

Geographical variations on medicinal plant uses are also evident in Table 23.1, for example, the use of *Lygodium flexuosum* in the Kumaun Himalaya region (expectorant, rheumatism, sprains, scabies…) differs from its use in Yunnan China region (rheumatoid lumbago, gallstone) (Lee et al. 2008; Upreti et al. 2009). Some specific regional medicinal uses reflect cultural roots such as plants particularly used for the umbilical cicatrisation of newborn children (Grepin and Grepin 1984; Pétard 1986; Bloomfield 2002).

For centuries, because of botanical and taxonomic confusion of closely related taxa, groups of plants that were known with the same vernacular name were used for the same medicinal indications. So, for example, "C*alaguala*" was the vernacular name attributed to different *Polypodium* species grown in the South American region, which are traditionally used for the same therapeutic indications (Pareja 1988). In the same way, "*Metuapua'a*" is the common vernacular name of the widely used Polynesian medicinal fern corresponding to, at least, two different species of *Microsorum* (*M. scolopendria* and *M. membranifolium*), which are also used for the same treatments (Ho et al. 2007, 2008). Such botanical confusion may cause safety warnings when toxic fern species are involved in these closely related species or when the presence or absence of the active principle may have healthy or harmful consequences. This question is closely related to the chemical composition of these medicinal ferns.

23.3 Fern Bioactive Components

Biological activity and phytochemical assessments of fern extracts have been carried out for some medicinal fern species over the world. For that purpose, different in vitro and in vivo bioassays were performed to evaluate the biological potency of whole extracts, fractions, or isolated compounds. The chemical constituent

identification network needed separation and isolation steps using various fractionation processes, including partitions and chromatographic techniques (LPCC, MPCC, HPLC, TLC), followed by the combined use of spectroscopic methods (UV, IR, MS, NMR), which depend mainly on the targeted compound chemical class. A wide variety of secondary metabolites were reported as active components of fern species and mainly belonging to the terpenoid class (triterpenoids, diterpenoids, sesquiterpenoids), the phenolic group (phenylpropanoid derivatives and others), the flavonoid class, and the alkaloid class. Examples of medicinal fern contents and their biological activities are compiled in Table 23.2. Examples of molecular structures of these fern active compounds are compiled in Figs. 23.1–23.4, showing the different degrees of glycosylation (with different sugars) of some molecules.

23.3.1 Terpenoids

Terpenoids constitute the largest chemical group found in ferns including mainly triterpenoids, diterpenoids, and sesquiterpenoids having various biological interests.

23.3.2 Triterpenoids

Triterpenoids compounds are well represented in fern as hydrocarbons or functionalized molecules belonging to the steroidal group of ecdysteroids (see Chap. 22). Hydrocarbons such as "fern-7-en" or filicene (Fig. 23.1a) are typical fern components, which could be used as a marker of fern biodiversity (Reddy et al. 2001; Nakane et al. 2002; Bresciani et al. 2003; Singh et al. 2008; Parihar et al. 2010). Ecdysteroids are very interesting metabolites due to their wide spectra of biological activities including their adaptogenic behavior which offers a large potential for their anabolic, hypoglycemic, hypocholesteremic, tonic, hepatoprotective, antidepressant, and purgative effects (Lafont and Dinan 2003). *M. scolopendria* and *M. membranifolium* are traditional medicine ferns grown in Polynesia that have been found to be a rich source of ecdysteroids containing, as major constituents, 20-hydroxyecdysone, ecdysone, 2-deoxy-20-hydroxyecdysone, and 2-deoxyecdysone (Fig. 23.1a) in their fronds and rhizomes. The use of the latter plants in Polynesian traditional medicine can be fully justified by the reported biological activities of ecdysteroids (Ho et al. 2007; Meybeck et al. 2010).

23.3.3 Diterpenoids

Diterpenoids components were reported in medicinal ferns, mostly found in *Pteris* species belonging to ent-kaurane, ent-atisane, and ent-pimarane types (Alonso-Amelot 2002). Ent-kaurane compounds such as pterikauranes (M1, M2, M3), constituents of the Chinese medicinal fern *Pteris multifida* are shown in Figure 23.1b (Ge et al. 2008).

Table 23.2 Bioactive molecules of ferns

Family	Genus	Species	Family compounds	Bioactive molecules	Activities	Literature
Adiantaceae	*Acrostichum*	*aureum*	Phenolic compounds	Catechin	Antimicrobial	Cambie and Ash (1994)
	Adiantum	*ceneatum*	Triterpenoids	Filicene, filicenal	Analgesic	Bresciani et al. (2003)
		capillus-veneris	Hopane triterpenoids	4-Hydroxyfilican-3-one; fern-9 (11)-en-12β-ol; olean-18-en-3-one; olean-12-en-3-one	Antibacterial	Nakane et al. (2002), Singh et al. (2008), Parihar et al. (2010)
		lunulatum	Triterpenoids	Fern-9 (11)-ene; fern-9 (11)-en-25-oic acid; fern-9 (11)-en-28-ol; 22,29-diepoxy-30-norhopane-13β-ol filicenol B; adiantone	Antibacterial	Reddy et al. (2001)
	Cheilanthes	*farinosa*	Phenolic compounds	Rutin, cinnamic acids, caffeic acid, quinic acid derivatives, chlorogenic acid	Anti-inflammatory, antinociceptive	Yonathan et al. (2006)
Aspleniaceae	*Asplenium*	*trichomanes*	Phenolic compounds	4-Vinyl-phenol; 4-vinyl-phenol-1-*O*-[α–L-rhamnopyranosyl-(1—>6) β–D-glucopyranose]; phylligenin; arctigenin	Estrogenic	Dall'Acqua et al. (2009)
Davalliaceae	*Davallia*	*mariesii*	Phenolic compounds	Procyanidin-B5; epicatechin (4β-8)-epicatechin-(4β-6)-epicatechin; epicatechin-(4β-8)-epicatechin-(4β-6)-epicatechin); davallialactone; caffeic acids	Inhibition of protein kinase C	Cui et al. (1990), Cui et al. (1992)
		solida	Phenolic compounds	4-0-β-Glucopyranosyl-2,6,4′-trihydroxybenzophenone	Reversal of ciguatoxin-1B effects on myelinated axons	Benoit et al. (2000)

Lycopodiaceae	*Huperzia*	*serrata*	Alkaloids	Huperzine A, B, and R; 8-β phlegmariurine B	Neuroprotective, A D treatment	Zangara (2003), Zhang et al. (2008)
	Lycopodiella	*cernua*	Alkaloids	Cernuine; lycocernuine; lycopodine; dihydroxycernuine	Hay fever treatment, antifungal, inhibition of candidiasis	Cambie and Ash (1994), Zhang et al. (2002)
Marattiaceae	*Angiopteris*	*evecta*	Flavonoids	Violanthin, isoviolanthin	Hypoglycemic (antidiabetic)	Nguyen (2005)
Polypodiaceae	*Microsorum*	*membranifolium*	Triterpenoids	Ecdysone; 20-hydroxyecdysone; 2dE, 2d20E, 2d20E 3-[4-(1β-D-glucopyranosyl)]-ferulate, 2dE 3-[4-(1β-D-glucopyranosyl)]-ferulate and 2dE 25-α-L-rhamnopyranoside (6-deoxy-α-L-mannopyranoside)	Antioxidant, for skin and hair care	Ho et al. (2007), Ho et al. (2008), Meybeck et al. (2010)
		scolopendria	Triterpenoids	Ecdysone; 20-hydroxyecdysone; makisterones A and C; inokosterone; amarasterone; posterone; 24,28-diepi-cyasterone; 20-deoxymakisterone A; 25-deoxyecdysone-22-glucoside	Antioxidant, for skin and haircare	Snogan et al. (2007), Ho et al. (2008), Meybeck et al. (2010)
	Phymatodes[a]	*scolopendria*	Phenolics	Coumarin	Bronchodilator activity	Ramanitrahasimbola et al. (2004)
	Polypodium	*angustifolium*	Phenolics	Anthraquinone	Synthesis of corticoid hormones	Pareja (1988)
		decumanum	Triterpenoids	fer-9(11)-ene,29-acetoxy-hopane	Antimalarial, antibacterial	Bustillos et al. (2002)

(continued)

Table 23.2 (continued)

Family	Genus	Species	Family compounds	Bioactive molecules	Activities	Literature
		leucotomos	Triterpenoids and Phenolic compounds	Ecdysone, ecdysterone; 3,4-dihydroxybenzoic acid; 4–hydroxybenzoic acid; chlorogenic acid; vanillic acid; caffeic acid; ferulic acid; 4-hydroxycinnamic acid; 4-hydroxycinnamoyl-quinic acid	Antioxidant, photoprotectant, anti-inflammatory, immunomodulator, anti-psoriasis	Garcia et al. (2006), Das and Einstein (2007)
		subpetiolatum	Triterpenoids	Fer-9(11)-ene; neohop-13(18)-ene; diploptene; fern-7-ene; hop-17(21)-ene; serratene	Antineoplastic	Anderson et al. (1979)
		vulgare	Triterpenoids and Flavonoids	Ecdysone- 20 *E*; abutasterone; polypodine B; inokosterone; 24-hydroxyecdysone; pterosterone; catechin; saponin; osladin; polypodin	Antioxidant, diuretic, hypocholesteremic	Grzybek (1983), Reixach et al. (1997), Duke (2001), Bagniewska-Zadworna et al. (2008)
Pteridaceae	*Pityrogramma*	*calomelanos*	Phenolic compounds	Dihydrochalcone	Cytotoxic, anticancer	Martin et al. (2006)
	Pteridium	*aquilinium*	Sesquiterpenoids	2,5,7-Trimethyl-indan-1-one; pterosin Z; acetyl-Δ-dehydropterosin B; ptaquiloside	Carcinogenic, cytotoxic	Yamada et al. (2007)), Ge et al. (2008)
	Pteris	*ensiformis*	Phenolic compounds	7-*O*-caffeoylhydroxymaltol3-*O*-β-D-glucopyranoside; hispidin4-*O*-b-D-glucopyranoside-[6-(3,4-dihydroxystyryl)-4-*O*-β-D-glucopyranoside-2-pyrone]	Atherosclerosis prevention	Wei et al. (2007)
		multifida	Diterpenoids	Entkaurane-2beta, 16 alpha-diol; entkaur-16-ene-2beta, 15alpha-diol; pterokaurane M1; pterokaurane M2; pterokaurane M3	Cytotoxic, antitumor	Woerdenbag et al. (1996), Hu et al. (2006), Zheng et al. (2008a, 2008b), Ge et al. (2008)

Flavonoids	Apigenin; apigenin-7-*O*-β-D-glucopyranosyl-4′-*O*-α-L-rhamnopyranoside; apigenin-7-*O*-β-D-glucopyranoside; apigenin-4′-*O*-β-L-rhamnopyranoside; apigenin-7-*O*-β-D-neohesperidoside; naringenin-7-*O*-β-D-neohesperidoside; luteolin; lueolin-7-*O*-β-D-glucopyranoside; naringenin-7-*O*-β-D-neohesperidoside; muxiangrine III	Cytotoxic, antitumor	Ouyang, Yang and Kong (2008), Wang and Zhang (2008), Ouyang et al. (2009)
Triterpenoids	Polyporusterone I; β-sitosterol; β-rosasterol; scaphopetalone	Cytotoxic, antitumor	Hu and Zheng (2004), Hu and Zheng (2005); Zheng, Hu and Hu (2008a)
Sesquiterpenoids	Multifidoside A; multifoside B; multifidoside C; (2*S*,3*S*)-pterosin C; (2*R*,3*S*)-pterosin C; (2S,3*S*)-pterosin S	cytotoxic, antitumor, anti-inflammatory	Ben Cao (1999), Hu et al. (2006), Ge et al. (2008), Ouyang, Yang and Kong (2008)
Phenolic compounds	Isovanillic acid; ferulic acid; licoagrochalcone D; vanillin; 5-(3″-methylbutyl)-8-methoxyfurocoumarin; 2-(3′-hydroxy-3′-methylbutyl)-4-hydroxy-5-methoxyphenol-1-*O*-b-D-glucopyranoside	Cytotoxic, antitumor	Hu and Zheng (2004), Hu and Zheng (2005), Ouyang, Yang and Kong (2008), Zheng, Hu and Hu (2008b)

[a]*Phymatodes scolopendria is a synonym of Microsorum scolopendria* (Nooteboom 1997)

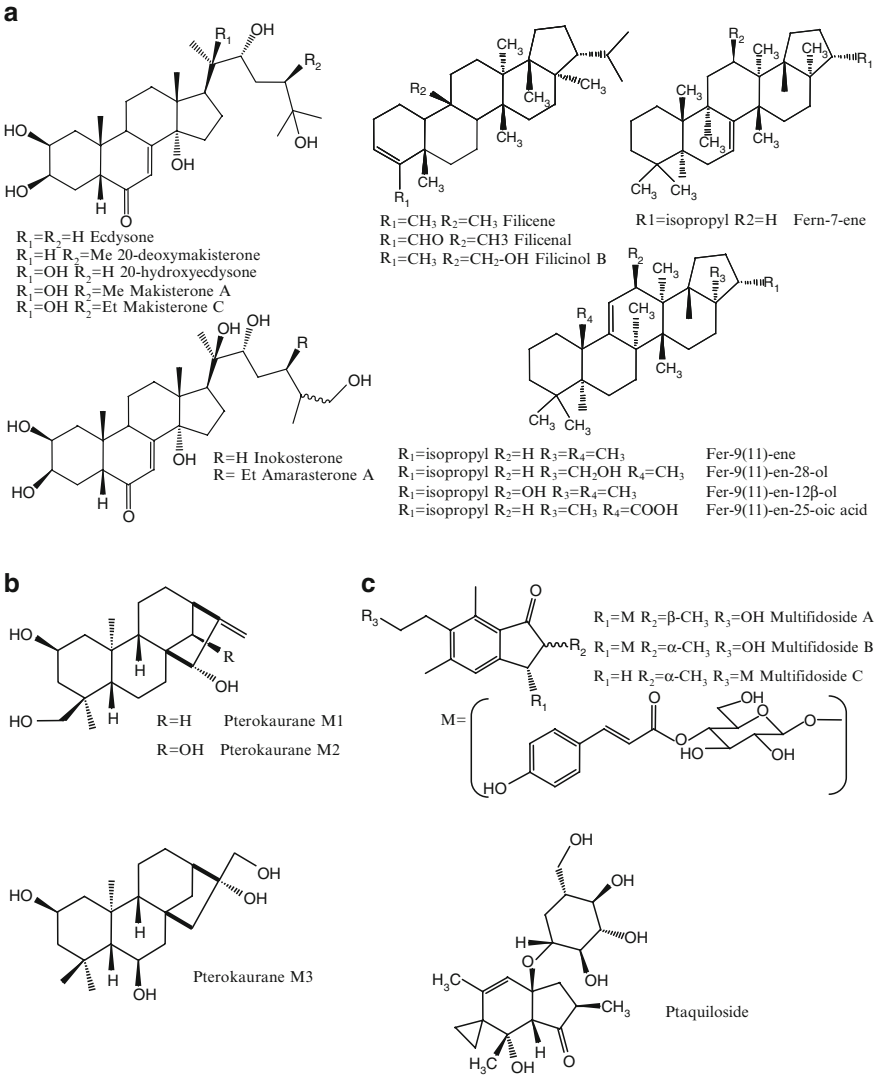

Fig. 23.1 (**a**) Examples of triterpenoid components present in ferns. (**b**) Examples of diterpenoid components present in ferns. (**c**) Examples of sesquiterpenoid components present in ferns

23.3.4 Sesquiterpenoids

Ferns contain sesquiterpenoid compounds belonging to different skeletons such as indane or cadinane. A specific group of nor-sesquiterpenoids, mainly represented by sesquiterpenyl indanones called pterosins and their glycosides pterosides, are known for their biological activities (Ben Cao 1999; Hu et al. 2006; Ge et al. 2008; Ouyang et al. 2008; Zheng et al. 2008a). These compounds are usually found in

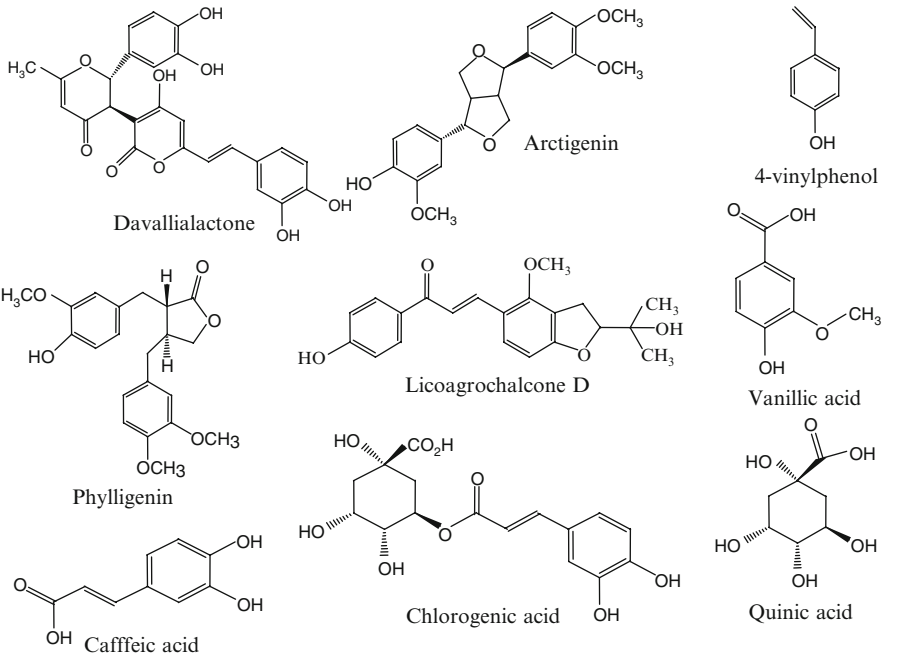

Fig. 23.2 Examples of phenolic molecules present in ferns

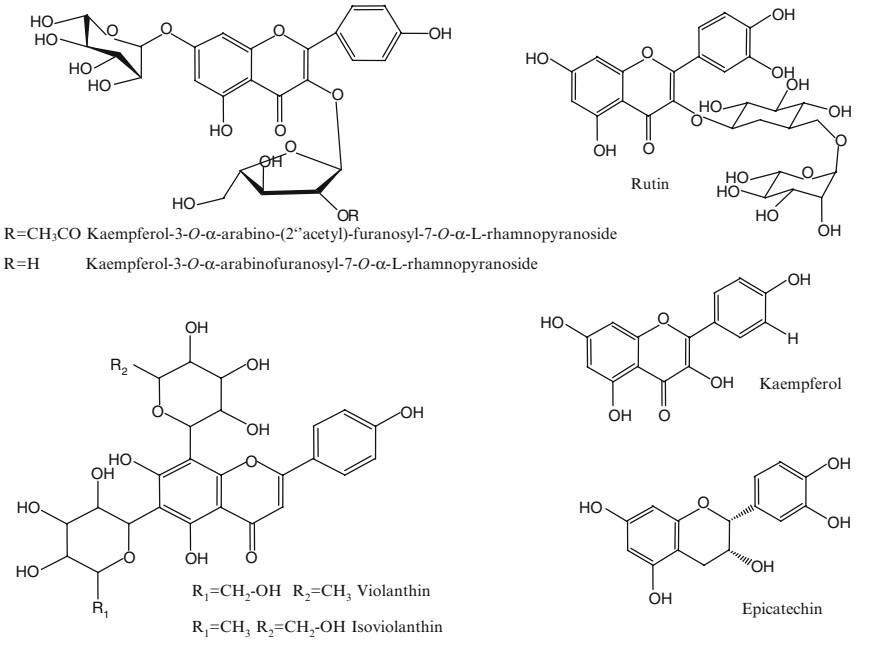

Fig. 23.3 Examples of flavonoid structures present in ferns

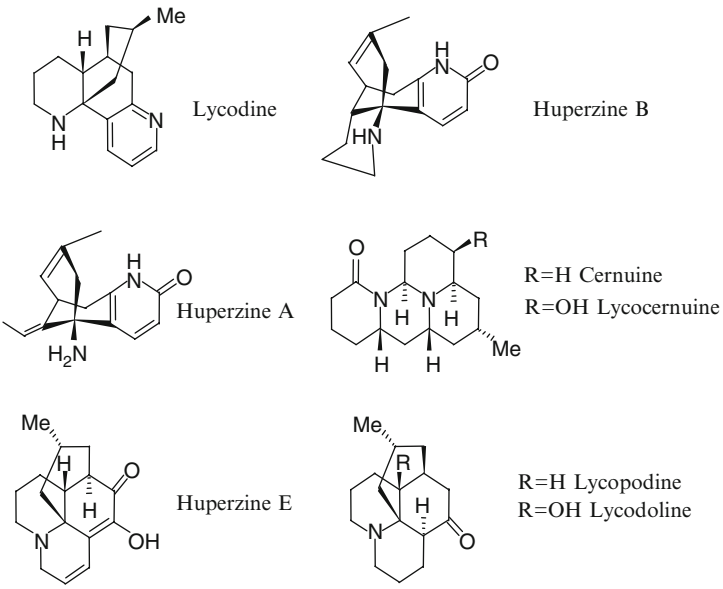

Fig. 23.4 Examples of alkaloid components of ferns

bracken species and polypodiaceous ferns, some of them being used in traditional medicine. For example, *Pteris multifida* contains multifidosides (A and B) (Figure 23.1c) having significant inhibitory effects against HepG2 cells and K562 cells with IC_{50} value at a micromolar level, which revealed that cytoxicity could be related to the antitumor activity of this medicinal plant (Ge et al. 2008; Ouyang et al. 2008). This latter case is a good example showing a coherence between the revealed pharmaceutical properties of the molecules contained in the plant and its traditional use.

Pteridium aquilinium, a bracken fern that was also used as a medicinal plant in some regions, contains ptaquiloside (Fig. 23.1c), a pteroside compound well known as a carcinogenic and toxic molecule causing severe health problems (Yamada et al. 2007). The need for pharmacovigilance measures must be pointed out to limit the medicinal use of such a hazardous plant (Alonso-Amelot 2002; Yamada et al. 2007).

23.3.5 Phenolic Compounds

Phenolic compounds like phenylpropanoid derivatives, whether glycosylated or not, are widely distributed in ferns as bioactive components. Compounds, such as chlorogenic, caffeic, ferulic, hydroxybenzoic, hydroxycinnamic, and vanillic acids, bearing

acidic functions, are frequently found in different species (Fig. 23.2). Various biological activities are attributed to these compounds, mainly antioxidant and photoprotectant activities reported for the medicinal fern *Polypodium leucotomos* that was also used as the active principle of skin care formulas, especially for psoriasis treatment (Lucca 1992; House et al. 1994; Gonzalez, Gilaberte and Philips 2010). Atherosclerosis prevention potential of *Pteris ensiformis* is supposedly attributed to its glycosylated phenolic acid constituent (7-O-caffeoylhydroxymaltol-3-β-D-glucopyranoside) (Wei et al. 2007).

Chalcone derivatives constitute a class of phenolic compounds found in medicinal ferns. *Pityrogramma calomelanos* contain the dihydrochalcone component, the cytotoxicity of which is supposed to be responsible of its anticancer potential (Martin et al. 2006). Licoagrochalcone D is also another chalcone compound present in *Pteris multifida* that may contribute to its overall biological activity (Hu and Zheng 2005).

The traditional medicine uses of *Asplenium trichomanes*, including as an emmenagogue and abortifacient (Negri 1979; Moerman 1998; Pomini 1990), were related to revealed in vitro estrogenic activity of the plant extracts and isolated compounds. Some of these estrogenic active components are phenolic derivatives such as 4-vinylphenol, phylligenin, and arctigenin shown in Fig. 23.2 (Dall'Acqua et al. 2009).

23.3.6 Flavonoids

Numerous flavonoid components were reported as medicinal fern constituents, like glycosylated or uncommon ones (apigenin, luteolin, naringenin, kaempferol), having strong multiple physiological and biological activities such as reducing blood lipid levels, protecting the liver, resisting inflammation, relaxing coronary arteries, and antibacterial activity, among many other ones. Specific ones such as violanthin and isoviolanthin (Fig. 23.3), contained in *Angiopteris evecta*, were supposed to be responsible for the hypoglycemic and antidiabetic effects of this medicinal plant (Nguyen 2005). Flavonoid components of *Pteris multifida* were extracted as active ingredients to treat hepatitis (Wang and Zhang 2008).

23.3.7 Alkaloids

Lycopodiaceae (fern in *sensu latu*) is a family that contains typical alkaloids, some of them contained in medicinal ferns. *Lycopodium* alkaloids comprise diverse compounds that often possess unusual skeletons but can be classified in four major structural groups: lycopodine, lycodine, fawcettimine, and miscellaneous (Ma et al. 2007). Huperzine A, a lycodine alkaloid having a quinolizidine skeleton, was isolated from the Chinese folk medicine *Huperzia serrata*, belonging to the Huperziaceae family, used to

cure schizophrenia (and other diseases such as contusions, strains, swellings, and myasthenia gravis) and was found to be a strong inhibitor of acetylcholinesterase and so having interesting neuroprotective effects as a potential treatment for Alzheimer disease and vascular dementia (Liu et al. 1986a, b; Ma et al. 2007; Hostettmann et al. 2006; Zhang et al. 2008). Revealed biological activity of huperzine A can be related to the traditional use of *Huperzia serrata* as a treatment against schizophrenia. Examples of *Lycopodium* alkaloids found in medicinal ferns belonging to the Lycopodiaceae family, are also shown in Fig. 23.4, some of them reported to possess acetylcholinesterase inhibition activity (Ma et al. 2007).

23.4 Pharmaceutical Development

Traditional phytomedicine partly benefits from its centuries-long use in terms of efficiency and safety, even though pharmaceutical development of plant-based compounds still remains a tough and long-winded procedure. So, finally, very few drug candidates can reach the stage of clinical trials and real industrial development. Examples of fern-derived drugs and medicinal formula are presented herein.

23.4.1 Huperzine A

Phytochemical research on traditional Chinese medicine (TCM) provided findings of new plant-derived molecules that were developed as modern drugs, like in the success story of Huperzine A and B. Since its first isolation from *Huperzia serrata*, huperzine A attracted worldwide scientific interests, which led to intensive research on naturally occurring analogous structures from related taxa (genus and species), biological activities, synthesis, pharmacological properties, and development (Ma et al. 2007). Total synthesis of huperzine A allowed further clinical trials and formulation of the first new drug huperzine A tablet called “Shuangyipin,” which has been marketed in China since 1996 (Tang 1996). Nevertheless, huperzine-containing plants were screened to find new natural resources to lower the pressure on *H. serrata* and related species, which became increasingly threatened plants as they were harvested from the wild. Then, ZT-1, a novel huperzine A synthetic derivate also called “schiprizine,” was selected as a new drug candidate over 100 huperzine derivatives and found to be more potent than donepezil and tacrine, which are synthetic drugs used for Alzheimer disease treatment (Zhu et al. 1999, 2000; Liu et al. 2009). This later compound was patented for its synthesis and application and submitted to clinical studies (Phase I) in China and Switzerland (Wei et al. 2006, Xu and Zhao 2005). A clinical Phase II treatment of Alzheimer’s disease is being conducted in patients with mild to moderate symptoms (Liu et al. 2009). Recent biological investigations on huperzine A showed its multiple neuroprotective effects (regulation of APP metabolism, alleviating Aβ-induced oxidative stress, apoptosis and mitochondrial failure, and anti-inflammation),

with promising therapeutic applications, and still motivate further investigations for a better understanding of its mode of action (Zhang et al. 2008).

23.4.2 Pharmaceutical Preparations from Polypodium leucotomos

P. leucotomos is a widespread fern, mainly in South America, and its traditional medicine uses and observed efficiency led to production, at industrial level, of standardized pharmaceutical preparations containing *P. leucotomos* extracts for different applications in complementary and alternative medicine. "Anapsos" is a Spanish pharmaceutical formulation (registered as "Regender" and "Armaya fuerte" by Spanish Health department) containing *P. leucotomos*, whose dermatological use for psoriasis and atopic dermatitis treatment was authorized after controlled clinical trials (Beltran et al. 1982; Jimenez et al. 1987, Lopez and Vargas 1988, Vasange-Tuominen et al. 1994, Sempere-Ortells et al. 2002). Further pharmacological studies showed other biological activities of "Anapsos," mainly as photoprotectant, immunomodulator, and antioxidant, which would offer new uses in nutraceutical and cosmetic fields (Gonzalez and Pathak 1996; Gombau et al. 2006). "Fernblock" is also a formulation of an aqueous extract of the aerial part of *P. leucotomos* for topical and oral photoprotective effects due to its antioxidant activity (Garcia et al. 2006).

In conclusion, ethnopharmacological data report numerous and various uses of ferns in traditional medicine all over the world for thousands of years and some of them were subjected to phytochemical and pharmaceutical investigations. Interesting biological activities of the studied ferns were also revealed, but they are rarely related to the reported traditional uses. Actually, the performed bioassays correspond to specific diseases that might chronologically not have been known at the period when the plant was used. For all the studied ferns, the bioactive constituent is rarely subjected to pharmaceutical development or any kind of valorization. From these above observations, we can point out the gap between modern pharmacological knowledge and traditional uses of ferns and the lack of development of the known active principles. So, more phytochemical and pharmaceutical studies are encouraged to be carried out for a better understanding of the medicinal uses of ferns, aiming at a better valorization of these plants.

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Chapter 24
Functional Activities of Ferns for Human Health

Cheol Hee Lee and So Lim Shin

24.1 Introduction

Most people think that there are limited uses for ferns. However, these plants have given many health benefits to humans since ancient times. Not surprisingly, herbal medicines of Chinese, Indian, and Native American peoples include ferns. These cultures have used them for food, tea, and drugs. In the present day, the functional activities of ferns and fern allies for human health have been studied using several advanced scientific technologies. Compared to flowering plants, ferns and fern allies have limited use to human health in modern times. So, various functional activities for human life and possibilities of industrial application of ferns and fern allies will be discussed in this chapter.

Plants normally produce various secondary metabolites not only to adapt to their environment but also to defend themselves against biotic or abiotic stress, such as high light intensity, extremely high or low temperature, high salinity, drought and natural enemies. To provide protection against adverse effects of their environment, plants have the tendency to produce many kinds of secondary metabolites in severe conditions (Bennett and Wallsgrove 2006). These metabolites are polyphenols, flavonoids, terpenoids, steroids, quinones, alkaloids, polysaccharides and so on (Swain 1977). These metabolites are also engaged with the color, flavor and aroma of plants. These functional metabolites have properties which prevent and cure various diseases as well as aging in mammals including humans.

Since ferns and fern allies have survived from Paleozoic times, they have adapted with many more various changes of environment than the other primitive vascular plants (Wallace et al. 1991). Therefore, ferns are expected to have many useful secondary metabolites than other plants. Ferns were reported to have many useful phytochemicals (secondary metabolites) such as flavonoids, steroids, alkaloids, phenols, triterpenoid compounds, varieties of amino acids and fatty acids (Zeng-fu et al. 2008).

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They also have some unique secondary metabolites which have not been discovered in higher plants (Zhao et al. 2007; Shinozaki et al. 2008).

Ferns have various types of useful phytochemicals. Polyphenols are useful phytochemicals which provide health benefits such as antioxidants. Antioxidant is generally recognized to reduce the risk factors of chronic disease. From experiments for screening of total polyphenol contents of 37 ferns and fern allies, *Polystichum lepidocaulon* and *Polystichum polyblepharum* were reported to have more than 13% of total polyphenols from dried materials of both fronds and rhizomes (Shin and Lee 2010; Shin 2010). In addition, fronds of *Davallia mariesii* and rhizomes of *Cyrtomium fortune*, *Dicranopteris pedata*, *Athyrium niponicum* and *Dryopteris nipponensis* showed more than 10% of total polyphenols from dried materials.

Due to an increased concern about human health, longevity and eco-friendly life style, the health supplement markets are expanding rapidly. Synthetic compounds were popular due to their cheap price and quick efficacy in the past. However many studies reported their side effects, such as carcinogenesis (Branen 1975). The preference of natural substances has increased rapidly worldwide (Nakatani 1992). In these circumstances, human interests for ferns and fern allies will be not only ornamental but also medicinal plants.

Ferns have lived with human beings for a long time. They influenced millions of human lives as traditional medicinal cures or treatments for ascarid disease, cold, diarrhea, burn, trauma bleeding, and more in many countries (May 1978; Wu 1990; Benjamin and Manickam 2007). Recently, high bioactivities of traditional medicinal ferns were analyzed (Table 24.1). They were reported to have various bioactivities, such as a antioxidant (Carcia et al. 2006; Chen et al. 2007; Ding et al. 2008; Shin and Lee 2010), antimicrobial (Maruzzella 1961; Banerjee and Sen 1980, Parihar and Bohra 2002; Singh et al. 2008a, b), antiviral (McCutcheon et al. 1995), anti-inflammatory (Liu et al. 1998; Punzon et al. 2003), antitumor (Konoshima et al. 1996) and anti-HIV (Mizushina et al. 1998) and so on. Out of all these, the most useful bioactivity for human life is antioxidant activity.

24.2 Natural Antioxidant

The most important function of antioxidants is reducing reactive oxygen species (ROS). ROS are a byproduct of respiration. They can be beneficial to the human body by removing pathogens and old proteins. However, an overproduction of ROS can cause various factors such as environmental pollutions, stresses, synthetic substances, etc. They could eventually be responsible for chronic diseases including aging, cancer, cardiovascular diseases, rheumatoid arthritis, atherosclerosis, etc. (Finkel and Holbrook 2000; Yildirim et al. 2000; Gulcin et al. 2002; Mau et al. 2002).

Plants have antioxidant systems for self-protection against biotic and abiotic stress conditions (Hossain et al. 2007). These antioxidant activities of plants have useful effects on human bodies. Ferns and fern allies exposed to several stresses are thought to be effective antioxidant agents for protecting against aging and chronic disease. Recently, efficient antioxidant properties of ferns were reported.

Table 24.1 Representative ferns expressed useful functional activities

Bioactivities	Ferns	Reference
Antioxidant	*Braomea insignis*	Ding et al. (2008)
	Nothoperanema hendersonii	Ding et al. (2008)
	Polypodium leucotomos	Carcia et al. (2006), Gombau et al. (2006)
	Polystichum semifertile	Ding et al. (2008)
	Pteris ensiformis	Chen et al. (2007)
Antimicrobial	*Adiantum capillus-veneris*	Guha et al. (2004)
	A. caudatum, A. peruvianum *A. venustum*	Singh et al. (2008a)
	A. incisum	Lakchmi and Pullaiah (2006)
	A. latifolium	Lakshmi et al. (2006)
	Dryopteris crassirizoma	Lee et al. (2009)
	Pteris biaurita	Dalli et al. (2007)
	P. multifida	Hum et al. (2008)
	P. vittata	Singh et al. (2008b)
Antiviral	*P. glycyrrhiza*	McCutcheon et al. (1993, 1995)
	P. vulgare, P. aureum	Husson et al. (1986)
Anti-inflammatory	*Blechnum occidentale*	Nonato et al. (2009)
	Dryopteris sp.	Otsuka et al. (1972)
	Phlebodium decumanum	Punzon et al. (2003)
	Polypodium sp.	Liu et al. (1998)
	P. ensiformis	Wu et al. (2005)
Antitumor	Triterpenoids hydrocarbons isolated from *Polypodium nipponica, P. formosanum, P. vulgare, P. fauriei, P. virginianum, Dryopteris crassirizoma, Adiantum monochlamys,* and *Oleandra wallichii*	Konoshima et al. (1996)
Anti-HIV	*Athyrium niponicum*	Mizushina et al. (1998)

Antioxidant activities (DPPH radical and ABTS radical cation scavenging) of frond and rhizome extracts of several genus such as *Davallia*, *Hypolepis*, *Pteridium*, *Cytominum*, *Dryopteris*, *Polystichum*, *Dicranopteris*, *Lycopodium*, *Osmunda*, *Adiatum*, *Coniogramme*, *Polypodium*, *Pyrrosia*, *Pteris*, *Lygodium*, *Selaginella*, *Thelypteris*, *Athyrium*, *Matteuccia*, *Onoclea* and *Woodsia* were analyzed (Shin 2010). As a result, several ferns showed vigorous antioxidant activities on scavenging of DPPH and ABTS radicals. Specially, Dryopteridaceae, Osmundaceae, Woodsiaceae exhibit powerful antioxidant activities. And some crude extracts obtained from ferns showed powerful antioxidant activities more than vitamin C or BHT (synthetic antioxidant) (Table 24.2). So, we think that most ferns have huge potential abilities as antioxidants. Thus, we expect that analyzing antioxidant activities in many ferns will result in the development of health-care products for aging and chronic disease by their high bioactivities.

Table 24.2 Fern's methanol extracts showed higher antioxidant activities than ascorbic acid or BHT (Shin 2010)

Family	Scientific name	Parts	DPPH radical scavenging RC_{50} (mg/mL)[a]	ABTS radical scavenging
	Ascorbic acid[b]		0.03	0.20
	BHT[c]		0.12	0.22
Davalliaceae	*Davallia mariesii*	Frond	0.05	0.06
		Rhizome	0.08	0.07
Dryopteridaceae	*Cyrtomium fortunei*	Rhizome	0.03	0.11
	Dryopteris crassirhizoma	Rhizome	0.11	0.11
	D. nipponensis	Frond	0.11	0.14
		Rhizome	0.05	0.06
	Polystichum lepidocaulon	Frond	0.05	0.09
		Rhizome	0.04	0.04
	P. polyblepharum	Frond	0.08	0.10
		Rhizome	0.02	0.03
Gleicheniaceae	*Dicranopteris pedata*	Rhizome	0.03	0.03
Osmundaceae	*Osmunda cinnamomea var. fokiensis*	Rhizome	0.06	0.08
	O. japonica	Rhizome	0.08	0.10
Parkeriaceae	*Adiantum pedatum*	Rhizome	0.06	0.07
Polypodicaceae	*Pyrrosia lingua*	Frond	0.11	0.08
Schizaeaceae	*Lygodium japonicum*	Rhizome	0.07	0.09
Thelypteridaceae	*Thelypteris acuminata*	Rhizome	0.06	0.06
Woodsiaceae	*A. niponicum*	Rhizome	0.04	0.07
	Matteuccia struthiopteris	Rhizome	0.11	0.14
	Onoclea sensibilis var. *interrupta*	Rhizome	0.11	0.15

[a]Concentration of the soluble solids which is required to DPPH and ABTS radical scavenging 50%
[b]Positive control as a natural antioxidant
[c]Positive control as a synthetic antioxidant

24.3 Foods

In Europe and America, a few ferns, such as ostrich fern, were used for food. However, in Oceania and Asia, there are so many ferns used as foods as main or side dishes and traded in market place (Table 24.3). Especially in Korea, the dried and steamed fiddle head of brackens are important as ancestral service food for Thanksgiving Day, New Year's Day, and home ceremony for ancestors every year.

It is possible to preserve the fiddle heads of ferns as food materials. The preservation methods for fiddleheads are extremely simple. Clean fiddleheads should be steam boiled in hot water with or without ash. Boiled ferns would be dried under sunny conditions or preserved in a salt layer. Then they could be preserved for 2–3 years. When they are needed, dried fiddleheads are boiled and washed with running water again. After draining the water, it would be cooked with mashed garlic, salt, sesame oil, and soybean sources. Salted fiddleheads should be washed again with running water and cooked like dried matter. In USA, boiled fiddleheads are commonly prepared with butter, cider or wine vinegar and a bit of pepper. In Northern New England, some people make pickles with fiddleheads. The cooked fiddleheads have a soft and rich taste; sometimes they taste a bit like asparagus but are much tougher.

Bracken fiddleheads are considered as a nutritionally rich food in Korea. They contain significant amounts of protein, fiber, vitamins, and minerals. However in many countries, brackens are known as poisoning plants because of their carcinogenic and antithiamin properties. The carcinogenic substance of bracken is ptaquiloside

Table 24.3 Edible ferns often used worldwide (Copeland 1942; Thakur et al. 1998; Shin 2010; Wei 2010)

Scientific name	English name	Countries	Edible parts
Athyrium acutipinnulum		Korea	Fiddlehead
A. brevifrons		Korea	Fiddlehead
A. distentifolium	Alpine Lady-Fern	Korea	Fiddlehead
A. esculentum	Vegetable fern, Pako fern	Asia, Oceania	Fiddlehead
Diplazium squamigerum		Japan	Fiddlehead
Matteuccia struthiopteris	Ostric fern	Canada, China, Europe, Malaysia, India, Japan, USA	Fiddlehead
Osmunda cinnamomea	Cinnamon fern	East Asis	Fiddlehead
O. japonica	Flowering fern	East Asia	Fiddlehead
O. regalis	Royal fern	Worldwide	Fiddlehead
Pteridium aquilinum var. *latiusculum*	Bracken	Worldwide	Fiddlehead
Stenochlaena palustris	High climbing fern	South Pacific, India.	Fiddlehead
Ceratopteris	Swamp fern, water lettuce, water sprite	Asia, Australia	Leaves
Cyathea	Tree fern	Oceania	Young leaves, terminal bud

(Hirono et al. 1984). Ptaquiloside is very carcinogenic in mammals, especially ruminants, which repetitively ingest huge amounts of bracken. However, bracken consumption does not lead to carcinogenesis in humans because people eat bracken in smaller quantities than animals, and do not eat the bracken repetitively. Several other foods also contain carcinogenic substances. Estragole is contained in basil, fennel and tarragon (Miller et al. 1983; Bender and Eisenbarth 2007), safrole in cinnamon, camphor, nutmeg, ginger, cocoa and pepper (Ioannides et al. 1981; Bender and Eisenbarth 2007), pyrrolizidine alkaloids in coltsfoot, comfrey and Indian plantain (Bender and Eisenbarth 2007), and agaritine in meadow mushroom (Bender and Eisenbarth 2007). Also, several vegetables, such as beets, celery, radishes, lettuce, spinach, etc., have nitrate (Wolff and Wasserman 1972). Nitrate is not a carcinogenic substance, but nitrosamine derived from nitrate is a strong carcinogenic in the human body (Du et al. 2007). Nevertheless, these products are regularly used as delicious and healthy foods. When people eat 350 g fiddlehead of bracken every day, it can cause cancer (Ham 2004). However, people could not get cancer by consuming bracken because nobody can eat more than 350 g of fiddlehead every day.

The antithiamin activity of bracken is extinguished during washing in running water after boiling with or without ashes or sodium hydrogen carbonate. Furthermore, the antithiamin substances in brackens, such as astragalin, isoquercitrin, rutin, caffeic acid, tannic acid, etc., are known as useful natural substances for anticancer or antioxidant in the present time (Kweon 1986; Cai et al. 2004; Katsube et al. 2006). So, the fiddlehead of bracken can be used as a tasty side dish helpful to human health.

While toxicities caused by carcinogenesis and antithiamin activities of bracken fern have been highlighted, the pharmacological effects of the fiddleheads or whole plants of ferns and fern allies are underestimated. However, several healthy effects of ferns and fern allies are currently well known. For example, the glycoprotein isolated from bracken fiddlehead has immune function (Park et al. 1998), and the acidic polysaccharides isolated from the hot water extract of dried bracken fiddlehead has anti-complementary activity (Oh et al. 1994). Also, the fiddlehead of *Athyrium acutipinulum* has strong antioxidant effects (Lee et al. 2005). So, we expect that more people will enjoy the taste and useful function of young fiddlehead of ferns in the future.

24.4 Natural Antimicrobial Agents

As global temperatures are rising steadily, the duration of the summer season is also increasing in many countries currently. Therefore, the optimal conditions for living of pathogenic bacteria is also increasing. Bacteria including *Listeria*, *Escherichia coli*, *Salmonella*, *Vibrio*, *Staphyococcus*, etc. are vigorously propagated in 37–40°C (Madigan et al. 1997). So, various side effects, such as food poisoning, infection, spoiled food, and more, are expected with the increase of harmful microbial activities. According to WHO, increasing resistance to antibiotics is a growing problem in

many countries even in developed countries. Thus, antimicrobial substances are being developed very rapidly. Especially, interest in natural antioxidant agents is rapidly increasing due to the side effects of synthetic substances.

The extracts obtained from ferns and fern allies have effective antimicrobial activities against gram positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*), gram negative bacteria (*E. coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*), as well as fungi (Banerjee and Sen 1980; Vincent and Kanna 2007; Lee et al. 2009). Especially, the genus *Dryopteris* showed vigorous antibiotic activities. *D. crassirhizoma* and *D. filix-mas* can be used against MRSA (Methicillin resistant *S. aureus*) (Lee et al. 2009), and *D. cochleata* against gram positive and negative bacteria and fungi (Banerjee and Sen 1980). Also *D. crassirhizoma* is patented as an anti-tooth decay substance (Park et al. 1995) because of its high activity against *Streptococcus* in Korea.

However, frond of *A. niponicum* and *Hypolepis punctata* are more efficient than *D. crassirhizoma* against *Streptococcus* such as *S. mutans* and *S. sobrinus* (Shin 2010). Several ferns and fern allies were also reported to have excellent antimicrobial activities in *S. mutans* and *S. sobrinus* similar to *D. crassirhizoma* (Table 24.4). Therefore, ferns and fern allies can be used as efficient natural antimicrobial ingredients against several harmful microbials. They could be developed into antibiotic sprays, packing material, toothpaste, hand wash, etc. for protecting the human body and our living environment from undesirable microbials. Most Pteridophytes are known to contain antimicrobial substances such as polyphenols and flavonoids (Francisco and Cooper-Driver 1984). It is possible that several non-analyzed ferns and fern allies could contain effective antibiotic substances. So, the more antibiotic activities of the Pteridophytes are analyzed, the more natural antibiotics could be developed.

Table 24.4 Ferns expressed higher anti-tooth decay activities in the same concentration of methanol extract than rhizome of *Dryopteris crassirhizoma* (Shin 2010)

		Clear zone (mm)	
Scientific name	Parts	*Streptococcus mutans*	*S. sobrinus*
Dryopteris crassirhizoma	Rhizome	16 ± 1.5	14 ± 0.7
A. niponicum	Rhizome	16 ± 1.0	15 ± 0.3
Dicranopteris pedata	Rhizome	15 ± 0.3	15 ± 0.3
Dryopteris fragrans var. *remotiuscula*	Frond	17 ± 0.6	14 ± 0.6
Lygodium japonicum	Rhizome	16 ± 0.3	15 ± 0.3
Osmunda japonica	Rhizome	17 ± 0.3	16 ± 0.7
Onoclea sensibilis var. *interrupta*	Rhizome	15 ± 0.9	16 ± 0.6
Polystichum lepidocaulon	Frond	18 ± 0.6	16 ± 0.7
Polystichum lepidocaulon	Rhizome	18 ± 0.0	18 ± 0.0
Pteris cretica	Rhizome	16 ± 1.2	15 ± 0.3
Thelypteris acuminata	Rhizome	16 ± 0.9	15 ± 0.6

24.5 Cosmetic Ingredient

Ferns can be used in cosmetic materials. For example, some *Dryopteris* spp. exhibit strong antimicrobial activities against *Propionibacterium acnes*, known as a main factor of acne (pimple) (Kim et al. 2006). Therefore, the extracts obtained from some ferns could be utilized as effective natural cosmetic ingredients for the treatment or prevention of acne. Furthermore, many ferns and fern allies normally contain more phenolic compounds than other plants. The phenolic compounds of plants are known to have beneficial skin care effects such as the prevention of UV-induced skin damage (Svobodová et al. 2003; Tanaka et al. 2004), anti-wrinkle (An et al. 2005), skin-whitening (Parvez et al. 2006), etc. Many plant extracts, which contain high phenolic compounds, are currently used as ingredients of natural body and facial cosmetics such as cleanser, toner, moisturizer, shampoo, conditioner, and so on.

Several ferns contain phytoecdysteroids which do not exist in most plants. The phytoecdysteroids, such as ecdysone, show the effects of cell regeneration, skin texture refinement and skin barrier strengthening (Lin and Lin 1989; Meybeck et al. 1997). So, it is used as a cosmetic ingredient. Since fern spores are not villains in hay fever (Moran 2004), the cosmetics including fern spores are patented in Korea. Such a cosmetic is a facial scrub product including spores of bracken (Jin et al. 2005). Due to the very small particle size of bracken spores, the scrub including spores does not cause the skin abrasion. Therefore scrubbing the face or body with spores could promote blood circulation and remove dead skin cells smoothly. Other patented uses include face mask, powder foundation and compact powder containing the ground or skimmed spores of *Lygodium japonicum* (Son et al. 1999). The face mask containing spores of *L. japonicum* could increase the effects of skin refinement and cleansing. The powder foundation or compact powder containing spores of *L. japonicum* could also increase color expression and control discoloration due to the absorption of sweat and sebum. So, various parts of ferns could be effectively used as natural cosmetic ingredients for skin healing, skin smoothening, anti-acne and protection from aging or UV damage. However, as there are fewer natural cosmetics with ferns as main ingredients than with flowering plants, more research of ferns for application to cosmetic material is required.

24.6 Air Purifier

Ferns are useful not only as ornamental plants but also as air cleaning plants. As ornamental plants, ferns have great value including their beautiful leaves and rhizomes. According to research by NASA and KRDA (Korea Rural Development Administration), many fern species also show strong air purification activities of volatile formaldehyde removal. For example, *Nephrolepis exaltata* (Boston fern) and *Nephrolepis obliterata* (Kimberly queen fern) are included in NASA's 50 air purifying plants for homes and offices, with their ranks as 9th and 13th, respectively. According to

Table 24.5 Ferns showed higher formaldehyde removal effect than *Dypsis lutescens* (KRDA 2006)

Scientific name	Amount of formaldehyde removal ($\mu g/m^3/cm^2$ plant volume)
Dypsis lutescens	0.81
Cyrtomium caryotideum var. *coreanum* Nakai	1.09
Davallia mariesii Moore ex Bak.	3.56
Microlepia strigosa (Thunb.) C. Presl	1.03
Osmunda japonica Thunb.	6.37
Polypodium formosanum Bak.	3.03
Pteris multifida Poir.	1.76
P. dispar Kunze	1.44
Sceptridium ternatum (Thunb.) Sw.	1.00
Selaginella tamariscina (P. Beauv.) Spring	4.24

NASA's report, *N. exaltata* and *N. obliterata* have benefits for reducing indoor air pollution such as formaldehyde, xylene and toluene. Furthermore, *N. exaltata* is reported as the most efficient species for removing formaldehyde.

Formaldehyde is the most common indoor volatile organic compound (VOC) with substantially high concentrations. Formaldehyde has several side effects to human beings such as nausea, sore throat, watery eyes, eye burning sensations, headaches, fatigue, and so on (Olsen and Dossing 1982; US CPSC 1997). Therefore, the formaldehyde-absorbing ability is one of the most effective functions of ornamental plants. Also according to KRDA, several ferns and fern allies show high efficiency of formaldehyde removal indoors (Table 24.5). They tested the formaldehyde removal effects of 84 species of plants. *Osmunda japonica* (similar to royal fern) showed the best formaldehyde removal in chamber. In addition, *Selaginella tamariscina*, *Davallia mariesii*, *Polypodium formosanum* and *Pteris multifida* have been ranked as highly efficient formaldehyde-removing plants. Other ferns, such as *Pteris dispar*, *Cyrtomium caryotideum* var. *koreanum*, and *Sceptrium ternatum*, showed better formaldehyde removal than areca palm tree (the best air purifying plant ranked by NASA). So, ferns could purify the air just by keeping them indoors or outdoors. Also we expect that many ferns in addition to the above-mentioned may have efficient formaldehyde removal activities. They also could remove many other volatile organic compounds (VOCs). So, more studies about the air purifying ability of ferns are necessary. Since ferns grow well in shady places, they can be adapted to indoor, even bedroom, restroom and bathroom use. Moreover, ferns are high quality ornamental plants.

24.7 The Future of Ferns and Fern Allies

Many people have been fascinated with ferns and fern allies for their beauty and medicinal properties for a long time. But in the present days, their value has been underestimated than in the past since people have discovered many other useful plants. Even though their useful functions for human have not yet been analyzed

thoroughly, ferns and fern allies have many efficient functional activities for human life. The leaves (fronds), spores, fiddle heads, rhizomes, and roots of ferns and fern allies have infinite potential for improving the quality of human life because of their biological activities. They can be applicable to commercial products such as food, medicine, cosmetics, ornamental materials, household products, and more. Negative effects of ferns, such as carcinogenesis, are more magnified than positive effects. In spite of their toxicity, many ferns and fern allies are more beneficial.

Ferns and fern allies also have other advantages for their propagation and culture. Due to adaptation to various environmental conditions for a long time, they are easier to propagate and cultivate than other plants in general (Lee 2004). They can be propagated rapidly using tissue culture techniques. For example, gametophytes are mass-propagated in vitro, and then the juvenile sporophytes are induced from in vitro cultured gametophytes in pots. Also, many ferns and fern allies grow rapidly and produce huge biomass in short periods. So, they can be used as cost-effective ingredients applied to the manufacturing of various functional goods and healthy products for human beings.

The use of ferns for personal health care and environmental esthetics is ecologically sound. Ferns are integral plants which provide food and medicine for all inhabitants. They are in every forest, jungle, rock, mountain and garden. They have a great biodiversity and are representatives of plant evolution level. Now they include wild and cultivated plants. We hope that many people join in studying the various bioactivities of ferns and fern allies for human life. As a result, more people could enjoy a healthy and eco-friendly life with ferns and fern allies.

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Chapter 25
Toxicological and Medicinal Aspects of the Most Frequent Fern Species, *Pteridium aquilinum* (L.) Kuhn

János Vetter

25.1 Introduction

Pteridium aquilinum (bracken fern) is the most widely and frequently distributed pteridophyte plant and belongs to the most common vascular weed species: it is being found on all continents (except Antarctica). This plant species was originally a member of the open forest plant communities, but its distribution has increased as a result of human activity. The bracken fern has been used by many cultures but the use has varied with time. The strong rhizomes of plant have been used directly as a food or as a source (component) of bread (by Australian, British, French, Japanese aboriginals or by Lapp and Siberian cultures; Veitch 1990). The fern was a favourite, edible plant for native Americans, who cooked, dried and ground the rhizomes to a meal, which was used in different ways. The rhizomes have high content of starch (about 45%) therefore they were used for preparation of glues and for brewing of beer. In Japan, the young shoots are considered a delicacy and used like asparagus. The bracken roots are dried and ground into a powder on the Canary Island, which is mixed with barley to produce goflo, a supplement in the diet of poorer farmers.

P. aquilinum has a place in the literature too: in Elizabethan England, it was believed the spores of the plant gave invisibility to the possessor ("We have the receipt of fern seed – we walk invisible" Shakespeare, W. in Henry IV).

The plant has been used routinely for animal bedding (and human bedding) since Roman and Viking times. There were and are many examples of the implementation of bracken fern as a fertilizer, recently it has been tested as mulch green manure and as a potash source for potato or as compost in horticulture. Our fern species has also been employed industrially: for soap and glass making, to fire brick and lime in kilns.

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25.1.1 Chemical Aspects of the Plant

The basis of bracken's toxicology is given by the occurrence of different molecules (mainly secondary plant metabolites) therefore we discuss firstly the chemical composition, chemical constituents of *P. aquilinum* (L.) Kuhn.

Considering the conventional, usual components of bracken: the crude protein content is (on dry weight base) 14–16%, the concentration of crude fibre is about 30–34%, the crude fat: 1.4%, ash 5.2% and the N-free extract: 44–45% (Williams and Evans 1959). The nutritional value of bracken ("bracken hay") is influenced not only by these conventional nutrients but by their digestibility. In one classical publication (William and Evans 1959) were compared the coefficients of digestibility for main nutrients without and after steaming. Steaming was a successful method for inactivation of some toxic factor, but the digestibility of proteins was remarkably decreased (54.3% and 28%, without and with steaming, respectively). The normally dried (un-steamed) bracken hay had a nutritional value similar to meadow hay.

The mineral element spectrum of bracken has been investigated in previous years (Cornara et al. 2007; Samecka-Cymerman et al. 2009). The average means of macro-element contents of plants from serpentine soils (in mg/kg) are 16,470 (N), 10,206 (K), 1,056 (P), 2,233 (S), 5,843 (Ca) and 5,767 (Mg). Regarding some – mainly the poisonous – microelements: iron manganese and zinc contents are high or moderately high (98, 123 and 25 mg/kg, respectively), the found levels of Cu, Cr and Cd are low (3.4, 0.66 and 0.14 mg/kg). There are no data about the accumulation of any poisonous element in bracken fern.

The number of isolated secondary metabolites is more than 100 (Fenwick 1988), i.e., the chemical composition of the bracken fern is very complicated, which may explain not only its extreme vitality and productivity but its distribution in a wide range of ecosystems.

25.2 Toxicological Aspects

The poisoning character of *P. aquilinum* has been known since the last years of the nineteenth century, for cattle and horses (Williams and Evans 1959), and the first experimentally produced poisonings were described in cattle, horses and in sheep in these years.

25.2.1 Compounds of Anti-thiamine Character

Few plants (among others bracken fern) show thiaminase activity, which can cause the decomposition – therefore the inactivation – of thiamine (vitamin B_1). The occurrence of thiaminase in *P. aquilinum* has been known since the 1940s, this information was based on characteristic animal symptoms (first of all poisoning of horses) induced by the fern. The thiaminase of bracken fern belongs to the enzyme type I (Meyer 1989), which is the most common type, found in few animals, plants and in some bacteria.

Feeding a diet of 15–30% rhizome mixed with green grass to sheep produced thiamine deficiency within a month (Meyer 1989). The thiaminase activity (content) is the highest in rhizomes and having a maximum in late summer – early autumn. Thiaminases require a co-substrate (usually an amine or sulphydryl containing compound such as proline or cysteine). The enzymes can cleave the thiamine into the inactive pyrimidine and thiazole molecules, the ingestion of significant content of thiaminase will induce thiamine deficiency. The thiaminase from bracken fern has a molecular mass about 93 kDa, the pH optima 8–9, has a stability between 3 and 12 pH, and the essential temperature for 50% denaturation is 63°C (Chick et al. 1989).

Other molecules of thiaminase character were registered and described in the mid of 1980s (Konishi and Ichijo 1984) but these molecules are not enzymes and are thermostable. Coming from this fact, thiamine contents in blood and urine of horses decreased, oppositely blood pyruvate and lactate concentrations increased and the animals had typical symptoms of bracken fern poisoning. The responsible thermostable compounds were identified as caffeic acid, astragalin and isoquercetin. The caffeic acid inhibits thiamine transport in rat intestines. Ponies fed a constant diet containing 0.9 mg thiamine/kg, in addition 300 mg caffeic acid given orally for 28 days produced significant increases in serum pyruvate and lactate (Bertone et al. 1984) compared to control animals.

25.2.2 Cyanogen Glycosides

The cyanogen glycosides are secondary plant products and are distributed differently among plant families and genera including some fern groups. The phenomenon of cyanogenesis is part of the anti-herbivore defence mechanism of plants. The main cyanogen glycoside of bracken fern is the prunasin (Fig. 25.1), its concentration varies between 10.4 and 61.3 mg/g fresh tissue (Alonso-Amelot and Oliveros 2000). The young fronds have higher prunasin concentrations than their older counterparts. The content of cyanogen glycosides and therefore the content of releasing HCN are not too high, thus HCN amount is by itself does not deter animals from feeding on bracken. Most of the glycosides probably remain in the gut of insects and must continue to release the HCN molecules (Alonso-Amelot et al. 2001).

No death of higher animals caused by cyanogen glycosides of bracken has been described. Higher contents of prunasin are found in shaded woodland rather than in open habitat. The cyanogenesis of bracken seems be correlated with climatologic factors such as temperature and rainfall (Hadfield and Dyer 1986).

25.2.3 Compounds of Illudane Skeleton

25.2.3.1 Ptaquiloside

First information about the carcinogen character of the boiling water bracken extract was published in 1978, isolations, independent of each other were carried

out, first in Japan by Yamada group and the second by van der Hoeven and co-workers in the Netherlands (Yamada et al. 2007). The new molecules found were named ptaquiloside (Yamada group) and aquilide A (van der Hoeven and co-workers), the isolated molecules were the same, the today used name is ptaquiloside (PT). PT is a colourless, amorphous compound (molecular formula $C_{20}H_{30}O_8$; the molecular weight is: 398.45), readily soluble in water. The molecule is stable at room temperature for more than a week and at low temperature (between 0°C and −20°C) for more than 6 months. PT belongs to the norsesquiterpene glycosides of the illudane type. The exact chemical name of this molecule is: Spiro [cyclopropane-1,5′-[5*H*] inden]-3′(2′h)-one,7′α-(β-D-glycopsranosloxy)-1′,3′α,4′,7′α-tetrahydro-4′ hydroxy-2′,4′,6′-trimethyl-, (2′*R*,3′α*R*,4′*S*,7′α*R*) (Fig. 25.1).

These terpenoids are relatively rare: such molecules (as illudine-M and S) are components of some poisonous mushrooms (*Omphalotus illudens*, *O. olearius*). PT is unstable in aqueous solution, it decomposes under acidic as well as alkaline conditions. Under alkaline conditions the products of hydrolysis are ptaquilosin (aglycon of PT) then a dienone is formed (after liberation of glucose, Fig. 25.1). Under acidic conditions aromatization process occurs and pterosin B is produced (Fig. 25.1). The PT molecules in aqueous solution are converted into glucose and pterosin B.

Other bioactive molecules identified from bracken fern are the *iso*-ptaquiloside, caudatoside, ptaquiloside Z, they are named ptaquiloside-like compounds (Castillo et al. 1997, 1998). These are also found in other species of the fern family Dennsteadtiaceae.

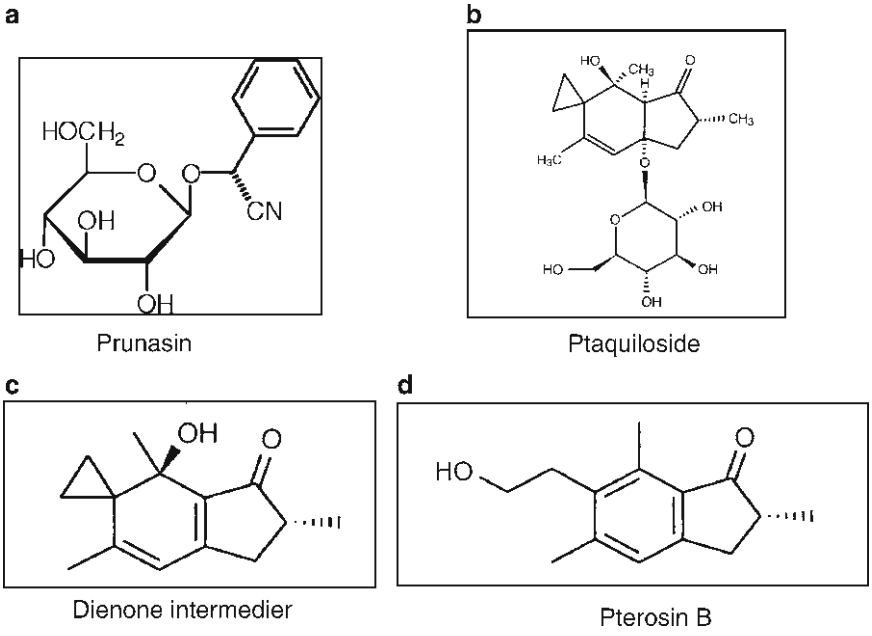

Fig. 25.1 Chemical structures of bracken components: (**a**) Prunasin, (**b**) Ptaquiloside, (**c**) Dienone intermedier and (**d**) Pterosin B

Bracken fern samples from Australia contained PT between 0 and 12,945 μg/g (Smith et al. 1994). Fifteen percent of same the samples had greater PT content than 5,000 μg/g. Plants from the cultivated bracken-clone collection had lower (0–9,776 μg/g) concentration.

PT distribution by plant organs:

(a) Rhizomes have in general lower (<1,200 μg/g) concentration during the frond growth season and higher concentration in plants with mature fronds. Storage-rhizomes had higher contents than the rhizomes bearing fronds.
(b) PT contents of roots are very low (5–230 μg/g).
(c) Fronds: according to new investigations (Rasmussen et al. 2008) the found average PT level was 3,800 μg/g (min: 280, max: 13,300 μg/g d.m.).
(d) PT content of spores is surprisingly very low, less than 20 μg/g (Rasmussen 2003).

Interesting questions are the small- and large-scale geographical variations of PT. The measured contents for fronds (from 12 stands of four geographically distinct habitats) were found between 210 and 2,150 μg/g, this variation was positively correlated with the development phases and the altitudes. Several studies have been carried out in New Zealand, Australia, Costa Rica and Venezuela. PT contents seemed to be correlated with altitude but the correlation is more complex than that, there are some contradictory results. The different ecological factors probably have direct or indirect role in the regulation of actual PT content. Edaphic stress (nutrient deficiency, water holding capacity of soil, etc.) may enhance PT level. High contents have been estimated in bracken leaves originating from stands subjected to mowing-grazing of animals (Smith et al. 1994). Precipitation (especially heavy showers) can cause decreased PT level, thus inducing wash out of molecules from the leaves.

PT molecules can occur not only in bracken fern but in other fern species (Somvanshi et al. 2006). *Onychium contiguum* has similarly high level of PT, while others have moderate or nil. Therefore a few other fern species can cause (alone or in combination with bracken fern) hazardous effects.

25.2.3.2 Pterosins

Formation of pterosins – common products of PT transformation – is present in rhizomes and fronds of bracken. The contents of pterosin B range between 10 and 100 μg/g, in rhizomes, 20–2,100 μg/g in fronds. The pterosides are pterosine-glycosides in the concentration ranges between 400 and 9,500 μg/g, 60–1,000 μg/g, and 20–80 μg/g for rhizomes, leaves and spores, respectively.

25.2.3.3 Other Chemical Components

Flavonoids. Different coloured compounds of flavonoid character (kaempferol, apigenin and quercetin) have been isolated; some of these are aglycons of certain flavonols. Their contents are in the range of 10,000–25,000 μg/g.

Phenoloids. Several derivatives of cinnamic- and benzoic acids were detected. The levels of these compounds are representative, depending on varieties of bracken. Complexes of phenoloid polymers (i.e., tannins) show high concentrations (up to 16% of d.m.). These compounds have a negative role in digestibility and they can be a deterrent against insects and other animals.

25.3 Effects on Animals

25.3.1 Non-ruminant Animals

The non-ruminant animals – first of all horses – fed bracken fern will show typically neurological dysfunctions. The bracken thiaminases (and other anti-thiamine factors) split the essential vitamin B_1, resulting decreased level in blood from 80–100 mg/L to 25–30 mg/L. Majority of clinical cases are related to ingestion of poor-quality hay. Typical signs of thiamine deficiency occur when the bracken content of forage is at least 10–20% and consumption is at least 3–4 weeks. Characteristic general symptoms of poisoning are: weight loss, lethargy, anaemia, anorexia, colic, convulsion, gait dysfunction (staggering), recumbency and loss of muscular control. In severe cases, tachycardia is present and death is preceded by convulsions, clonic spasms and opisthothonus. Rectal temperature can be normal or may reach as high as 40°C. The symptoms of vitamin B_1 deficiency can be induced by bracken extract (Konishi and Ichijo 1984) or by feeding bracken itself (Fernandes et al. 1990). The ruminant animals are practically resistant to thiamine deficiency since rumen microbes can synthesize it.

25.3.1.1 Swine

Reports of acute bracken poisoning in swine are relatively rare. The symptoms are: dyspnoea, recumbency, elevated blood pyruvate content. As consequences one can observe: enlarged, mottled heart, congestive heart failure, slowed heart rate, degeneration of cardiac muscle, oedematous lungs and enlarged gall bladder. Feeding experiments with fern rhizomes produced loss of appetite (after 8 weeks), later rapid deterioration and death. Some piglets died after ingestion of bracken by the pregnant sows (Fenwick 1988).

25.3.1.2 Rabbit

Fern fed rabbits (Gounalan et al. 1999) showed significant increases in serum enzyme (SGOT, SGPT, ALP) activities, in urea and creatinine levels. The animals showed anaemia, leucopoenia, heterophilia and vascular changes in visceral organs,

as well vacuolar degenerative changes in hepatocytes, hypersecretory activity in intestine, degenerative changes in renal tubular cells.

25.3.1.3 Guinea Pig

Biochemical factors of bracken (30%) fed animals was evaluated (Kumar et al. 1999, 2000). Decreases in total protein and albumin contents were observed in serum, but creatinine and LDH showed increases. Haematological studies established leucopoenia, eosinophilia and decrease in packed cell volume. Possible induction of carcinomas was confirmed in different experiments (Krishna and Dawara 1994; Bringuier et al. 1995).

25.3.1.4 Rat and Mice

Rats as model animals were used in different experiments to establish biological-toxicological effects of bracken (or PT). The fern fed rats showed progressive anaemia, leucopoenia, lymphopenia, neutrophilia, brain oedema, vascular changes and hypersecretory activity of intestine. Activities of some serum enzymes as well as the concentration of urea and creatinine were increased (Gounalan et al. 1999).

Carcinogenic effect of bracken spores has also been recognized. The fern spores can cause leukaemia, gastric, mammary and lung tumours in mice (Siman et al. 2000). The spores (or spore-extract) can induce DNA damages in human cell cultures. PT content of spores seems to be low or the lowest among the bracken plant organs, consequently the spores may contain other, unidentified carcinogenic agents.

25.3.2 Ruminant Animals

25.3.2.1 Cattle

The Bovine Enzootic Haemature

This is a chronic disease of cattle. It can be characterised by different bleedings (haemorrhages) and later tumours in the urinary bladder and by blood in the urine. The BEH syndrome occurs in distinct parts of the world: mainly in South America (Brazil, Bolivia, Venezuela, Argentina, etc.), in Asia (India, China, Japan); in Europe (Wales, French); in Australia and New Zealand. Epidemiological studies have stated unambiguous correlation between occurrence of BEH and PT content of bracken. Clinical symptoms of BEH are: degradation of condition, progressive emaciation, dyspnoea and noisy breathing, relative disphagia, salivation, halitosis and haemature. The histopathological observations showed epithelian and mesenchymal tumours in the urinary bladder. Haematological status of the affected animals can be

characterised by low haemoglobin content (6.88 g/dL), packed cell volume, total erythrocyte count of $4.43 \pm 0.4 \times 10^6/\mu L$. The animals had hypoglycaemia, hypocalcaemia and hyphophosphataemia. Blood nitrogen and creatinine levels were elevated (Wadhwa et al. 2001). The lesions of the affected urinary bladders have three types (capillary haemangioma, haemangiosarcoma and squamos cell carcinoma; Souto et al. 2006). Outside of the lesions of urinary bladder, metastasis of adenocarcinomas occurred in lung, liver and heart of some animals. In some cases, carcinoma of kidney, proliferative glomerulonecrosis and hyaline deposition in blood vessels were observed (Krishna et al. 1991). Characteristic changes were identified after 30 days at bracken-fed animals: increases of urea, uric acid, creatinine and acidic phosphatase activity. The urine samples contained epithelial cells, amorphous phosphate and calcium oxalate crystals, after 45 days (Singh et al. 1987).

Haemorrhagic Syndromes (Acute Haemorrhagic Disease)

This problem occurs mainly in weaned calves but is also known for older cattle (and rare for sheep). The megakaryocytes producing the platelets are depleted and trombocytopeania occurs, which causes the acute haemorrhagic crisis (Smith 2004). Ingestion of significant dose of bracken results in bone marrow damage, causing decrease in its normal function. Number of platelets is extremely low (as low as 40,000/mm^3) compared to the normal level (500,000/mm^3), count of white blood cells is low. Haemorrhagic symptoms: bleeding is observable from the nose, vagina and membranes around the mouth and eyes. Oedema of the throat region can be developed in young animals. The affected cattle are weak, pyrexic (41–43°C), have pale mucosae and bleedings on skin (petechiae). The blood of animals fails to clot normally, often clots of blood can be in the faeces.

This acute haemorrhagic syndrome can cause death; the post-mortem observations demonstrate bleedings in lung, heart, stomach and intestines (Fenwick 1988). There is frank blood and ulcers in the gastrointestinal tract. The bone marrow shows hypoplasia, it is yellow and fatty.

Upper Alimentary Carcinoma

Different forms of carcinomas in oral cavity, oesophagus and fore-stomach have been reported from different parts of world mainly for cattle (rare in sheep). In most of these cases malignant transformation occurs, caused by the virus (BVP 4) papillomata. The bracken fern is a source of different immunosuppressants and mutagens. The bracken-fed cattle become immunosuppressed and cannot give adequate immune response against the virus. *P. aquilinum* has an important content of quercetin (see earlier), which molecules can cause breaks and rearrangement in DNA and chromosomal damages (Beniston et al. 2001). Exact details of these processes are only partly understood, but the involvement of bracken quercetin (and/or other chemical components) seems to be very likely.

25.3.2.2 Sheep

This species is in general less susceptible to different poisonous factors than cattle. Despite this, mass poisoning was induced in Merino sheep by young bracken fronds in Australia (Sundermann 1987). One hundred and twenty animals had died out of 450. There was no sign of bone marrow self-repair in the poisoned animals 10 weeks after they had been removed from the bracken-infested paddock. Three months after the outbreak some sheep were still anaemic and lymphopenic.

A progressive degeneration of the retina (bright blindness) was developed in some areas of the UK. This fact was first described in the middle of the 1960s (Watson et al. 1965). Progressive retinal atrophy occurs in bracken-fed animals older than 18 months. The symptoms are: blindness, high-stepping gait and dilated pupils with poor light reflex. Animals develop a degeneration of neuroepithelium of the retina, low blood platelets and white blood cells counts.

25.4 Effects in Humans

Bracken fern can influence the human health in direct or indirect ways. Epidemiological studies in man have shown close correlation between bracken consumption and cancer of (upper) alimentary tract in Japan, Brazil, Venezuela, Costa Rica, Wales, etc. (Alonso-Amelot and Avendano 2002). The occurrence of oesophageal and gastric cancer is fivefold and eightfold higher, respectively in people in Brazil with a daily, recurring bracken intake than in the people not eating this plant (Marliere et al. 1998). Pamukcu et al. (1978) first reported about the possibility of excretion of PT in the milk of bracken-fed animals. Publication of Alonso-Amelot et al. (1996) confirmed this finding, PT was detected in milk 38 h after starting the feeding experiment and the quantity of secreted PT was about 8.6% of PT intake. Daily consumption of 0.5 L milk from an animal consuming about 5,000 mg PT daily would result in a human daily intake of about 1.75 ± 1.45 mg PT.

25.5 Working Mechanism of Ptaquiloside

The possible mechanism of carcinogenesis is in close connection with the chemical-biochemical properties of the molecule. Under alkaline conditions, PT can produce ptaquilosin (Fig. 25.2); soon this ptaquilosin can generate an unstable dienon intermediate (Fig. 25.1). This compound has an extreme reactivity and can yield a series of stable aromatic molecules (as pterosin B) (see earlier Fig. 25.1), or can form covalent adducts with DNA (Fig. 25.2) and can induce DNA strands to break. These reactions were first proposed by Ojika (Ojika et al. 1987) as the basics of carcinogen effects. Nowadays it is believed: PT molecules can alkylate N3 of adenine mononucleotide, modified structures of codons lead to different genetic changes (point mutations), which

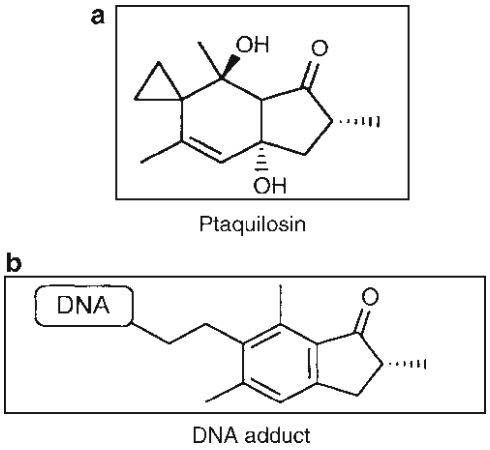

Fig. 25.2 Chemical structures of bracken components: (**a**) Ptaquilosin and (**b**) DNA adduct

can create protein (enzyme) molecules of harmful effects. The explained molecular changes caused by PT or its derivatives can be the first steps of carcinogenesis. Studies regarding to carcinogenicity of cattle have been shown that DNA adducts were formed due PT in the ileum and urinary bladder. These DNA adducts can result mutations via adenine alkylation in codon 61, causing adenine → guanine transition or adenine → pyrimidin (T or C) transversion (Prakash et al. 1996).

The problem of carcinogenesis induced by bracken (PT) is not fully understood, human and veterinary sciences of medicine have different questions and problems. Several DNA adducts were isolated from tissues of mice, fed with bracken and these were partly different than adducts produced by PT (Freitas et al. 2001). These facts suggest that *P. aquilinum* has probable other carcinogen (genotoxic) substances as well. Different materials of phenoloid character can have such role in mutagenesis and/or in carcinogenesis. In the recent review of Roperto et al. (2010), new data is presented about the role of oxidative stress in the fern-induced urinary bladder carcinogenesis. Further studies are needed to better understand and explain these highly important problems.

PT molecule is assigned currently to group of possibly carcinogenic to humans (2B group) based of epidemiological and experimental evidences from IARC (International Agency for Research on Cancer: Roperto et al. 2010). Cattle living on fern-infested habitat are useful models for the deeper understanding of chemical carcinogenesis.

25.6 Presence of Ptaquiloside in Soil and Water

PT molecules are not only poisonous compounds of bracken but they may be transported into the environment (soil, water) by different means: by leaching (from living and dead leaves, roots, rhizomes by rain) or transfer by the animals (from fern in

soil by urine). Examinations of Rasmussen and co-workers proved that the different soil layers and the litter include different quantities of PT (in Denmark, Scotland and Sweden: Rasmussen 2003, Rasmussen et al. 2005). Logical questions are: the role and "fate" of PT molecules (i.e., decomposes or not?) what environmental and toxicological consequences are arising from these facts? According to measurements and calculations: PT production of bracken is at least 500 mg/m². PT molecules are transferred into the underlying soil and even deeper into the water table. Leaching of PT will be most extensive on sandy soil (pH > 4, organic matter content is low). PT has the lowest hydrolytic rate at slightly acidic pH and low temperature. PT appears to be relatively stable in all soil-solution types and the risk of PT leaching is controlled mainly by the microbiological status and chemical properties (organic matter, clay silicates, etc.) of soil (Ovesen et al. 2008). Further studies (better analytical methods) are required for the deeper understanding of the PT transport means and the incidental environmental risks.

25.7 Medicinal Uses of *P. aquilinum*

The plant has been used as a medicine, as an antihelminthic agent, but there were at least 21 uses including: burned wounds, galled cattle, ulcers, against worms, pain of spleen, nose bleeds, in case of general wounds, etc. It has been used as a cure for rickets and as an aphrodisiac (Rymer 1976). The bracken leaves have a characteristic smell, therefore it is a repellent to insects. It is also used to wrap-up (package) plant products (vegetables, fruits).

The use of some plant extracts for treatment of gastrointestinal parasites in animals and humans has a long history going back to Greek times. The Nordic countries of Europe have an ancient and rich culture of plant usage as antihelminthic medicaments. Decoction of bracken rhizome was used against helminths and cestodes of humans (Waller et al. 2001). Moerman (1998) noted a number of medicinal uses by North American Indian tribes: thus the rhizomes were utilized as antiemeticum, antihaemorrhagicum, analgeticum and tonicum. The extract of fronds was employed to cure liver, urinary and venereal illnesses. Compress of leaves were good for skin sores. In India and Pakistan, the bracken fern (together with other fern species) had (and have) different ethno medicinal uses. Infusion of rhizome and fronds is taken orally at bedtime against worms (Rout et al. 2009) and is used in the case of stomach cramps. In Northern Pakistan, the bracken extract is used for cure of stomach disorders.

25.8 Conclusions

1. Bracken fern (*P. aquilinum*) belongs to one of the most frequently occurring weeds of our ecosystem and it is a problematic, dangerous and poisonous plant due to its special chemical composition.

2. The fern species has the vitamin B_1 decomposer thiaminase enzyme and other, thermostable organic molecules of antithiamin character. Bracken-fed monogastric animals show characteristic neurotoxic symptoms of vitamin B_1 deficiency, the horses are very susceptible. Different cardiac problems for swine, biochemical blood changes and carcinomas for rabbit and for guinea pig can be observed.
3. Bracken fern can cause three main disorder groups in ruminant animals (first of all in cattle).
   (a) Most frequent problem is the bovine enzootic haemature (BEH), which means bleedings (and other clinical signs) and (later) tumours in fern fed animals.
   (b) Damages of bone marrow cause the second main problem: the acute haemorrhagic disease (changes in blood composition, bleedings, blood in urine, in faeces). The responsible molecules for these two problems are the norsesquiterpene glycoside PT and products of its decomposition.
   (c) Different carcinoma types can be developed in upper part of alimentary canal caused by some immunosuppressant and/or mutagen substances (quercetin).
4. Progressive degeneration of retina occurs sporadically in sheep caused by PT.
5. Different types of human carcinomas (oesophageal, gastric cancers, etc.) are produced by ingestion of bracken fern or of milk yielded by bracken fern fed cattle. The calculated daily ingested dose of this molecule (1.7–3.2 mg daily/person) can be dangerous if exposed to it for a long period of time (some years or more). The probable mode of carcinogen action of PT is: an unstable dienone derivative is produced from PT by hydrolysis, and it can form a covalent adduct of DNA. The modified DNA structures lead to genetic, later to biochemical changes and finally to carcinogenesis.
6. Examinations have established, that PT molecules may leach (diffuse) into to underlying soil, litter and water. Further investigations are needed because the possibilities of passage (transport) of such especially poisonous molecule mean great danger for our ecosystem including a wide range animals and humans alike.

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