l Organ Culture 73: 1–13, 2003.
emic Publishers. Printed in the Netherlands.

hnology and Applied Genetics

in vitro culture of ornamental ferns

H. Fernández* & M.A. Revilla
*Department of B.O.S., Laboratory of Plant Physiology, c) Catedrático R. Uría s/n 33071, Oviedo, Spain (*requests for offprints; Fax: +34-98-510-4867; E-mail: helenaf@sci.cpd.uniovi.es)*

Received 12 June 2002; accepted in revised form 25 November 2002

Key words: fern, gametophyte, *in vitro* culture, micropropagation, spore, sporophyte

Abstract

Increasing demand for ferns means that more knowledge of propagation techniques be developed to guarantee the continuous supply of these plants from commercial ornamental companies. *In vitro* tissue culture techniques show a certain promise with respect to a future increase in production. This review summarises all the research that has been conducted with ornamental ferns using *in vitro* techniques at different phases in their life cycle, starting from spores, to gametophytes and sporophytes. Nutritional, environmental and other factors affecting growth and development in each phase of the life cycle of the fern are discussed, highlighting some protocols that enhance their morphogenic and reproductive capacity.

Introduction

Ferns have been with us for more than 300 million years. Their diversity of forms is enormous and they thrive in many habitats. During the Carboniferous Period (the age of ferns), they were the dominant part of the vegetation. While most of them became extinct, some evolved into our modern ferns, which currently number around 12000 species all over the world.

Ferns pass through two distinct phases during their life cycle: the small, simple, haploid gamete-producing phase (gametophyte) and the large, morphologically complex, diploid spore-producing phase (sporophyte). The transition times from gametophyte to sporophyte and vice versa coincide with fertilisation and sporogenesis (Figure 1).

Spores are produced in the sporangia located in clusters on the underside of the fronds. In certain species of ferns, such as *Blechnum spicant* and *Osmunda regalis*, the fronds are dimorphic and spores are formed in smaller, fertile fronds that contain sporangia. In many ferns, spore formation occurs at certain time of the year, e.g. early spring, but in others, this may occur all year round. Sporogenesis combines meiosis, providing an opportunity for genetic recombination, with the switch in gene activation that initiates the change in phase from sporophyte to gametophyte.

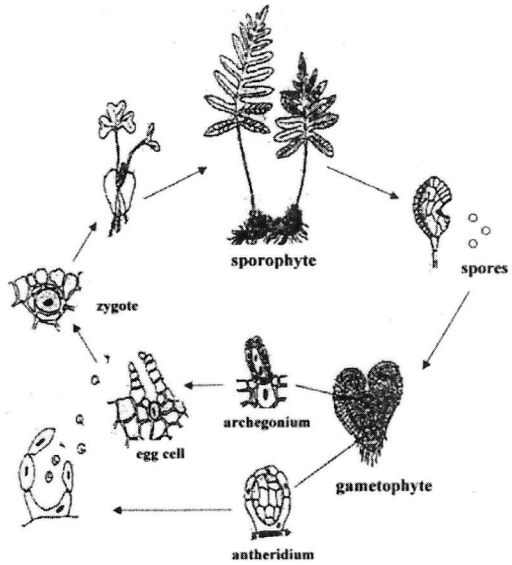


Figure 1. Fern life cycle.

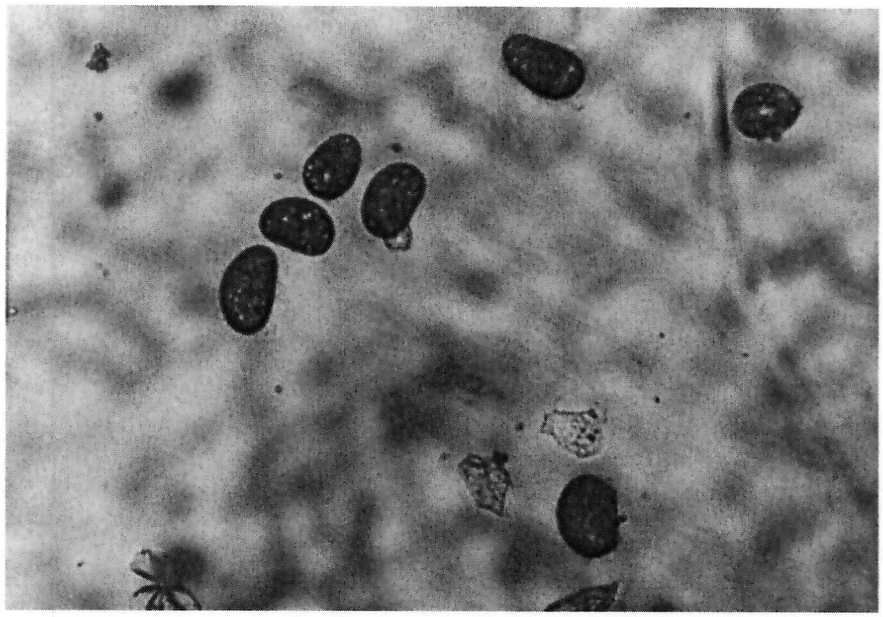


Figure 2. Spore culture in Knudson (1946).

The gametophyte generation begins precisely with spore germination (Figure 2). The most reliable criterion of germination is the outgrowth of the primary rhizoid or protonemal cell, or both. The gametophyte is named prothallus due to its simple organization, and its morphological appearance passes through different stages: filamentous, spatulate and heart shaped (Figure 3). The mature gametophyte is a dorsiventral structure of rather irregular form, differentiated into a massive basal region poor in chlorophyll and a number of distal green lobes with a well-defined meristematic area called the apical notch. Gametophytes are tiny (less that 1 cm wide), thalloid, short-lived structures. There is a thick, elongated cushion in the middle, bearing the sexual organs, antheridia and archegonia, which form the male and female gametes, respectively, and rhizoids on its lower surface. Antheridia are formed by the basal, ring and cap sterile cells that surround the sperm (antherozoids or spermatozoids) (Figure 4a). In *Blechnum spicant*, the basal cell becomes elongated, a feature of this species. Archegonia for the advanced leptosporangiate ferns are long and markedly curved away from the notch region. The neck is composed of four rows of cells, each one being 4–5 cells high (Figure 4b), and inside this structure is to be found the egg cell which gives rise to a new sporophyte after being fertilised by an antherozoid. In watery medium, antheridia swell up until bursting, releasing antherozoids which will swim via a droplet of water to the egg produced by the archegonium.

The main economic value of ferns is as popular ornamental foliage plants. Ferns are conventionally propagated by both the sexual as well as the vegetative method. The vegetative method of propagation involves the use of rhizomes or other vegetative organs as planting material. This is a reliable method to produce plants that are genetically identical to the mother plant. 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. The sexual method of propagation involves raising plants from spores. Linsay (1994) reported a reliable method of propagating ferns via spores, considered more advantageous than the vegetative mode of propagation for economic and transport reasons. However, the production of

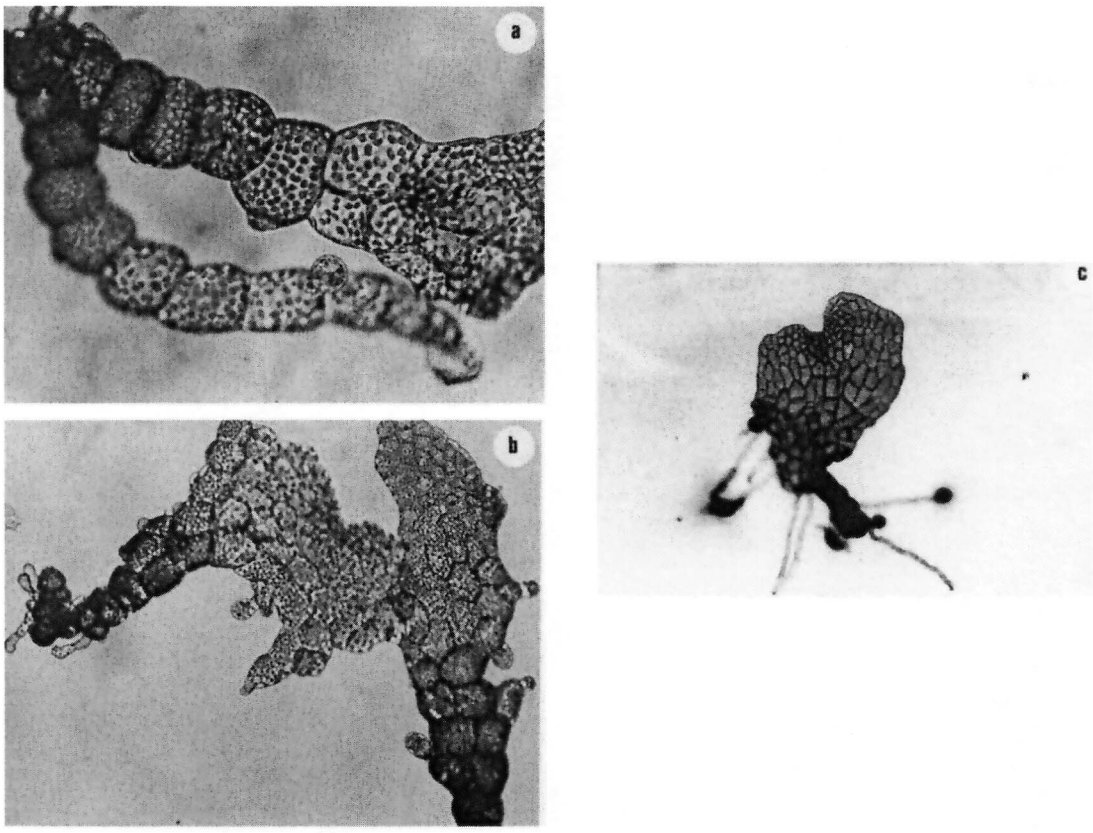


Figure 3. Different aspects in gametophyte development: (a) Filamentous, (b) Spatulate and (c) Heart shaped.

plants from spores depends on several factors such as viability and storage of spores, media and soil surfaces, sterilisation of spores and soil, size of the spore, density of spore sowing, temperature, pH range and gametophyte-sporophyte interaction (Kaur, 1991).

In vitro methods of propagation are now used for large-scale production of ornamental plants to meet the growing demand in both the domestic and the export market. According to Pierik (1991), 157 million plants, i.e. 74% of the total production of micropropagated plants, were of ornamental species. Of these, approximately 40 million plants were important pot plants. The list was headed by the fern *Nephrolepis*, at 17.8 million plants, considered the largest among *in vitro* propagated plants. Hedge and D'Souza (2000) summarise the reports of tissue culture work carried out to date with details of fern species propagated, source of explant, type of culture medium, results and authors.

Spore culture

Fern tissue culture began with the culture of spores on artificial substrates, first under partially sterile conditions such as sterile sand banks and then under fully axenic conditions on agar-plated petridishes. Spore germination using tissue culture methods permits a spore population free from contamination by spores of other species as well as infection by bacteria, fungi, algae and mosses, a major problem when growing under normal field conditions (Deberg, 1994). Among various surface sterilants, the one most commonly used for ferns has been sodium hypochlorite. The concentration used and length of exposure vary greatly depending on the type of plant material. A surfactant such as Tween or an antiseptic soap solution is normally included when some tissues require more exacting sterilization conditions. Other sterilants that have been used include calcium hypochlorite, mer-

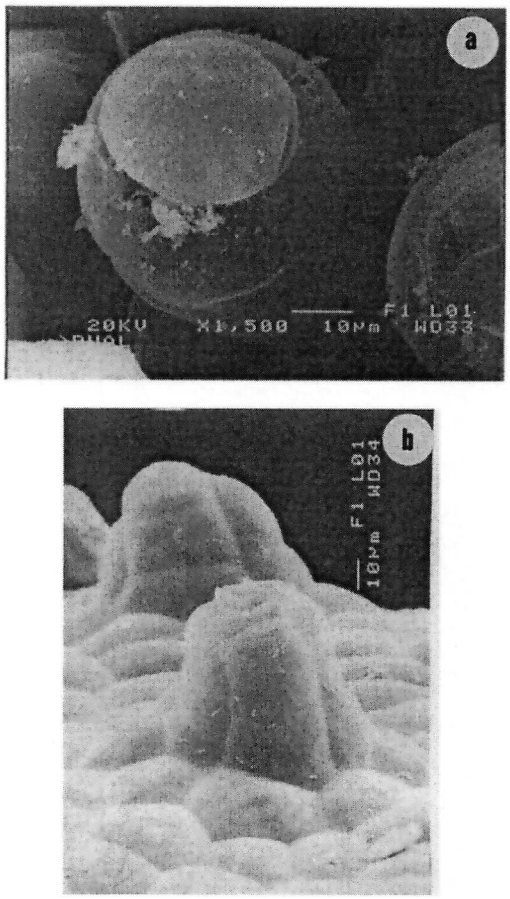


Figure 4. Sexual organs in *Asplenium nidus* gametophyte. (a) Detail of an antheridium with the basal, ring and cap cells. (b) Detail of an archegonium. Cryo SEM.

curic chloride and hydrogen peroxide (Fay, 1994). Spore sterilization methods are conducted by sterilising these along with the fronds (Hedge, 1998) or by isolating the spores from the fronds and sterilising them either in filter bags/packets (Pierik et al., 1986) or by suspending them in sterilising solution. The suspended spores are later harvested in sterile distilled water by centrifugation (Fernández et al., 1993).

Among the various media used for spore germination, the most popular ones are Knop (1865) and Knudson (1946) media. Variation in mineral content, nitrogen source and temperature are needed to promote or allow gametophyte formation from spores in some species (Whittier, 1981; Melan and Whittier, 1990). Carbohydrates were originally considered very critical for spores (Whittier, 1965). Later, the role of the osmotic potential of the media was proven to be of greater significance by Whittier (1975). The presence of sucrose is known to encourage the subsequent growth of gametophytes and sometimes even apogamy (Whittier and Steeves, 1960; Whittier, 1962). The spores of most ferns require exposure to light to germinate and grow (Dyer, 1979). Some species of ferns, such as *Botrychium* and *Ophioglossum*, require darkness to germinate (Whittier, 1981). The ability to germinate in the dark may vary with specimen age, exposure to different temperatures and plant hormone treatment (Miller, 1968; Dyer, 1979). Studies using spores of leptosporangiate ferns have shown that the best germination occurs at a slightly acidic or neutral pH (Miller, 1968). Douglas and Sheffield (1992) have reported extensive studies on favourable conditions for *in vitro* spore germination and gametophyte development. The germination time for fern spores has been found to vary from a few days to a year. *Nephrolepis* spores, as reported by Smith and Yee (1975), germinate in 3–4 days in culture, while in *Ophioglossaceae* germination, this is known to occur at the end of 6 months of incubation in the dark (Whittier, 1981). Certain Helminthostachys species are known to take 8 months to germinate (Hedge and D'Souza, 2000).

Gametophyte culture

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

Nutritional requirements

Nutrients, as well as other physical and chemical factors such as light, pH, physical state of medium and plant growth regulators, affect all the processes involved in the growth and development of prothallium (Hotta and Osawa, 1958; Mohr, 1962; Kato, 1964; Bopp, 1968; Miller, 1968; Swami and Raghavan, 1980; Sheffield and Bell, 1987; Fernández et al., 1996b, 1997a, 1997b, 1999b).

Nutritional requirements may differ depending on the species of ferns. For leptosporangiate ferns such as *Asplenium nidus, Blechnum spicant, Dryopteris affinis, Pteris ensiformis* and *Woodwardia virginica*,

media rich in nutrients, such as the full strength Murashige and Skoog (1962) medium (MS), produced a high increase in dry weight. In contrast, gametophytes of *Osmunda regalis,* a protoleptosporangiate fern, grew better in the Knop mineral medium (1865), which is low in nutrients, than in richer media. The growth of these gametophytes cultured in the Knop medium supplemented with several concentrations of mannitol, at the same osmotic values as full or half strength MS, was not inhibited. As the osmotic factor was not the one responsible for growth inhibition, media composition was considered in order to explain these results. In this sense, the addition of ammonium to the Knop medium inhibited the growth of *Osmunda regalis* gametophytes (Fernández et al., 1997a). Therefore, the high ammonium concentration of the MS medium may be responsible for gametophyte growth inhibition. On the other hand, the presence of ammonium in the culture medium was needed to grow *Pteris ensiformis* and *Blechnum spicant* gametophytes. Ammonium has been reported as an excellent nitrogen source in plants needing heterotrophic nitrogen nutrition, such as the gametophytes of *Psilotum* (Whittier, 1989) and *Bothrychium* (Melan and Whittier, 1990).

Sucrose is the most widely used carbon source in *in vitro* culture (Dyer, 1979) and when added to the nutrient media promotes gametophyte growth of leptosporangiate ferns. The optimum sucrose 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 the sucrose concentration of the culture medium increases. However, differences among species are observed and the gametophyte of *Osmunda regalis* is able to grow independently of the presence of sucrose in the culture medium, indicating autotrophy of this organism when cultured *in vitro* (Fernández et al., 1997a).

In the species of ferns with sexual reproduction, the formation and maturation of antheridia and archegonia, the male and female sexual organs respectively, occurs in different parts of the prothalli. Antheridia may be found, under varying circumstances, of any size whatsoever, ranging from one-celled sporelings to large meristematic prothalli, whereas the formation of archegonia is apparently limited to plants having at least one well-differentiated meristem (Miller, 1968). It is widely accepted that archegonia need rich media to develop. However, when culturing *Osmunda regalis* gametophytes, we observed that female sexual organs are also present when gametophytes are cultured on a medium without sucrose. In general, antheridia can develop in less complex culture media.

Environmental requirements

In addition to its effect on spore germination, light controls the subsequent development of the gametophyte (Miller, 1968). Even though light is considered necessary for the bidimensional growth of fern gametophytes, the gametophyte of *Osmunda regalis* develops a heart-shaped morphology even in darkness (Fernández et al., 1997a), as well as the gametophyte of *Asplenium nidus*, which grows better under low light intensities (Fernández et al., 1997c).

In vitro culture of *Asplenium nidus* and *Blechnum spicant* gametophytes showed a great tolerance to pH changes from 4.2 to 8.7 units of pH (Fernández et al., 1997b, 1997c), *Osmunda regalis* showed an optimum growth at pH 5.7 (Fernández et al., 1997a) and *Dryopteris affinins* sp. *affinis* growth was inhibited in a media with 8.2 units of pH (Fernández et al., 1996a).

Gametophytic growth was affected by the physical state of the culture medium: solid or liquid. In this sense, the dry weight of *Osmunda regalis* gametophytes cultured in a medium supplemented with 0.35% agar increased with respect to solid cultures (0.7% agar) (Fernández et al., 1997a), and in *Blechnum spicant*, liquid medium favoured gametophytic growth but the gametophytes showed hyperhydricity (Fernández et al., 1997c).

Vegetative reproduction of gametophytes

Gametophytes undergo vegetative reproduction by two means: via gemma or by 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 *Psilotum*, globular gemma of up to 20 parenchymatic cells are produced by growing apices of both sporophyte rhizomes and gametophytes (Hughes, 1971). These are apparently differentiated from rhizoid initials and are capable of developing into new plants when detached. In gametophytes of Vittariaceae ferns, mature gemmae consist of a uniseriate filament of four body cells, derived from gemminiferous cells initiated by the apical meristem, which will change to a bidimensional structure (Farrar, 1974; Sheffield and Farrar, 1988). Farrar and Johnson-Groh

(1990) working with *Botrychium* in populations growing under natural conditions reported that the habitat may induce this phenomenon. Fernández et al. (1997b) were the first to describe a gemmation process in *Osmunda regalis* gametophytes cultured *in vitro*. There is no data for *in vivo* gemmation in this species; it spreads by sexual reproduction and a high genetic load was reported by Klekowski (1973). In this case, it must be emphasised that sexual and asexual behaviour takes place under the same *in vitro* conditions. However, gemmation is inhibited in the absence of sucrose and/or light. *In vitro* culture represents a useful tool for studying how archegonia and gemma differentiation are regulated in the royal fern *O.regalis*. In contrast, in *Vitaria lineata* (Emigh and Farrar, 1977), gemmae contributed to spreading male gametes. Gemma formation might increase the probability of intergametophytic mating, as only antheridia are formed (Klekowski, 1969), thus avoiding negative effects caused by accumulation of lethal genes.

In the second type of vegetative multiplication of gametophytes or branching, a new gametophyte begins as a one-dimensional filament that soon becomes two-dimensional, subsequently taking on the typical shape of gametophytes produced from spores. This is the most frequent way for gametophytes to propagate themselves *in vitro*.

Sporophyte formation

Sporophyte formation occurs by sexual or asexual means. Fernández et al. (1999a) reported some aspects related to sporophyte formation. First of all, differences in the time period from spore culture to sporophyte formation are perceivable among species. The work conducted in our laboratory reveals that there are species, such as *Woodwardia virginica*, that have a fast life cycle, taking approximately 1 month to form sporophytes, and others species, such as *Asplenium nidus*, that have a longer life cycle, taking between 6 and 8 months to form sporophytes when cultured on Murashige and Skoog medium. Secondly, we have noticed differences among species based on both gametophyte growth and sporophyte formation. In this sense, in *A. nidus* and *Dryopteris affinis* sp. *affinis*, gametophyte growth ceased when sporophyte formation occurred. With *Woodwardia virginica*, gametophytic growth continued after sporophytes were formed. With *Blechnum spicant, Osmunda regalis* and *Pteris ensiformis*, sporophyte formation and gametophyte growth occurred at the same time.

Fernández et al. (1999a) also reported on the excellent multiplication capacity of gametophytes cultured *in vitro*, which was in contrast with the low number of sporophytes observed in *B. spicant* and *P. ensiformis* gametophytes. On the one hand, the lack of genetic diversity in the gametophytic colonies obtained by asexual reproduction could increase the presence of homozygous individuals and therefore affect their survival in certain species. On the other hand, we might assume nutritional competition between gametophyte and sporophyte development. As embryo formation occurs near to the apical notch and gametophyte multiplication in the basal region, the nutrients are taken up by the rhizoids, in the basal region, to supply a great number of gametophytes formed by branching actively, so sporophyte formation could be affected.

Sexual reproduction

In Pteridophyta, sporophyte formation occurs by means of the fusion of sexual cells, either of the same or different prothalli, and we can distinguish between intro- and intergametophytic crosses, respectively (Klekowski and Lloyd, 1968). Natural systems have been reported to favour intergametophytic crosses, promoting genetic interchange at different levels, at for instance a morphological, population or genetic level. In many ferns, a chemical messenger, antheridiogen, controls the onset of antheridium formation in young gametophytes (Döpp, 1950; Näf et al., 1969; Cousens, 1979; Näf, 1979; Fernández et al., 1997c)

Antheridiogen has been associated with gibberellins, though its chemical nature is not well defined. Yamane et al. (1979), using mass spectrometry, found a methyl ester of GA_9 regulating the formation of reproductive organs in *Lygodium japonicum*. Corey et al. (1986) likewise identified an antheridiogen in *Anemia phyllitidis* as an ent-kaureno derivative compound. The presence of an operate antheridiogen system has also been reported in *Blechnum spicant* by Cousens (1979). Fernández et al. (1999b) recently observed that no antheridia were formed when gametophyte colonies were changed to a fresh medium each month, and that the gametophytes remained female. However, when spores germinated in

a medium supplemented with the extract of a culture medium that had supported gametophyte growth for 3 months, they developed antheridia, which were present in all stages of gametophyte development: filamentous, spatulate or heart shaped. Fernández et al. (1999b) observed that on applying a protocol to isolate and purify gibberellins following Fernández et al. (1997d) to an extract obtained from a culture medium that had supported growth of *Blechnum spicant* gametophytes for 3 months, a fraction was found that included the most apolar compounds after the HPLC step, which inhibited gametophyte growth and promoted antheridia formation when it was incorporated into the culture medium. In the absence of this fraction, gametophyte growth was enhanced and archegonia formation took place, leading to a population entirely composed of female gametophytes.

Asexual reproduction: apogamy

When the sporophyte originates from somatic gametophyte cells, i.e. without the intervention of sexual organs, it is termed apogamy. The term 'obligate apogamy' describes a cycle in which a sporophyte is regularly produced from the gametophyte without sexual fusion. Approximately 10% of ferns and an unknown proportion of other Pteridophyta have life cycles of this kind (Sheffield and Bell, 1987).

A good example of this is the apogamous fern *Dryopteris affinis* sp. *affinis* (Fernández et al., 1996a). In this species, spore germination takes place during the first week of culture, whereby each spore produces one gametophyte whose growth evolves from the one-dimensional filamentous stage to the two-dimensional heart-shaped stage, with many glandular hairs on its surface and with many functional antheridia, but no archegonia. Two-month-old gametophytes, cultured on full-strength MS medium showed a typical developmental pattern and were composed entirely of parenchyma-type cells. One month later, a brown meristematic area developed near the apical notch and produced one sporophyte per gametophyte (Figure 5). It must be emphasised that this process was strictly apogamic, since sexual reproduction was not possible due to the absence of archegonia.

Apogamy can be induced by changes in *in vitro* culture conditions (Bell, 1959; Whittier and Steeves, 1960; Fernández et al., 1996a). For instance, when *D. affinis* sp. *affinis* gametophytes were cultured on full-strength MS medium, there was an increase in the number of sporophytes formed. Besides, sucrose 1–3% (w/v) was needed to trigger apogamy and furthermore promoted the formation of apogamous sporophytes in this species. Whittier and Steeves (1960, 1962) described the formation of apogamous plants on fern gametophytes in response to suitable concentrations of sugar in the medium. Initially, only the nutritional effect of the carbohydrate had been considered important, although Whittier (1975) showed the importance in *Pteridium* of osmotic potential. In considering the effect of dry media on apogamy, it has been suggested that obligate apogamy may be an adaptation to a xerophytic habit. Sperm requires free water for access to the archegonia, and obligate apogamy would obviate the need for water for sporophyte formation. However, there is no evidence to induce apogamous sporophytes in response to dry media in *D. affinis* sp. *affinis* (Fernández et al., 1996a) which is agree with what was reported by Whittier (1965) in the obligate apogamous species *Cheilantes tomentosa* and *C.alabamiensis*.

Apogamy may be considered the organogenesis of sporophyte from gametophytic tissue, and as is well known, promotion or inhibition of organogenesis in plant tissues is affected by growth substances (Skoog and Miller, 1957). Whittier (1966), studying the influence of growth substances on the induction of apogamy in *Pteridium* gametophytes, found that a relatively low auxin/cytokinin balance promoted shoot formation in this species. Gametophytic cells of *Drypteris affinis* sp. *affinis* showed a great morphogenetic capacity after their dedifferentiation in the presence of a relatively low auxin/cytokinin ratio, being able to develop different organisation patterns, such as callus, gametophyte or sporophyte, as a function of the growth regulators added to the culture medium after callus induction (Fernández et al., 1996a). In this report, the culture of gametophytes during 1 month in the presence of a low auxin/cytokinin ratio favoured sporophyte organisation, while gametophyte regeneration was possible after 2 months of culture in a medium supplemented with 6-benzyladenine (BA). Auxin and cytokinin are direct triggers of expression of sporophytic and gametophytic genes in the gametophyte of *D. affinis* sp. *affinis*; these growth regulators played a key role in, at least, the activation of these genes. Kuriyama et al. (1990) reported that exogenously supplied BA influenced the gametophyte of *Equisetum arvensis* to

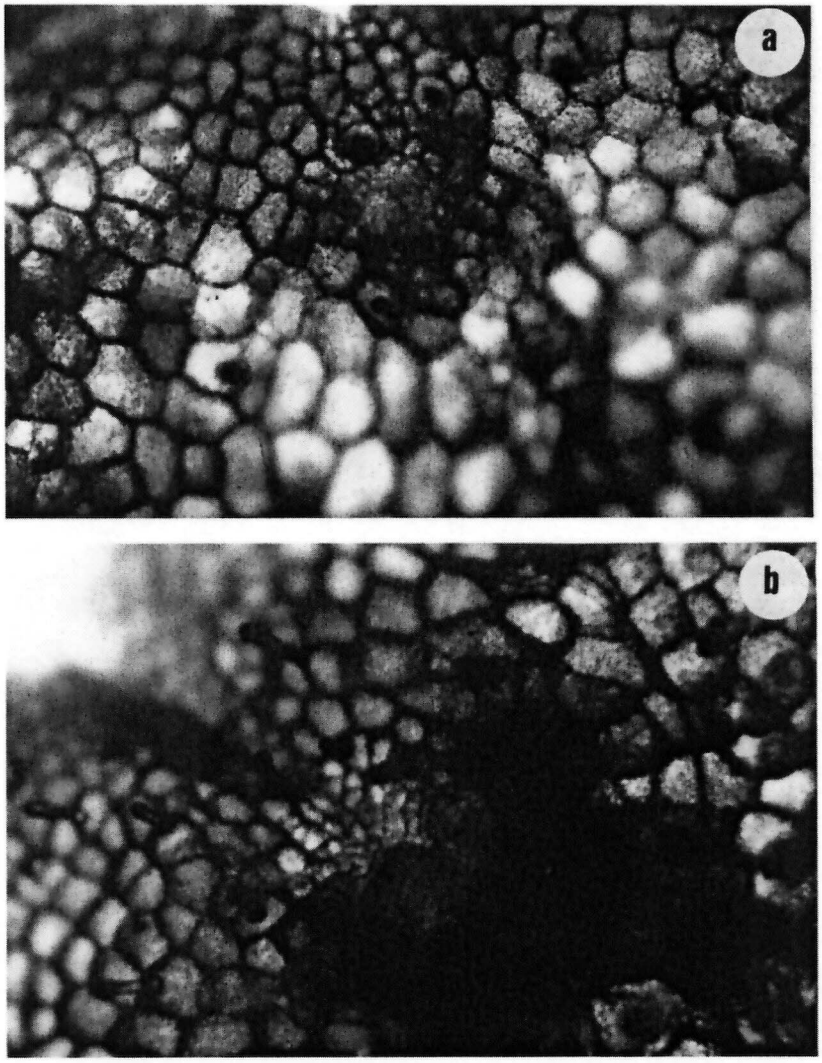


Figure 5. Apogamy in *Dryopteris affinins* sp. *affinins*. (a) Meristematic area located near to the apical notch. (b) Primordia of an sporophyte (×120).

produce sporophytes and callus. Such callus differentiated sporophyte only when cultured in a BA-free medium; gametophytes were never recovered from such callus or sporophyte.

Nutritional stress

A significant increase in sporophyte formation took place after 2 months of culturing gametophytes of *Osmunda regalis* and *Pteris ensiformis* in the absence of nutrients, in a medium containing just water and 0.7% agar (Fernández et al., 1999b). The sole presence of sucrose in the culture medium, without mineral salts, inhibited gametophyte development of both species, which became necrotic. In *P. ensiformis*, the half-strength MS basal medium without sucrose favoured leaf expansion of sporophytes.

Morphogenesis in juvenile sporophyte

Sporophytes are produced from cultured tissues either by axillary shoot proliferation (Murashige, 1974) or as adventitious shoots formed directly or from callus (Amaki and Higuchi, 1991). The effect of cytokinins, especially N_6-benzyladenine (BA), on bud formation, yielding a high number of sporophytes, has been previously reported by several authors (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, 1997b). In the last report, juvenile sporophytes cultured in the presence of a low auxin/cytokinin ratio show a characteristic swelling of the rhizome as a result of the presence of proliferation centres located both on epidermal and subepidermal layers, as well as on the inner parenchyma due to cell division in all planes (Figure 6). The organisation pattern they follow depends on the supply of growth regulators and the contact period between explants and growth regulators, (Fernández et al., 1997b). Subculture of rhizomes for 1 month on Murashige and Skoog medium without growth regulators induced bud formation from inner meristematic centres, making their way through the parenchyma until regenerating a new sporophyte. Explants exposed to BA for 2 months induced phase change (apospory) and gametophytes are formed from surface-located proliferation centres. The role of cytokinins on apospory is not clear and more experimental work is needed to understand how the phase change in ferns occurs *in vitro*. The subculture of BA-pretreated rhizomes in media containing NAA induced different responses. In *Blechnum spicant,* NAA induced green globular bodies (GGB), which form sporophytes actively. In *Pteris ensiformis*, both callogenesis and rhizogenesis processes occurred (Fernández et al., 1997b).

Bertrand et al. (1999) cultured explants from the rhizome, frond, petiole and root tip of juvenile sporophytes of *Polypodium cambricum* on media containing BA or kinetin alone or combined with NAA. Root tip explants exhibited the lowest capacity for organogenesis.

Thus, cytokinins such as BA must be used carefully and the length of the culture period must be controlled. Certainly, cells of both gametophyte and sporophyte are totipotent, and the genetic information stored in the nucleus of cells of either generation can be switched to the expression of gametophytic or sporophytic tissue, simply by manipulating the environmental conditions that plant tissues are exposed to.

Homogenised cultures

Reports by Knauss (1976), Finnie and van Staden (1987) and Cooke (1979) that gametophyte or sporophyte homogenates provide a simpler propagation system than the traditional methods are only partially true. In our laboratory, approximately 2000 sporophytes were obtained by homogenisation of *Woodwardia virginica* gametophytes after 2 months of culture. While this method is recommended for species with a short life cycle such as *W. virginica*, the results cannot be extended to species with longer life cycles. Furthermore, re-homogenisation of gametophytes that have been regenerated by homogenisation gave rise to different responses depending on the species. In *W. virginica*, for instance, sporophytes formed at the same time as the initial homogenate culture. On the other hand, with *Osmunda regalis*, repeated homogenisation inhibited sporophyte formation.

Homogenates from juvenile sporophytes of *Asplenium nidus* regenerated both gametophytes and sporophytes, but the amount of sporophytes was lower than gametophytes (Fernández et al., 1993). As mentioned above, cytokinins induced bud proliferation in the rhizome of ferns, but this effect increased after homogenisation of this organ (Fernández et al., 1997b) and from each explant several buds were formed which were able to regenerate sporophytes (Figure 7). This system allows the improvement of the propagation rates of this species and may be extended to other species of ferns. Moreover, the culture of homogenates in liquid medium is amenable to automation and to establishing large-scale cultures in bioreactors, reducing handling and also improving plant production.

In summary, *in vitro* culture of the fern's life cycle represents a powerful tool for dissecting the mechanisms underlying plant development. To deep on phase change in this plant group, or gametophyte-sporophyte transition, would throw light to know about the complex rules of plant physiology by means of easier and accurated experimental systems. This knowledge has important practical repercusions such as to get higher production of species having a great economical value for ornamental industry.

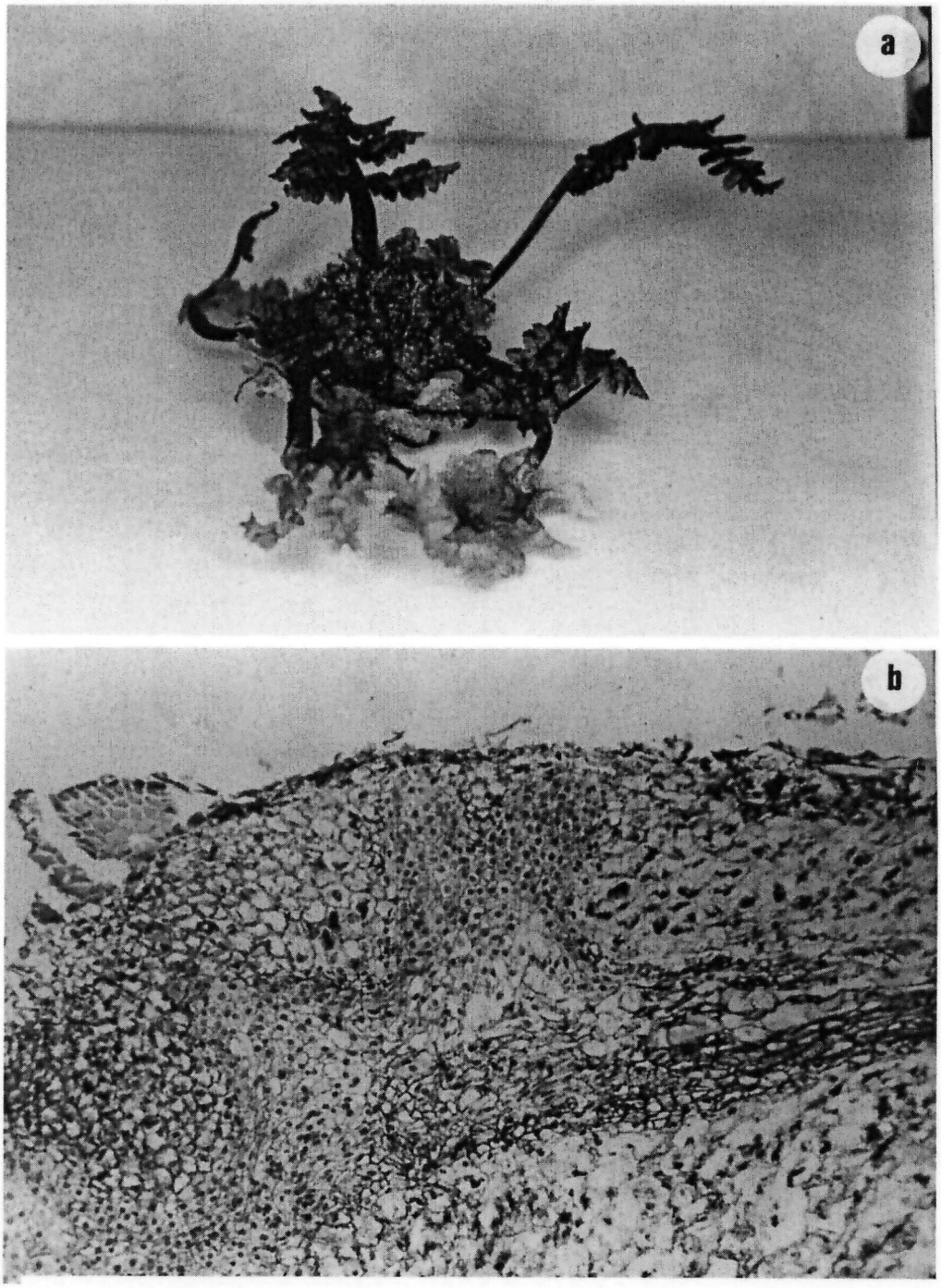


Figure 6. Effect of growth regulators on juvenile sporophytes of *Woodwardia virginica*. (a) Swelling of rhizome after one moth of culture on MS medium with 4.4 μM BA. (b) Detail of the inner parenchyma differentiating meristematic cells actively.

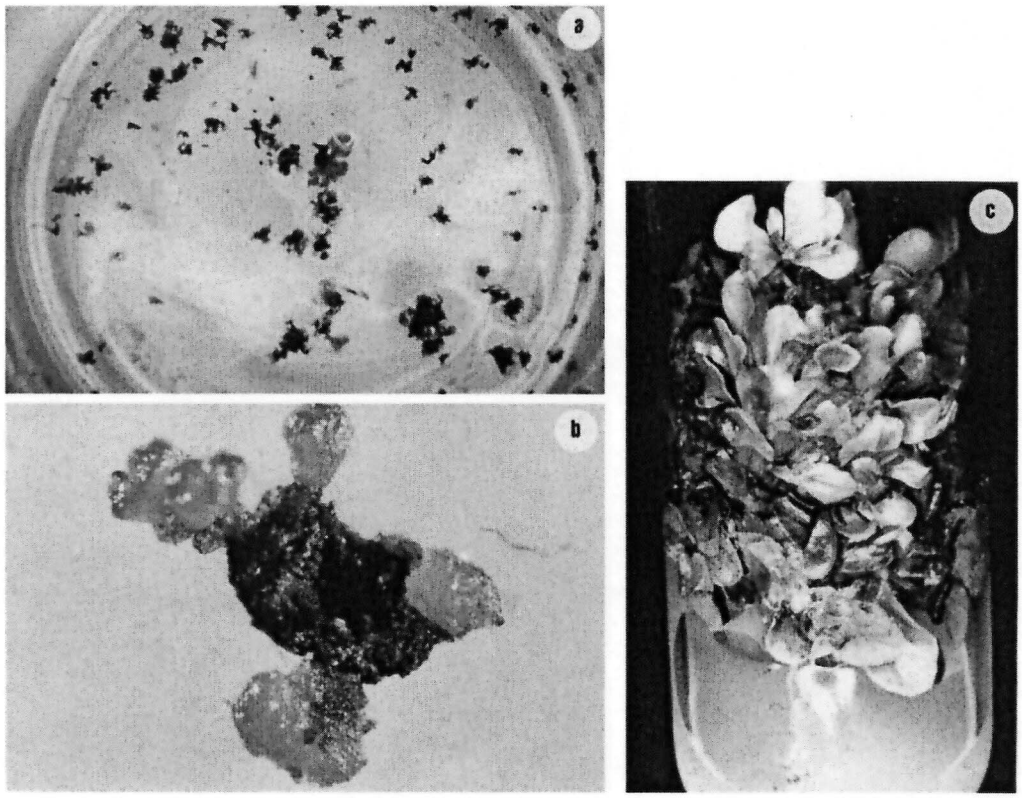


Figure 7. Homogenised cultures in *Asplenium nidus* L. (a) Initial explants from rhizome pretreated with BA. (b) Meristemoids developed in homogenized cultures obtained from rhizome explants. (c) Sporophytes derived from homogenized cultures.

References

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

Beck MJ & Caponetti JD (1983) The effects of kinetin and naphtaleneacetic acid *in vitro* shoot multiplication and rooting in the fishtail fern. Am. J. Bot. 70: 1–7

Bell PD (1959) The experimental investigation of the pteridophyte life cycle. J. Linn. Soc. Bot. London 56: 188–202

Bertrand AM, Albuerne MA, Fernández H, González A & Sánchez--Tamés R (1999) *In vitro* organogenesis of *Polypodium cambricum*. Plant Cell Tiss. Org. Cult. 57: 65–69

Bopp M (1968) Control of differentiation in fern allies and bryophytes. Ann. Rev. Plant Physiol. 19: 361–380

Cooke RC (1979) Homogenization as an aid in tissue culture propagation of *Platycerium* and *Davallia*. HortSci. 14: 21–22

Corey EJ, Myers AG, Takahashi N, Yamane H & Schraudolf H (1986) Constitution of antheridium-inducing factor of *Anemia phyllitidis*. Tetrahedron. Lett. 27: 5083–5084

Cousens MI (1979) Gametophytic ontogeny, sex expression and genetic load as measures of population divergence in *Blechnum spicant*. Am. J. Bot. 66: 116–132

Deberg P (1994) *In vitro* culture of ornamentals. In: Vasil IK & Thorpe TA (eds) Plant Cell and Tissue Culture (pp. 561–573). Kluwer Academic Publishers, Dordrecht, The Netherlands

Döpp W (1950) Eine die Antheridienbildung bei farnen fördernde substanz in den prothallien von *Pteridium aquilinum* (L.) Kuhn. Ver Deut. Bot. Ges. 63: 139

Douglas GE & Sheffield E (1992) The investigation of existing and novel artificial growth systems for the production of fern gametophytes. In: Ide JM, Jermy CA & Paul AM (eds) Fern Horticulture: Past. Present and Future Perspectives (pp. 183–187). Intercept, Andover

Dyer AF (1979) The culture of fern gametophytes for experimental investigation. In: Dyer AF (ed) The Experimental Biology of Ferns (pp. 253–305). Academic Press, London

Emigh VD & Farrar DR (1977) Gemmae: a role in sexual reproduction in the fern genus *Vittaria*. Science 198: 297–298

Farrar DR (1974) Gemmiferous fern gametophytes *Vittariaceae*. Am. J. Bot. 61: 146–155

Farrar DR & Johnson-Groh CL (1990) Subterranean sporophytic gemmae in moowort ferns, *Botrychium subgenus Botrychium*. Am. J. Bot. 77: 1168–1175

Fay MF (1994) In what situations is *in vitro* culture appropriate to plant conservation? Biodiversity and Conservation 3: 176–183

Fernández H, Bertrand AM & Sánchez Tamés R (1993) *In vitro* regeneration of *Asplenium nidus* L. from gametophytic and sporophytic tissue. Sci. Hort. 56: 71–77

Fernández H, Bertrand AM & Sánchez-Tamés R (1996a) Influence of tissue culture conditions on apogamy in *Dryopteris affinis* sp. *affinis*. Plant Cell Tiss. Org. Cult. 45: 93–97

Fernández H, Bertrand AM & Sánchez-Tamés R (1996b) Micropropagation and phase change in *Blechnum spicant* and *Pteris ensiformis*. Plant Cell Tiss. Org. Cult. 44: 261–265

Fernández H, Bertrand AM & Sánchez-Tamés R (1997a) Gemmation in *Osmunda regalis* L. gametophyte cultured *in vitro*. Plant Cell Rep. 16: 358–362

Fernández H, Bertrand AM & Sánchez-Tamés R (1997b) Plantlet regeneration in *Asplenium nidus* L. and *Pteris ensiformis* L. by homogenisation of BA treated rhizomes. Sci. Hort. 68: 243–247

Fernández H, Bertrand AM, Feito I & Sánchez-Tamés R (1997c) Gametophyte culture *in vitro* and antheridiogen activity in *Blechnum spicant*. Plant Cell Tiss. Org. Cult. 50: 71–74

Fernández H, Doumas P & Masimbert M (1997d) Quantification of GA_1, GA_3, GA_4, GA_7, GA_9, GA_{19} and GA_{20}, and GA_{20} metabolism in dormant and non-dormant beechnuts. Plant Growth Reg. 22: 29–35

Fernández H, Bertrand AM & Sánchez-Tamés R (1999a) Biological and nutritional aspects involved in fern multiplication. Plant Cell Tiss. Org. Cult. 56: 211–214

Fernández H, Bertrand AM, Sierra MI & Sánchez-Tamés R (1999b) An apolar GA-like compound responsible for the antheridiogen activity in *Blechnum spicant*. Plant Growth Reg. 28: 143–144

Finnie JF & van Staden J (1987) Multiplication of the tree fern *Cyathea degrei*. HortSci. 22: 665

Harper K (1976) Asexual Multiplication of Leptosporangiatae Ferns Through Tissue Culture. MSC Thesis. University of California, Riverside (pp. 168)

Hedge S (1998) Propagation of Some Native Ornamental Plants using Biotechnology, Ph.D Thesis, Mangalore University, Mangalore, India

Hedge S & D'Souza L (2000) Recent advances in biotechnology of ferns. In: Trivedi PC (ed) Plant Technology,. Panima Publishing Corporation, New Delhi, Bangalore

Hicks G & von Aderkas P (1986) A tissue culture of the ostrich fern *Matteuccia struthiopteris* L. Todaro. Plant Cell Tiss. Org. Cult. 5: 199–204

Higuchi H & Amaki W (1989) Effects of 6-benzylaminopurine on the organogenesis of *Asplenium nidus* L. through *in vitro* propagation. Sci. Hort. 37: 351–359

Higuchi H, Amaki W & Suzuki S (1987) *In vitro* propagation of *Nephrolepis cordiflora*. Sci. Hort. 32: 105–113

Hotta Y & Osawa S (1958) Control of differentiation in the fern gametophyte by amino acid analogs and 8-azaguanine. Exp. Cell Res. 15: 85–94

Hughes SJ (1971) On conidia of fungi and gemmae of algae, bryophytes and pteridophytes. Can. J. Bot. 49: 1319–1339

Kato Y (1964) Physiological and morphogenetic studies of fern gametophytes in aseptic culture II. One and two-dimensional growth in sugar media. Bot. Gaz. 125: 33–37

Kaur S (1991) Ferns and fern allies. Their domestication and conservation. In: Bharadwaj TN & Gena CB (eds) Perspectives in Pteridology: Present and Future. Today & Tomorrow's Printers and Publishers, New Delhi (India), Vol. 13 (pp. 83–89). Aspects of Plant Sciences.

Klekowski EJ Jr. (1969) Reproductive biology of the Pteridophyta. II. Theoretical considerations. J. Linn. Soc. (Bot.). 62: 347–359

Klekowski EJ Jr. (1973) Genetic load in *Osmunda regalis* populations. Am. J. Bot. 60: 146–154

Klekowski EJ Jr. & Lloyd RM (1968) Reproductive biology of the Pteridophyta I. General considerations and a study of *Onoclea sensibilis* L. J. Linn. Soc. (Bot.) 60: 315–324

Knauss JF (1976) A partial tissue culture method for pathogen-free propagation of selected ferns from spores. Proc. Fla State Hort. Soc. 89: 363–365

Knop W (1865) Quantitative untersuchungen uber die ernahrungsprozesse der pflanzen. Landwirtsch Vers Stn. 7: 93–107

Knudson L (1946) A nutrient solution for the germination of orchid seed. Bull. Am. Orchid Soc. 15: 214–217

Kuriyama A, Sugawara Y, Matsushima H & Takeuchi M (1990) Production of sporophytic structures from gametophytes by cytokinin in *Equisetum arvense*. Naturwissenschften 77: 31–32

Linsay J (1994) Account of germination and raising of ferns from the seed. Trans. Linn. Soc. London 2: 93–100

Loescher WH & Albrecht CN (1979) Development *in vitro* of *Nephrolepis exaltata* "Bostoniensis' runner tissue. Physiol. Plant 47: 250–254

Melan MA & Whittier DP (1990) Effects of inorganic nitrogen sources on spore germination and gametophyte growth in *Botrychium dissectum*. Plant Cell Environ. 13: 477–482

Miller JH (1968) Fern gametophytes as experimental material. Bot. Rev. 34: 361–440

Mohr H (1962) The influence of visible radiation on the germination of archegoniate spores and the growth of the fern protonema. J. Linn. Soc. Bot. 58: 287–296

Murashige T (1974) Current status of plant cell and organ culture. Hort. Sci. 12: 3–6

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15: 473–497

Näf U (1979) Antheridiogens and antheridial development. In: Dyer AF (ed) Exp Biol Ferns,. Academic Press, London

Näf U, Sullivan J & Cummins M (1969) New antheridiogen from the fern *Onocleasensibilis*. Science 163: 1357

Peck JH, Peck CJ & Farrar DR (1990) Influences of life history attributes on formation of local and distant fern populations. Am. Fern. J. 80: 126–142

Pierik RLM (1991) Commercial aspects of micropropagation. In: Prakash J & Pierik RML (eds) Horticulture – New Technologies and Applications (pp. 141–153). Kluwer Academic Publishers, Dordrecht, The Netherlands

Pierik RML, Oerlemans APC, Verhoeven MAP, Mentjox MA & Molenberg H (1986) Zaai van varens in kweekbuizen. Vakblad Bloemisterij 10: 48–49

Sheffield E & Bell PR (1987) Current studies of the pteridophyte life cycle. Bot. Rev. 53: 442–490

Sheffield E & Farrar DR (1988) Cryo SEM examination of gemma formation in *Vittaria raminifolia*. Am. J. Bot. 75: 894–899

Skoog F & Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Proc. Symp. Soc. Exp. Biol. 11: 118–131

Smith CW & Yee RNS (1975) The effect of coconut milk on the germination and growth of spores of *Nephrolepis hirsutula*. Am. Fern. J. 65: 13–18

Swami P & Raghavan V (1980) Control of morphogenesis in the gametophyte of a fern by light and growth hormones. Can. J. Bot. 58: 1464–1473

Whittier DP (1962) The origin and development of apogamous structures in the gametophyte of *Pteridium* in sterile culture. Phytomorphology 12: 11–20

Whittier DP (1965) Obligate apogamy in *Cheilantes tomentosa* & *C. alabamiensis*. Bot. Gaz. 126: 275–281

Whittier DP (1966) The influence of growth substances on the induction of apogamy in *Pteridium* gametophytes. Am. J. Bot. 53: 882–886

Whittier DP (1975) The influence of osmotic conditions on induced apogamy in *Pteridium* gametophytes. Phytomorphology 25: 246–249

Whittier DP (1981) Spore germination and young gametophyte development of Botrychium and Ophioglossum in actinic culture. Am. Fern. J. 71: 13–19

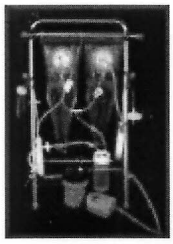
Whittier DP (1989) Effects of nitrogen source on spore germination and gametophyte growth in *Psilotum*. Bot. Gaz. 151: 50–53

Whittier DP & Steeves TA (1960) The induction of apogamy in the bracken fern. Can. J. Bot. 38: 925–930

Whittier DP & Steeves TA (1962) Further studies on induced apogamy in ferns. Can. J. Bot. 40: 1525–1531

Yamane H, Takahashi N, Takeno K & Furuya M (1979) Identification of gibberellin A_9 methyl ester as a natural substance regulating formation of reproductive organs in *Lygodium japonicum*. Planta 147: 251–256

The Osmotek LIFE LINE
Advanced Products for Plant Tissue Culture



Bioreactor multiplication.
nodules, meristem clumps, somatic embryos and cells, bulbs ,boston fern,potato, corms or microtubers.

The LifeGuard

plant growth vessels and aseptic membrane vented lids are ideal for plant tissue culture. These unique vented vessels allow complete control of aeration while prevecontamination from external sources from entering.

Methods and Apparatus for liquid Media and Semi - Automated Micropropagation

Robert Levin Yekutiel Alper
Osmoiek Ltd POB 550 Agricultural Eng.
Kiryat Weizmann Institute
Rehovot, Israel ARO, Bet Dagan
Israel

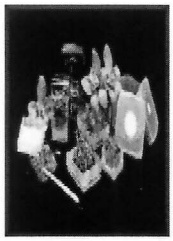
Rani Slav and Abed A. Watad
Department of Ornamental
Horticulture
ARO Bet Dagan
Trap.I

Additional index words: **Banana, potato, clusters, microtubers, automation.**

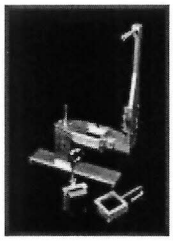
A method and equipment for **mechanized micropropagation of potato and banana** is described.
The method employs multiplication of meristematic or bud clusters on interfacial membrane rafts and in plastic film air lift bioreactors, separation of the clusters by a mechanical cutting device and subculture to multiplication or to a growth medium on an interfacial membrane raft. For potato the production of microtubers in a tuber induction medium is also reported.

1.Introduction

Significant expansion of commercial micropropagation will require the implementation of scale-up and automation technology (Vasil, 1991; Vasil 1994).
For developed countries, where labor costs are around 70% of the costs of production (George, 1996), the cost reduction resulting from the use of mechanization would permit cost effective micropropagation of transplant vegetable and forestry crops.
This cost reduction may also permit the commercial micropropagation of genetically modified plants in which desirable characteristics could be lost



The LifeRaft
unique multiplication system of nodules and meristem or shoot clumps,as well as the growth of shoots, microtubers, bulbs and root culture on liquid medium.



The VitroCut
mechanical plant culture separator randomly cuts culture material on a knife grid with a unique self cleaning anvil. Cut plant material falls directly into a sterile vessel.

during sexual reproduction (Levin and Vasil, 1989).
In countries where labor costs are low, mechanization could standardize production and reduce contamination introduced during manual operations.
A number of cost reduction techniques have been described including the use of liquid medium and automated media transfer, growth and multiplication of culture in bioreactors, automated media preparation and vessel handling, nodule culture, hedging and homogenization (Aitken-Christie, 1991, George, 1996, Ziv et al., 1996).
In addition, a number of mechanized, robotic and automated systems for micropropagation have been described (Kurata, 1995).
However, there have been very few reports of mechanization of micropropagation using commercially available component systems.
The following is description of a procedure for mechanized micropropagation of potato and banana employing equipment produced and marketed by Osmotek Ltd., Israel.

2.Materials and Methods

2.1 Description of the membrane raft system The rafts system (LifeRafts™, Osmotek, Rehovot, Israel) includes a microporous membrane raft and a buoyant float.
The membrane supports the plant tissue and the float maintains the membrane raft at the air/liquid interface.

2.2 Description of the bubble reactor The bubble reactor was I liter working volume air lift reactor similar to that reported by Ziv and Hadar (1991).
The reactor consisted of a sealed glass cylinder with a sparger at the bottom for bubbling air, a scalable port for filling and emptying the reactor, and an port for releasing overpressure in the reactor.
Air entering the reactor was filtered through a 0.2(.i.m sterilizing filter (Gelman Sciences,Aero 50) then passed through a humidifier and a one way check valve.
Air exiting the reactor passed through a drier and a 0.4 urn filter (Gelman Sciences, Aero 50) to minimize environmental contamination entering the reactor through the overpressure release port.
2.3 Description of the plastic film bioreactor The plastic film bioreactor (LifeReactor™, Osmotek, Rehovot, Israel) was a 1.5 liter working volume vessel fabricated from clear plastic film with a 6 cm dia. inoculation port.
The vessel included an autoclaveable port cap with two port channels for air inlet and air outlet, and two additional channels for the control of culture conditions (pH, nutrient supplement, sampling etc.) and medium recirculation through a filter as described above.
A multiple use glass sparger was connected by silicon tubing and

connectors to the air inlet port. The air inlet and air outlet system was as described for the bubble reactor.
2.4 Description of the vented vessels The vessels were IOcm x 10 cm polypropylene containers (LifeGuard™, Osmotek, Rehovot, Israel) with a 16mm or 40mm microporous membrane vent with a nominal pore size of0.3)-im.
2.5 Description of the mechanical cutting device The cutting device was the VitroCut™ (Osmotek, Rehovot, Israel).
This apparatus is an autoclavable stainless steel knife grid with a manually activated piston like head which engages the knife grid.
Multiplying plant tissue culture clusters are placed on the knife grid , the head lowered with the separated clusters falling into a vessel positioned below the knife grid. For the present experiments the knife grid was a 5 mm or 7 mm mesh.
2.6 Medium, plant material and culture procedures for banana The liquid proliferation medium for the bioreactor experiments included MS minerals (Sigma) with 380 mg/l NaH2P04, 100 mg/l, 160 mg/l adenine sulphate, 200 mg/l tyrosine, 1000 mg/l casein hydrolyzate, 1.0 mg/l thiamine HCL, 3% sucrose, I - 5 mg/l ancymidol (Elanco) 5mg/l benzyl adenine (BA) and 2 mg/l indole acetic acid (IAA).
The proliferation medium for the raft elongation experiments employed was and included 2 mg/l Ancymidol, but omitted casein hydrolyztae.
The liquid elongation medium for the raft experiments and the agar solidified medium for elongation after the bioreactor used 5mg/l kinetin instead ofBA and omitted Ancymidol.
The bioreactor experiments were performed in the laboratory of M. Ziv and reported in Ziv et al. (1996).
The raft experiments were performed in a commercial laboratory Banana plantlets (cv 'Williams'or 'Grand Nain' were supplied *m vitro* by a commercial propagator.
The leaves were removed and the basal part of the stem (approximately 2mm long) was inoculated into vessels containing proliferation medium,For the bioreactor experiments the stems were inoculated into 80 ml of liquid proliferation medium in 500 ml Erienmeyer flasks closed with cellulose plu"s and incubated in the dark at 24 ± 1°C on a gyratory shaker at 85-90 rpm for 25 - 28 days.
The clusters which were formed were inoculated into the plastic film bioreactor with 1.2 liters of proliferation medium.
The bioreactors were maintained in the dark and aerated at 1.0 -1.5 wrn.
Clusters from the bioreactor were randomly separated by a mechanical cutting device (VitroCut, Osmotek Ltd. Rehovot, Israel) into approximately 5 x 5 cm sections.
A mass of propagules weighing 6.0 grams fresh weight was spread over the surface of agar solidified elongation medium in Magenta GA7 vessels and cultured at 24 ± 1°C with 16 hours of light supplied by cool white

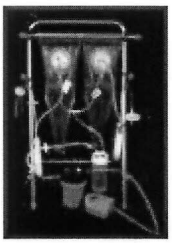
fluorescent lamps giving 40 Limoles.m'".s' .
For the raft experiments stems were inoculated into vented polypropylene vessels (16mm vent) with 150 ml of agar solidified proliferation medium and incubated in the dark at 26 ± 2°C for 25 -28 days.
The clusters which were formed were randomly separated by the VitroCut into approximately 7 x 7 mm sections and 20 grams reinoculated on proliferation medium as before.
After three subculture generations 20 grams of whole clusters were placed in polypropylene containers with 40mm vents (LiferGuard ™ Vessels, Osmotek Ltd., Rehovot, Israel) with 150 ml of liquid elongation medium and supported by a membrane raft system (LifeRaft™, Osmotek Ltd., Rehovot).
The vessels were cultured at 24 ± 1°C with 16 hours of light supplied by cool white fluorescent lamps giving 40 ~moles .m .s' .
2.7 Medium, plant material and culture procedures for potato Liquid medium was employed for proliferation in all experiments.
The flask proliferation (PF) medium included MS basal slats and vitamins, 0.4 mg/l thiamine HCI, 0.01 mg/l NAA, 2 mg/l pantothenic acid, 0.25 mg/l GA3, and 20 g/l sucrose.
The bioreactor proliferation medium (PB) included MS basal salts and vitamins 0.4 mg/l thiamine HCI, 20 mg/l pantothenic acid, and 0.4 mg/l GA3, 20 g/l sucrose and 1.5 mg/l ancymidol.
The elongation medium for the bioreactor experiments was PF medium solidified with agar.
The liquid proliferation and elongation medium for the raft experiments included MS basal salts and vitamins, 20 mg/l pantothenic acid, I mg/l ancymidol, 20 g/l sucrose and I mg/l Kinetin.
The tuberization (Tl) medium consisted of MS basal salts and vitamins, 80 g/l sucrose, 20 mg/l panothenic acid, I mg/l ancymidol, 5 mg/l BA and 1.3 mg/l CCC.
For the bioreactor experiments 20 to 30 single node explants were removed agar cultured potato stock plants (cv Desiree) and subcultured to 500 ml Erienmeyer flasks containing 200 ml of PF medium and sealed with aluminum foil and parafilm.
Flasks were incubated on an orbital shaker at 90- 100 rpm for 21 days with light cycle of a 16 hours light (60 *1 -imoles* .m~Z, s") 8 hours dark at 25 ±2°C. Bud clusters

|Page1|Page2|

| Home | Introduction | Products Descriptions | Product Catalog|Instruction Manuals| Applications | PTC System| Literature|Links|

CONTACT US

The Osmotek LIFELINE
Advanced Products for Plant Tissue Culture



Bioreactor multiplication.
nodules, meristem clumps, somatic embryos and cells, bulbs ,boston fern,potato, corms or microtubers.

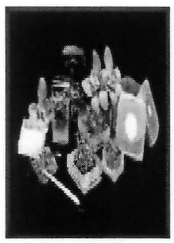
The LifeGuard

plant growth vessels and aseptic membrane vented lids are ideal for plant tissue culture. These unique vented vessels allow complete control of aeration while prevecontamination from external sources from entering.

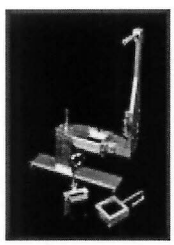
produced in the flasks were chopped in a blender for six seconds, washed in a sieve and inoculated into bubble reactors with I liter of PB medium.
The bubble reactors were incubated under constant light (70 (.lmoles.m~.s') at 25 ±2°C for 21 days for two cycles.
Bud clusters harvested from the bubble reactors were chopped in a blender, again for six seconds and washed in a sieve.
Between 4.0 and 4.5 g of the chopped clusters were inoculated on 100 ml of elongation medium in unvented LifeGuard™ vessels and incubated for 21 -28 days with 16 hours of light (60 l.l*m*oles.m`2.~) at 25 ±2°C.
For the raft experiments 70 - 90 single nodes of cv Desiree were subcultured from in vitro stock plantlets to LifeGuard™ vented vessel (40 mm vents) with 150 ml of liquid medium supported by the microporous membrane raft system (the LifeRaft™ system).
The vessels and rafts were incubated for 30 days with 16 hours of light (60 [.lmoles.m~.s") at 25 i2"C After incubation compact shoot clusters with very small leaves were produced.
These shoot clusters were cut mechanically with the VitroCut™ (7mm x 7mm grid) and reinoculated into raft multiplication culture for two additional multiplication cycles.
Culture produced on the third multiplication cycle was transferred to tuberization (Tl) medium by moving a raft to LifeGuard™ vented vessels (40 mm vents) containing 150 ml ofTI medium.
Incubation was at 19 ±l°Cfor nine weeks. For the first 10 days incubation was with 16 hours light (60 Limoles.m-2.s-l) after which incubation was in the dark.

3.Results

3.1 Results of the Banana Procedures The fresh weight of cluster biomass in liquid shake cultures with 2mg/l ancymidol increased from 10.Og to 35.2 g in 25 days.
The biomass of the clusters consisted 21.4 g of nodular meristems and 13.8 g expanded leaves. When the cluster were transferred to a plastic film bioreactor with 2 mg/l. ancymidol in the medium 20 g of fresh weight biomass increased to 170 g after 30 days with meristernoid clusters making up 40% of the biomass. When the ancmyidol level was increased to 4 mg/l the meristernoid cluster proportion of the biomass increased to



The LifeRaft
unique multiplication system of nodules and meristem or shoot clumps,as well as the growth of shoots, microtubers, bulbs and root culture on liquid medium.



The VitroCut
mechanical plant culture separator randomly cuts culture material on a knife grid with a unique self cleaning anvil. Cut plant material falls directly into a sterile vessel.

69% and there was very little necrotic degenerated tissue.
Plantlets developed when the bioreactor proliferated clusters were mechanically separated by the VitroCut™ into 5 x 5 cm sections and 6.0 got the propagules spread on the surface of agar solidified elongation medium in a culture vessel.
After 24 days. each vessel contained 28 -36 plantlets ranging from 0.9 to 2.8 cm in height.
When clusters were proliferated on agar solidified medium in LifeGuard™ vessels with 16mm vents and separated by the Vitrocut™, the fresh weight of cluster biomass increased from 20 g to 39g in 28 days.
When 20 g of whole clusters were transferred to liquid elongation medium supported by the microporous membrane raft (LifeRaft™) system in LifeGuard™ vessels with 40 mm vents an average of 40 commercially viable plantlets per vessel were produced.
3. 2 Results of the Potato Procedures Flasks prepared for the bioreactor experiments produced 10 - 12 grams of bud cluster after three weeks. 20 grams of chopped and washed flask buds increased in the bioreactors to 200 g after the second three week cycle.
The 4 to 4.5 g of chopped and washed buds from the bubble reactors produced about 60 plants per vessels on elongation medium.
In the raft experiments, single nodes placed on the LifeRaft ™ produced a dense mat of shoot clusters after 3 - 4 weeks incubation. Each raft produced shoot clusters which when divided by the VitroCut™ produced inoculum for three new rafts.
After the third cycle, the rafts produced an average of 94 plants . When a raft with regenerated plants was placed on Tl medium the raft produced 140 microtubers with 43 microtuber weighing more than 500 mg.

4.Discussion

The results of the banana experiments indicate that liquid medium provides a higher proliferation rate than semi-solid medium. This supports a number of observations (George, 1996; Watad et al; 1995, 1996). that liquid medium often results in faster rates of growth and multiplication than semi-solid medium.
The above results also suggest that a plant tissue culture mechanization system based on multiplication of bud or shoot clusters and using commercially available equipment can be applied to commercially relevant crops. This system, and parts of this system , are currently being tested in quite a few commercial laboratories, for a number of crops. These crops include *Aiitheriun-l, Apathiphyllum, Swgoniuni, Diffenbachia, African violets, Alstronieria* and others. Should these tests lead to the adoption of this procedure in these laboratories, it will be a first practical step in implementation of automation procedures for micropropagation.

References

Aitken-Christie, J. Automation. In: Debergh, P.C. and Zimmerman, R.H. (Eds.) Micropropagation, p. 363-388.
George, E.F, 1996. Plant propagation by tissue culture. Part 2, in practice .Exegetics, Ltd. Edington, Wilts., England.
Kurata, K., 1995. Automated systems for organogenesis. In: Atiken-Christie, J., Kozai, T. and Smith, M.A.L. (Eds.) Automation and environmental control in Plant Tissue Culture, p. 257-272. Kluver Academic Publishers, Dordrecht, Netherlands.
Levin, R & Vasil, 1.K., 1989. Progress in reducing the cost of micropropagation.

IAPTC Newsletter, 59, 2 - 12.
Vasil, 1.K., 1991. Rationale for the scale-up and automation of plant propagation. In:
Vasil, 1.K. (Ed.) Scale -up and automation in plant propagation (p. 1-6).Academic Press. New York, USA.
Vasil, 1.K., Automation of plant propagation. Plant cell, tissue and organ culture 39,105-108.
Watad, A.A., Kochba, M., Nissim, A. and Gaba, V., 1995. Improvement of *Acorntum fiapellus* micropropagation by liquid culture on floating membrane rafts. Plant Cell Reports 14, 345 - 348.
Watad, A.A., Ahroni, A., Zuker, A., Shejtmen, H., Nissim, A. and Vainstein, A., 1996.
Adventitious shoot formation from carnation stem segments: a comparison of different culture procedures. Scintia Horticulturae, 65, 313-320.
Ziv, M andHadar,A.,1991.Morphogenicpattern *ofNephrolepsisexalfacv.* Bostoniensis in agar or liquid cultures: implications for micropropagation. Isr.J..Bot. 40,7-16.
Ziv, M., Ronen, G. and Raviv M., 1996. Disposable presterilized bioreactors for large- scale micropropagation of plants. In vitro cellular & developmental biology (in press)

|Page1|Page2|

| Home | Introduction | Product Descriptions | Product Catalog|Instruction Manuals| Applications | Ptc System| Literature|Links|

E-Mail:Osmotek@netvision.net.il
To contact us: Tel: 972-8-931-5556,
Fax: 972-8-931-5177

CONTACT US