ROLE OF PHYTOHORMONES IN *IN VITRO* PROPAGATION OF PTERIDOPHYTES : A REVIEW

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***Abstract* :** Pteridophytes, the first land plants with vascular system are facing numerous threats including habitat degradation and climate fluctuations due to their specific microclimatic requirements. The increasing demand for pteridophytes as ornamental plants and medicinal resources has resulted in their depletion from natural ecosystems, highlighting the need for innovative conservation techniques such as *in vitro* propagation. This review consolidates existing research on the effects of phytohormones on pteridophyte propagation, providing insights into optimal hormone combinations and concentrations for successful in vitro culture. Additionally, it highlights the importance of considering species-specific variations and the influence of environmental factors in designing effective propagation protocols. Understanding the hormonal requirements and responses of pteridophytes during *in vitro* propagation is crucial for conservation efforts, sustainable utilization, and the establishment of large-scale production systems. Further research in this field can contribute to the preservation and commercialization of threatened pteridophyte species while reducing the pressure on their natural populations.

IndexTerms: Pteridophytes, conservation, phytohormones, *in vitro* propagation

INTRODUCTION

Pteridophytes constitute approximately 4% of the global floral diversity (Chapman, 2009). These plant taxa possess specific microclimatic requirements, rendering them highly vulnerable to habitat degradation, invasive species, and climate fluctuations (Arcand and Ranker, 2008). The aesthetic appeal of their foliage has spurred an escalating demand for pteridophytes as indoor ornamentals and floral embellishments, necessitating a continuous supply within the commercial ornamental sectors (Fernandez and Revilla, 2003). Furthermore, pteridophytes exhibit notable medicinal properties and are employed as edible resources in diverse regions (Liu *et al*., 2012). These combined factors have resulted in significant depletion of pteridophytes from their natural ecosystems, compelling the development of innovative techniques like *in vitro* propagation to expedite the production of these rare and sought-after species in substantial quantities.

Phytohormones, also known as plant hormones, are chemical substances produced naturally by plants that regulate various physiological processes. In the context of *in vitro* propagation, phytohormones play a crucial role in promoting the growth and development of plant tissues cultured in artificial environments. Auxins, such as indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), are commonly used in *in vitro* propagation to induce root formation in plant cuttings or to stimulate the initiation and multiplication of cells in tissue culture.

Cytokinins, such as kinetin and benzyladenine (BA), are used to promote shoot proliferation and the formation of multiple shoots from explants.

Gibberellins (GAs) are involved in regulating plant growth and development, including stem elongation, germination, and flowering. In *in vitro* propagation, GA_3 is often used to enhance seed germination, induce shoot elongation, and stimulate the development of certain plant structures.Ethylene and abscisic acid (ABA) are phytohormones associated with stress responses in plants. In *in vitro* propagation, ethylene can influence spore germination, cell division, and the development of prothalli in pteridophytes. ABA, on the other hand, plays a role in regulating seed dormancy, germination, and vegetative growth. It can affect antheridia formation and cell division in pteridophytes during in vitro culture.

In conclusion, phytohormones are essential tools in the field of *in vitro* propagation and by carefully selecting and applying specific phytohormones, researchers and plant propagators can optimize the success rate of *in vitro* propagation techniques and produce a large number of healthy plantlets for various purposes such as plant breeding, conservation, and commercial production.The life cycle of ferns encompasses distinct phases: a haploid gametophyte and a diploid sporophyte, both of which serve as explants for *in vitro* propagation. This comprehensive review critically assesses a range of investigations conducted on the *in vitro* propagation of ferns and related taxa, elucidating the differential impacts of five fundamental phytohormones (auxin, cytokinin, gibberellin, ethylene, and abscisic acid) on the growth and development of pteridophytes under controlled *in vitro* conditions.

1. *In vitro* propagation in pteridophytes

Pteridophyte *in vitro* propagation comprises two distinct methodologies: vegetative propagation and sexual propagation (Fernandez and Revilla, 2003). Sexual propagation involves the cultivation of spores obtained from selected pteridophyte species in a growth medium, leading to spore germination and the subsequent development of haploid gametophytes. These gametophytes produce archegonia and antheridia, which give rise to haploid gametes that eventually fuse, resulting in the formation of diploid sporophytes, thereby completing the sexual reproductive cycle.

Conversely, vegetative propagation entails the employment of both gametophyte and sporophyte explants to generate sporophytes, with the progeny being genetically identical to the parent plant. In gametophyte cultures, suitable gametophytes are cultivated *in vitro* and subsequently subjected to homogenization procedures such as grinding, blending, or enzymatic digestion. These processes facilitate the breakdown of gametophytic tissue into a uniform mass. Under meticulously regulated laboratory conditions, the homogenized gametophytes undergo proliferation and differentiation, leading to the emergence of specialized cell types, including rhizoids, protonemal filaments, and leafy gametophytes. These structures serve as foundational elements for the subsequent regeneration of new sporophytes.

Explant culture plays a pivotal role in pteridophyte *in vitro* propagation, wherein various types of explants such as leaf primordia, crozier, leaf blade, rhizome, scales, bulbils, and frond tips are employed. These explants are

cultured in a suitable growth medium, allowing them to produce callus, green globular Bodies, or aposporous gametophytes. These intermediary structures are then transferred to a growth medium supplemented with essential nutrients and plant growth regulators, facilitating the progression towards sporophyte development. Depending on the specific pteridophyte species and the *in vitro* conditions, the explants may also directly generate new shoots or roots (fig.1).

By employing these propagation techniques, pteridophytes can be efficiently multiplied and regenerated within controlled laboratory environments. This not only contributes to the conservation and preservation of rare or endangered fern species but also facilitates scientific investigations pertaining to their physiological and genetic attributes. Moreover, the comprehension and manipulation of growth conditions and regulators in the culture medium enable the induction of specific morphological or biochemical modifications in the propagated plants.

2.1 Sexual propagation

2.1.1. *In vitro* propagation using spores in pteridophytes

Spore culture has become the primary method for the commercial production of ferns due to its advantages in storage and transportation (Fay, 1994; Lindsay, 1994). Successful spore culture relies on various factors, including spore viability, appropriate culture medium selection, effective sterilization methods, optimal pH conditions, the use of plant growth regulators, and suitable light exposure (Kaur, 1991). Controlling the culture conditions enhances spore survival rates by preventing bacterial, fungal, and algal contamination (Debergh, 1994). To obtain spores for propagation, sporophylls are collected and placed in a paper bag at room temperature. After three to four days, the sporangia release the spores, which are then filtered to remove sporangial cells, sterilized, and sown in a suitable medium. Spore sterilization commonly involves the use of sodium hypochlorite, calcium hypochlorite, mercuric chloride, or hydrogen peroxide, with sodium hypochlorite being the most widely used sterilant due to its effectiveness in eliminating contaminants (Fernandez and Revilla, 2003). It is recommended to include a surfactant like Tween 20 and thoroughly rinse the spores with double distilled water to remove any remaining traces of the sterilant.

Different media have been employed for spore germination, such as Murashige and Skoog (MS medium), Knop (1865), Knudson (1946), Schenk and Hildebrandt (SH), and Parker & Thompson medium. The duration of germination varies among species, ranging from three days in *Nephrolepis falcata* to six months in *Ophioglossum pycnostichum* (Silverio, 2015; Whittier, 1981). In the process of spore culture, spores are cultured in a suitable nutrient medium with the necessary plant growth regulators (PGRs) until gametophyte formation occurs. Heterosporous species like *Selaginella* require a two-step approach, involving culturing megaspores for three weeks and then introducing surface-sterilized microspores for fertilization. However, the fertilization success rate for *Selaginella* species is only 12% (Yu et al., 2017a).

Herbarium-stored spores are also utilized for *in vitro* propagation of pteridophytes. Some species, like *Marsilea*, can maintain spore viability for up to a century, while *Pellea truncata* Godding can retain viability for around 50 years (Johnson, 1986; Windham *et al*., 1986). Nevertheless, the endeavour to propagate *Anogramma ascensionis* using herbarium-stored spores proved unsuccessful due to the loss of viability (Aragon and Pangua,

2004). The germination rate of eight-year-old spores from *Cheilanthes acrostica* and *Cosentinia vellea* was observed to be below 50%, attributed to the preservation treatments aimed at minimizing insect infestation (Magrini, 2011).

Taken together, these findings demonstrate that spore culture is a viable technique for the *in vitro* production of pteridophytes. It offers the advantage of generating a significant number of individual plants from a plentiful supply of spores obtained from a single sporophyll. By considering various factors and employing appropriate techniques, successful spore germination and the development of gametophytes and sporophytes can be achieved. However, it is essential to tailor the approach based on the specific requirements of different species and consider the viability limitations associated with herbarium-stored spores.

2.2 Vegetative propagation

2.2.1 Gametophyte culture

Vegetative propagation is a suitable technique for producing plants that are genetically identical to the parent plant. In nature, the survival of gametophytes (the reproductive structures of plants) depends on various environmental factors. However, in controlled *in vitro* culture, factors such as moisture, pH, medium composition, and plant growth regulators can be easily adjusted (Hotta and Osawa, 1958; Mohr, 1962; Kato, 1964; Bopp, 1968; Swami and Raghavan, 1980; Fernandez *et al*., 1996b, 1997a, 1997b; Bertrand *et al*.,1999b). The specific medium used for gametophyte culture varies depending on the species. For example, *Osmunda regalis* prefers a low-nutrient medium like Knop medium, while *Pteris ensiformis* and *Blechnum spicant* L. gametophytes thrive in a high-nutrient medium like MS medium (Fernandez and Revilla, 2003).

Homogenized cultures of gametophytes are particularly useful for species with short life cycles, as they can efficiently produce sporophytes. For instance, *Woodwardia virginica* has been successfully propagated through this method, with sporophytes obtained from gametophytes cultured for just two months (Fernandez *et al*., 1999a). It should be noted that re-homogenization of gametophytes produced through initial homogenization can inhibit sporophyte formation. This phenomenon has been observed in various studies and should be taken into consideration when applying vegetative propagation techniques.

2.2.2 Propagation using sporophyte plant parts

Propagation of pteridophytes through spore culture alone is not always reliable due to the limited availability of spores throughout the year. Consequently, alternative plant parts have been utilized for mass *in vitro* production. Various plant materials, such as gametophytes, leaf primordia, croziers, leaf blades, rhizomes, scales, and bulbils, have been commonly employed as explants for the *in vitro* propagation of pteridophytes (Wojciech *et al*., 2013). Vegetative propagation is often preferred in genera like *Lycopodium* and *Huperzia*, where spore germination and gametophyte development are time-consuming. In such cases, bulbils are frequently used as explants (Wojciech *et al*., 2013).

Leaf primordia have been utilized as explant materials for pteridophyte *in vitro* propagation since the early 1960s (Torres, 1989). The young circinate part of the foliage, before foliar expansion, is a commonly employed explant

for *in vitro* propagation. For example, in *Diplazium esculentum*, mature fronds were unsuccessful in producing callus or gametophytes, leading to the utilization of croziers as explants. The sterilized young croziers were grown in a medium supplemented with varying concentrations of cytokinin and auxin, resulting in successful propagation (Nair *et al.*, 2013).

In *Cyathea delgadii*, leaf stipes from five-month-old *in vitro* grown sporophytes were used as explants, leading to somatic embryogenesis and apospory (Mikula *et al*., 2015). *Platycerium bifurcatum* was propagated using 12 mm long juvenile leaves obtained from *in vitro* culture. In the presence of cytokinin, these leaves produced adventitious buds and rhizoids without the formation of callus. The adventitious buds were subsequently cultured in an auxin-containing medium for rooting (Camloha *et al*., 1994). The rhizome is a robust explant widely employed for fern propagation. It has been used as an explant in species such as *Marsilea quadrifolia* (Shekhawat and Manokari, 2015), *Polypodium cambricum* (Bertrand *et al.*, 1999), *Athyrium nidus*, and *Pteris ensiformis* (Fernandez *et al*., 1997b).

3. Phytohormones used in *in vitro* propagation of pteridophytes

Phytohormones are crucial molecules that act as internal and external stimuli in plants, playing a vital role in their growth and development. They can be categorized into five classes: auxin, abscisic acid, gibberellins, cytokinins, and ethylene. Among these, auxin and cytokinin are particularly important for the proper development of plant tissues and organs, while abscisic acid, gibberellins, and ethylene primarily have regulatory roles (Evans *et al*., 1981; Vasil and Thorpe, 1994). The study of phytohormones in pteridophytes began in the early 1900s. The first species in which the effect of a phytohormone, specifically ammonium 2,4-dichlorophenoxyacetate, was investigated, was *Pteris longifolia* (Strickler, 1946). In the context of *in vitro* propagation, both natural and synthetic forms of phytohormones are utilized. These hormones are added to the culture medium to provide the necessary signals for the growth and differentiation of plant tissues. Furthermore, the interactions between different phytohormones are complex and can have synergistic or antagonistic effects on plant growth. Manipulating the concentrations and ratios of phytohormones in the culture medium allows for precise control over the growth and development of plants *in vitro*. This knowledge has greatly contributed to the successful propagation and manipulation of ferns through tissue culture techniques.

3.1. Auxin

Auxin, the first plant growth regulator to be discovered, was initially described in Charles and Francis Darwin's book "The Power of Movement in Plants" in 1880. This influential work shed light on the role of auxin in promoting positive phototropism in grass coleoptiles (Darwin and Darwin, 1880). Since then, extensive research has been conducted to unravel the complex effects of auxin on various cellular processes such as cell division, differentiation, and elongation, although our understanding is still incomplete (Teale *et al*., 2006). In the realm of *in vitro* propagation, auxins are widely utilized to manipulate plant growth and development. Some commonly used auxins include indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 1-naphthaleneacetic acid (NAA). The specific impact of auxin on plants is highly dependent on the

type and concentration of the auxin employed (Teale *et al*., 2006). For instance, 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin, is frequently employed as a weedicide due to its ability to selectively control unwanted plant growth, underscoring the diverse effects of auxin across different plant species. Through careful manipulation of auxin levels and application methods, researchers and plant tissue culture practitioners can harness the potential of auxin to induce specific responses in plant tissues. This may include stimulating root development, promoting callus formation, or regulating the growth of shoot structures. Consequently, understanding the intricate interactions between auxin and other phytohormones is crucial for successful plant tissue culture and propagation.

In the development of *in vitro* propagation strategies for *Dipteris wallichii* and *Polypodium hesperium*, it was observed that IAA reduced spore germination percentage. However, when the germinated spores were cultured in a medium supplemented with IAA, the fresh weight of the prothalli significantly increased compared to the hormone-free medium (Singha *et al*., 2013; Haddad and Bayerly, 2014). In *Onoclea sensibilis* and *Pteridium aquilinum*, low concentrations of IAA promoted cell division in the protonema, resulting in its elongation. On the other hand, higher concentrations of IAA led to reduced cell division and a compact protonema (Miller, 1961; Sobota and Partanen, 1967). In *Lygodium japonicum*, IAA and 2,4-D induced cell division in the protonema, leading to the formation of a filamentous thallus. In *Ceratopteris richardii*, auxins such as NAA and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) at low concentrations activated the lateral meristem and caused the formation of marginal rhizoids (Swami and Raghavan, 1980; Gregorich and Fisher, 2006).

Auxin's role in rooting has also been extensively studied. In *Athyrium nidus*, root induction was successfully achieved in a medium supplemented with NAA, resulting in a 93% induction rate compared to the hormone-free medium. Low concentrations of NAA stimulated root induction and growth, but higher concentrations inhibited root induction and instead led to callus formation (Gupta, 1986; Vuylsteke, 1989; Haddad and Bayerly, 2014). Additionally, exogenous application of NAA and GA_3 (Gibberellin A_3) caused the formation of apogamous sporophytes in *Pteridium aquilinum* (Whittier, 1966). Auxin plays a crucial role in various aspects of plant growth and development, including root development, shoot growth, apical dominance, tropic responses, and organogenesis. It regulates gene expression, cell division, elongation, and differentiation, making it a vital tool in *in vitro* propagation techniques and plant tissue culture research.

3.2 Cytokinin

Cytokinins are a class of phytohormones known for their crucial role in promoting cell multiplication in root and shoot meristems, as well as their involvement in stress responses, leaf senescence, and chloroplast differentiation (Mok, 1994; Werner and Schmulling, 2009; Rolli *et al*., 2015). In the context of *in vitro* propagation, cytokinins serve as vital plant growth regulators, and several types are commonly used in micropropagation techniques, including kinetin, zeatin riboside (ZR), N6-2-isopentenyl adenine (2iP), thiadazuron (TDZ), diphenylurea, and 6-Benzylaminopurine (BAP).

Rolli *et al*. in 2015 investigated the effects of BAP, ZR, and 2iP on the *in vitro* propagation of *Marsilea quadrifolia* rhizomes. The researchers compared the growth of explants treated with various cytokinin

concentrations to those grown in a hormone-free medium. Interestingly, both BAP and ZR exhibited a significant inhibitory effect on node formation. As the concentrations and exposure duration of these two cytokinins increased, the number of nodes formed from the rhizome decreased noticeably. In contrast, 2iP had a positive impact, leading to an increased number of nodes formed as the supplemented concentrations and exposure period were raised. Bonomo *et al*. (2013) explored the influence of various concentrations of 2,4-D and BAP on the gametophytic morphogenesis of *Alsophila odonelliana*. The study revealed that 2,4-D negatively affected gametophyte growth, leading to a reduction in chlorophyll content, limited production of antheridia and gametangia, and deformation in the resulting cells. On the other hand, BAP positively influenced the filamentous stage, inducing numerous submarginal proliferations in the laminar stage. Eventually, gametophytes produced gametangia, although the cells were larger and appeared deformed.

In another experiment conducted by Ravi *et al*. in 2015, three-month-old gametophytes of *Pteris tripartita* were cultured in a half-strength MS medium supplemented with BAP or Kinetin at a concentration of 4 mg/L. The maximum number of sporophytes was induced under these conditions.

Cytokinins, with their diverse effects on plant growth and development, offer valuable tools for plant tissue culture and micropropagation. Their ability to influence cell division and differentiation makes them essential components for manipulating various aspects of plant tissue growth *in vitro*. By understanding the specific responses of different plant species to various cytokinins, researchers can tailor micropropagation techniques to suit the needs of individual plants, facilitating efficient and successful plant propagation for research, conservation, and agricultural applications.

3.3 Synergistic action of auxin and cytokinin

The successful *in vitro* propagation of plants relies on the synergistic action of auxin and cytokinin. While each phytohormone has specific effects on plant tissue growth, their combined interaction is essential for the comprehensive development of a fully functional plant. Cytokinins, when present at high concentrations, stimulate the growth of prothalli by activating RNA synthesis and regulating protein synthesis (Kulaeva, 1980; Haddad and Bayerly, 2014). However, at low concentrations, cytokinins can negatively impact cell division. In contrast, auxins are beneficial in low concentrations as they initiate and multiply cells, but higher concentrations of auxin can inhibit proper cell growth. This inhibitory effect may be attributed to the ability of auxins to induce high rates of DNA methylation, resulting in cell reprogramming (Lo Schiavo *et al*., 1989).

The interplay between auxin and cytokinin is crucial for shoot multiplication. Combining high levels of cytokinin with a low concentration of auxin has been found to be effective in promoting shoot growth (Peaud-Lenoel and Jouanneau, 1980). Studies on *Dipteris wallichii*, *Polypodium hesperium*, and *Athyrium nidus* have shown that these plants exhibited better growth rates when cultured in a medium supplemented with high kinetin (a cytokinin) and low IAA/IBA (auxins) compared to a hormone-free medium (Singha *et al*., 2013; Haddad and Bayerly, 2014). In the case of *Diplazium esculentum*, callus formation was observed when the medium was supplemented with either BAP (a cytokinin) or 2,4-D (an auxin) at specific concentrations. Interestingly, a higher concentration of 2,4-D induced root formation. However, simultaneous supplementation of both phytohormones

in the medium resulted in the induction of either roots or shoots, depending on the specific concentrations of each hormone (Nair *et al*., 2013).

The intricate balance between auxin and cytokinin is crucial at various stages of *in vitro* propagation, including shoot multiplication, root induction, and callus formation. By understanding and carefully manipulating this balance, researchers and plant tissue culture practitioners can optimize micropropagation techniques to produce healthy and robust plants for research, agriculture, and conservation purposes. The combined action of these phytohormones unlocks the full potential of *in vitro* propagation, enabling the efficient production of a diverse range of plants with desirable traits.

3.4 Gibberellins

Gibberellins, tetracyclic diterpene phytohormones, play a crucial role in the development of various organisms such as plants, fungi, algae, lichens, bryophytes, and bacteria. They are involved in promoting germination, stem elongation, transition to flowering, and seed development (Olszewski *et al.*, 2002; Sun and Gubler, 2004). The discovery of gibberellin can be attributed to Eiichi Kurosawa in 1926, who isolated it from the fungus *Gibberella fujikuroi*. Among the numerous types of gibberellins identified, GA_1 (Gibberellin A_1) and GA_4 (Gibberellin A_4) are the bioactive forms present in most plants (Olszewski *et al*., 2002).

Gibberellins exert diverse effects on plant growth and development. *Pteris tripartita* gametophytes treated with GA_3 in the MS medium displayed increased production of juvenile sporophytes at a concentration of 4 mg/L (Ravi *et al*., 2015). Spore germination in fern species like *Pteridium aquilinum*, *Blechnum spicant*, *Polystichum munitum*, and *Polypodium feei* was positively influenced by GA_3 (Fernandez *et al*., 1997; Weinberg and Voeller, 1969). However, the impact of GA_3 on *Polystichum aculeatum* spore germination was inhibitory, while no significant response was observed in *Sphenomeris chinensis* (Babenko *et al*., 2018; Ren *et al*., 2008). Gibberellins, including GA_3, GA_4, GA_7, and GA_{13}, enhanced the germination rates of *Anemia mexicana* and *Anemia phyllitidis* spores in the dark (Nester and Coolbaugh, 1986). It was proposed that gibberellins are activated when spores are exposed to red light, leading to improved germination rates (Kagawa and Michizo, 1991).

The effects of GA_3 on gametophytes varied among different species. *B. spicant* gametophytes exhibited hindered development and the absence of sporophyte formation when exposed to GA_3 (Fernandez *et al.*, 1997). High concentrations of GA_3 led to reduced cell division rates and smaller thalli in *A. phyllitidis* and *Osmundastrum cinnamomeum* (Kazmierczak, 1998, 2003; Hollingsworth *et al*., 2012). GA_3 also influenced thallus shape in *P. aculeatum*, causing the formation of small elongated thalli (Babenko *et al*., 2018). Additionally, gibberellins were found to activate early antheridia formation in gametophytes (Fernandez *et al*., 1997; Kazmierczak, 1998, 2003; Menendez *et al*., 2006a). GA_5 and GA_8 were identified as the most active gibberellins involved in antheridia formation in *A. phyllitidis*, while GA_4, GA_7, and GA_1 exhibited lower activity levels (Schraudolf, 1964; Kazmierczak, 1998, 2003). Studies on *Lygodium japonicum* demonstrated that GA_4, GA_7, GA_9, and GA_3 acted as effective stimulants for antheridia development (Takeno and Furuya, 1977).

The complex actions of gibberellins highlight their significance in regulating various developmental processes in plants. They exhibit species-specific effects, influencing factors such as germination, growth, differentiation, and antheridia formation. Understanding the intricate mechanisms underlying the actions of gibberellins is crucial for unravelling the complexities of plant growth and development.

3.5. Ethylene and Abscisic acid

Ethylene and Abscisic acid are two significant phytohormones involved in plant growth and development, particularly in response to various stresses. Ethylene, a gaseous plant hormone, exhibits dual effects on plant growth, depending on its concentration, plant age, and species. In the context of *in vitro* propagation of pteridophytes, research from the 1970s shed light on the effects of ethylene.

Investigations on *Osmunda sensibilis* demonstrated that ethylene inhibited spore germination in the absence of light but promoted germination under illumination and CO_2 treatment, indicating an antagonistic relationship between ethylene and CO_2 (Edwards and Miller, 1972; Fisher and Miller, 1975; Edwards, 1977; Miller et al., 1970). Additionally, ethylene was found to suppress cell division while promoting cell elongation during prothallus development, resulting in elongated prothalli (Edwards and Miller, 1972). *Pteridium aquilinum* exhibited ethylene-induced apogamy, further illustrating the hormone's influence (Elmore and Whittier, 1973). The effects of ethylene producer 2-chloroethylphosphoric acid varied depending on spore maturity in *Ceratopteris richardii*, inhibiting the growth of long-stored and matured spores but promoting the growth of immature and fresh spores (Hickok *et al*., 1987).

Another significant phytohormone, Abscisic acid (ABA), plays a vital role in seed dormancy, germination, and vegetative plant growth. In *C. richardii*, ABA was found to suppress antheridia formation even in the presence of antheridiogens, showcasing its regulatory role (Banks, 1999; Hickock, 1983). *L. japonicum* displayed inhibited spore germination in the presence of ABA at a concentration of 5 mg/L, while *Mohria caffrorum* and *Matteuccia struthiopteris* exhibited negligible responses to ABA (Swami and Raghavan, 1980; Chia and Raghavan, 1982; Jarvis and Wilkins, 1973). Higher concentrations of ABA resulted in reduced cell division and deformation of thalli from their normal heart-like structure to flat elongated forms in *L. japonicum* and *C. richardii* (Swami and Raghavan, 1980; Hickok, 1983). ABA also induced rhizoid formation at lower concentrations and had varying effects on antheridia development depending on its concentration (Hickok, 1983).

Ethylene and ABA play crucial roles in regulating various aspects of plant growth and development. Ethylene can have contrasting effects on plant growth depending on its concentration and the specific plant species, influencing processes such as seed germination, cell division, and prothallus development. ABA, on the other hand, is involved in seed dormancy, germination, and thallus development, affecting antheridia formation and cell division in pteridophytes. The intricate actions of these phytohormones contribute to the ability of plants to adapt and survive under challenging environmental conditions.

CONCLUSION

The decline in pteridophyte species due to factors like forest encroachment, human activities, and changing climate has put many of them at risk. Additionally, the demand for these plants for medicinal and ornamental purposes has led to their eradication from their natural habitats. Consequently, there is a growing need to develop *in vitro* propagation techniques for pteridophytes. While spores are commonly used for this purpose because of their easy storage, their availability is limited throughout the year. As a result, there is a rising interest in exploring other explant materials for propagation.

In this review, we have examined various studies conducted over the last two decades that focus on *in vitro* propagation of 32 species of ferns and fern-allies. These studies have also investigated the effects of phytohormones on pteridophyte *in vitro* propagation. The most widely phytohormones for the *in vitro* propagation include auxin, cytokinin and gibberellins. A careful combination of auxin and cytokinin has proven effective in establishing *in vitro* protocols for many pteridophytes. Using a medium supplemented with low auxin concentration and high cytokinin concentration has shown to enhance the proliferation rate in most species. The ratio of auxin to cytokinin in the medium determines whether the explant will induce gametophore/bud growth, callus formation, root development, shoot growth, or both root and shoot growth. Additionally, gibberellins have been found to promote spore germination in most species. However, there is limited research on the influence of ethylene and ABA, and understanding their role is crucial for developing new *in vitro* propagation methods to conserve threatened pteridophyte species in their natural environments.

REFERENCES

Aragon CF, Pangua E 2004. Spore viability under different storage conditions in four *Rupicolous Asplenium* L. Taxa. Am. Fern. J. 94(1):28-38.

Arcand N, Ranker T 2008. Conservation Biology In: Biology and Evolution of Ferns and Lycophytes. Cambridge Academic Press.

Babenko LM, Romanenko KO, Shcherbatiuk MM, Vasheka OV, Romanenko PO, Negretsky VA, Kosakivska IV (2018). Effects of exogenous phytohormones on spore germination and morphogenesis of *Polystichum aculeatum* (L.) Roth gametophyte *in vitro*culture. Cytol. Genet. 52(2):117-126.

Banks JA 1999. Gametophyte development in ferns. Annu Rev Plant Physiol Plant Mol Biol 50:163-186.

Bertrand AM, Sierra MI, Snchez-Tames R. 1999. An apolar GA-like compoundresponsible for the antheridiogen activity in *Blechnum spicant*. Plant Growth Regul 28(2):143-144.

Bonomo MC, Martinez OG, Tanco ME, Avilles Z. 2013. Spores' germination and gametophytes of *Alsophila odonelliana* (Cyatheaceae) in different sterile media. Phyton(B Aires) 82(1):119-126.

Bopp M 1968. Control of differentiation in fern allies and bryophytes. Annu. Rev. Plant Physiol. 19(1):361-380.

Camloha M, Gogala N, Rode J 1994. Plant regeneration from leaf explants of the fern *Platycerium bifurcatum in vitro*. Sci Hortic 56(3):257-266.

Chia S-GE, Raghavan V 1982. Abscisic acid effects on spore germination andprotonemal growth in the fern, *Mohria caffrorum*. New Phytol 92(1):31-37.

Darwin C, Darwin F 1880. The power of movement in plants: John Murray.

Debergh P 1994. *In vitro* culture of ornamentals. In: IK Vasil, Thorpe TA, eds. Plant cell and tissue culture: Dordrecht: Springer.

Edwards ME 1977. Carbon dioxide and ethylene control of spore germination in *Onoclea sensibilis* L. Plant Physiol 59(4):756-758.

Edwards ME, Miller JH .1972. Growth regulation by ethylene in fern gametophytes. III.Inhibition of spore germination. Am J Bot 59(5):458-465.

Elmore HW, Whittier DP 1973. The role of ethylene in the induction of apogamous buds in Pteridium gametophytes. *Planta, 111*(1), 85-90..

Evans DA, Sharp WR, Flick CE. 1981. Growth and behavior of cell cultures: Embryogenesis and organogenesis. In: TA Thorpe, ed., Plant cell culture: Methods and applications in agriculture (pp. 45-113): Academic Press.

Fay MF 1994. In what situations is *in vitro* culture appropriate to plant conservations? In: Biodivers Conserv 3(2):176-183.

Fernandez H, Bertrand AM, Sanchez-Tames R 1996b. Micropropagation and phase change in *Blechnum spicant* and *Pteris ensiformis*. Plant Cell Tiss Org Cult 44:261-265.

Fernandez H, Bertrand AM, Sanchez-Tames R 1997b. Plantlet regeneration in *Asplenium nidus* L. and *Pteris ensiformis* L. by homogenization of BA treated rhizomes.Sci Hortic 68(1-4):243-247.

Fernandez H, Bertrand AM, Sanchez-Tames R 1997a. Gemmation in culturedgametophytes of *Osmunda regalis*. Plant Cell Rep 16(5):358-362.

Fernandez H, Revilla MA (2003) *In vitro* culture of ornamental ferns. Plant CellTissue Organ Cult 73(1):1-13

Fernandez H, Bertrand AM, Sanchez-Tames R 1999a. Biological and nutritional aspects involved in fern multiplication. Plant Cell Tissue Organ Cult 56(3):211-214.

Fernandez H, Bertrand AM, Feito I, Sanchez-Tames R 1997. Gametophyte culture *in vitro* and antheridiogen activity in. Plant Cell Tissue Organ Cult 50(1):71-74.

Fisher RW, Miller JH 1975. Growth regulation by ethylene in fern gametophytes. IV. involvement of photosynthesis in overcoming ethylene inhibition of spore germination.Am J Bot 62(10):1104-1111.

Gregorich M, Fisher R 2006. Auxin regulates lateral meristem activation in developing gametophytes of *Ceratopteris richardii*. Can J Bot 84(10):1520-1530.

Gupta PP 1986. Eradication of mosaic disease and rapid clonal multiplication of bananas and plantains through meristem tip culture. Plant Cell Tiss Organ Cult 6(1):33-39.

Haddad S, Bayerly R 2014. *In vitro* propagation of ferns (*Asplenium nidus*) via spore culture. Jordan J Agric Sci 10(1):144-153.

Hickok LG 1983. Abscisic acid blocks antheridiogen-induced antheridium formation ingametophytes of the fern *Ceratopteris*. Can J Bot 61(3):888-892.

Hickok LG, Warne TR, Slocum MK 1987. *Ceratopteris richardii*: Applications for experimental plant biology. Am J Bot 74(8):1304-1316.

Hollingsworth SN, Andres EA, Greer GK 2012. Pheromonal interactions among gametophytes of *Osmundastrum cinnamomeum* and the origins of antheridiogen systemsin leptosporangiate ferns. Int J Plant Sci 173(4):382-390.

Jarvis SJ, Wilkins MB 1973. Photoresponses of *Matteuccia struihiopteris* (L.) Todaro.I. Germination. J Exp Bot 24:1149-1157.

Johnson DM 1986. Systematics of the New World species of *Marsilea* (Marsileaceae). Syst Bot Monogr 11:1-87.

Kato Y 1964. Physiological and morphogenetic studies of fern gametophytes in asepticculture II. One and two-dimensional growth in sugar media. Botanical Gazette 125(1):33-37.

Kaur S 1991. Ferns and fern allies. Their domestication and Conservation. In: TN Bharadwaj, Gena CB, eds. Perspectives in pteridology: Present and future. Today & tomorrow's printers and publishers, New Delhi (India).

Kazmierczak A 1998. Studies on morphology and metabolism of prothalli during GA3-induced formation of antheridia in *Anemia phyllitidis*. *Acta Physiol. Plant* 20(3):277-283.

Kazmierczak A 2003. Induction of cell division and cell expansion at the beginning of gibberellin A3-induced precocious antheridia formation in *Anemia phyllitidis* gametophytes. *Plant Sci*. 165(5):933-939.

Kulaeva ON 1980. Cytokinin action on enzyme activities in plants. In: *Proceedings in Life Science*. Proceedings of the in life sciences F Skoog, ed.:119-128.

Kurosawa E 1926. Experimental studies on the nature of the substance secreted by the"bakanae" fungus. N Atl Hist Soc Formosa 16:213-227.

Lindsay J 1994. Account of germination and raising of ferns from the seed. Trans Linn Soc Lond 2:93-100.

Liu Y, Wujisguleng W, Long C 2012. Food uses of ferns in China: A review. ActaSoc Bot Pol 81(4):263-270.

Lo Schiavo F, Pitto L, Giuljano G, Torti G, Nuti-Ronchi V, Marazziti D, Vergara R, Orsele S, Tereze M 1989. DNA methylation of embryogenic carrot cell cultures and itsvariations as caused by mutation, differentiation, hormones and hypomethylating drugs. *Theor Appl Genet*. 77(3):325-331.

Magrini S 2011. Herbaria as useful spore banks for integrated conservation strategies ofpteridophytic diversity. Plant Biosyst An Int J Dealing Aspects Plant Biol 145(3):635- 637.

Menendez V, Revilla MA, Bernard P, Gotor V, Fernandez H. 2006a. Gibberellins andantheridiogen on sex in *Blechnum spicant* L. Plant Cell Rep 25(10):1104-1110.
Mikula A, Pozoga M, Grzyb M, Rybczynski JJ. 2015. An unique system of somatic embryogenesis in the tree fern *Cyathea delgadii* Sternb.: The importance of explant type,and physical and chemical factors. Plant Cell Tiss Organ Cult 123(3):467-478.

Miller JH.1961. The effect of auxin and guanine on cell expansion and cell division inthe gametophyte of the fern, *Onoclea sensibilis*. Am J Bot 48(9):816-819.

Miller PM, Sweet HC, Miller JH 1970. Growth regulation by ethylene in fern gametophytes. I. Effects on protonemal and rhizoidal growth and interaction with auxin.Am J Bot 57(2):212-217.

Mohr H 1962. The influence of visible radiation on the germination of archegoniatespores and the growth of the fern protonema. J Linn Soc Bot 58:287-296.

Mok MC 1994. Cytokinin and plant development—An overview. In: Mok DWS, Mokeds MC, eds., Cytokinin: Chemistry: Boca Raton: Activity andFunction, CRC Press, 155-166.

Nair AG, Pradeesh S, Nikhila GS, Sangeetha G, Mini I, Swapna TS. 2013. *In vitro*propagation of a rare medicinal fern of Western Ghats--*Diplazium esculentum* (Reytz.) Indian J Exp Biol 51(11):919-923.
Nester JE, Coolbaugh RC 1986. Factors influencing spore germination and earlygametophyte development in *Anemia mexicana* and *Anemia phyllitidis*. Plant Physiol 82(1):230-235.

Olszewski N, Sun TP, Gubler F 2002. Gibberellin signaling: Biosynthesis, catabolism,and response pathways. Plant Cell 14(suppl.): S61-S80.

Peaud-Lenoel C, Jouanneau JP. 1980. Presence and possible functions of cytokinins inRNA. In: Skoog F, ed. Plant growth Substances 1979. Proceedings of the in life sciences: Berlin, Heidelberg: Springer

Ravi BX, Varuvel GVA, kilimas R, Robert J 2015. Apogamous sporophyte development through spore reproduction of a South Asia's critically endangered fern:Pteris tripartita Sw. Asian Pac J Reprod 4(2):135-139.

Ren BR, Xia B, Li WL, Wei-Lin, WU Ju-Lan, ZHAO You-Yi 2008. Investigation on spore germination of *Sphenomeris chinensis* (Lindsaeaceae). Acta Bot Yunnanica 30(6):713-717.

Rolli E, Brunoni F, Marieschi M, Torelli A, Ricci A 2015. *In vitro* micropropagation ofthe aquatic fern *Marsilea quadrifolia* L. and genetic stability assessment by RAPD markers. Plant Biosyst An Int J Dealing Aspects Plant Biol 149(1):7-14.

Schraudolf H 1964. Relative activity of gibberellins in antheridium induction in *Anemia phyllitidis*. Nature 201(4914):98-99.

Shekhawat MS, Manokari M 2015. Direct organogenesis from rhizome explants in*Marsilea quadrifolia* L.: A threatened fern species. Adv Biol 2015:1-6.

Singha KB, Choudhury D, Manabendra, Mazumdar PB 2013. *In vitro* propagation ofDipteris wallichii (R.Br.)

T. Moore: A hope for conservation of an endangered pteridophyte. Int Res J Pharm 4(3):215-219.

Sobota AE, Partanen CR 1967.The growth and division of cells in relation to morphogenesis in fern gametophytes: II. The effect of biochemical agents on the growthand development of *Pteridium aquilinum*. Can J Bot 45(5):595-603.

Strickler B 1946. Responses of *Pteris longifolia* to applications of ammonium 2,4-dichlorophenoxyacetate. Botanical Gazette 108(1):101-114.

Sun TP, Gubler F 2004. Molecular mechanism of gibberellin signaling in plants. Annu Rev Plant Biol 55:197-223.

Swami P, Raghavan V 1980. Control of morphogenesis in the gametophyte of a fern bylight and growth hormones. Can J Bot 58(13):1464-1473.

Takeno K, Furuya M 1977. Inhibitory effect of gibberellins on archegonial differentiation in *Lygodium japonicum*. Physiol Plant *39*(2):135-138.

Teale WD, Paponov IA, Palme K 2006. Auxin in action: Signalling, transport and thecontrol of plant growth and development. Nat Rev Mol Cell Biol *7*(11):847-859.

Torres KC 1989. Application of tissue culture techniques to horticultural crops. In: Tissue Culture Techniques for Horticultural Crops. Springer, Boston, MA.

Vasil IK, Thorpe TA 1994. Plant cell and tissue culture: Dordrecht: Kluwer Academic.

Vuylsteke D 1989. Shoot-tip culture for the propagation, conservation, and exchange of Musa Germplasm: Practical Manuals for Handling Crop Germplasm *in vitro* 2: International Board for Plant Genetic Resources.

Weinberg ES, Voeller BR 1969. External factors inducing germination of fern spores. Am Fern J 59(4):153-167.

Werner T, Schmulling T 2009. Cytokinin action in plant development. Curr Opin PlantBiol 12(5):527-538.

Whittier DP 1966. The influence of growth substances on the induction of apogamy in*Pteridium* gametophytes. Am J Bot 53(9):882-886.

Whittier P 1981. Spore germination and young gametophyte development of*Botrychium* and *Ophioglossum* in axenic culture. Am Fern J 71(1):13.

Windham MD, Wolf PG, Ranker TA.1986 Factors affecting prolonged spore viabilityin herbarium collections of three species of *Pellaea*. Am Fern J 76(3):141.

Wojciech J. Szypuła, Paulina Mistrzak, Olga Olszowska (2013).A new and fast method to obtain in vitro cultures of Huperzia selago (Huperziaceae) sporophytes, a club moss which is a source of huperzine A. Acta Soc Bot Pol 82(4):313–320.

Yu R, Baniaga AE, Jorgensen SA, Barker MS (2017a) A successful *in vitro* propagation technique for resurrection plants of the Selaginellaceae. Am Fern J 107(2):96-104.

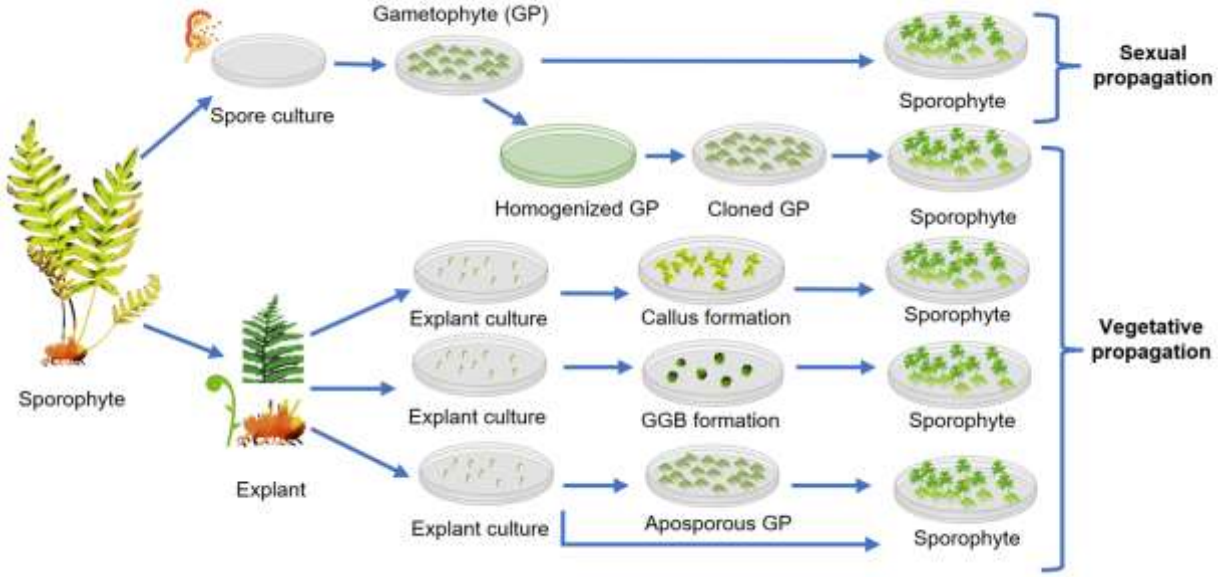

Figure 1: *In vitro* propagation in pteridophytes

Abbreviation: GP – Gametophyte, GGB – Green Globular Body