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Prothallia development of two fern species: Cyrtomium falcatum and Nephrolepis sp.

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1. Abstract

Ferns or Pteridophytes are considered the most diverse and widely distributed group of spore-bearing plants. They serve as an important evolutionary link between bryophytes and seed plants with a conserved life cycle defined by an independent alternation of generations. While the sporophyte is the dominant phase, the haploid gametophyte and its development, consisting of a series of morphological events, is an essential model for studying cell differentiation and plant development. Through a series of photographs, this thesis documents the successive developmental stages of the gametophytes of two homosporous fern species, *Cyrtomium falcatum* and *Nephrolepis* sp., identifying both conserved patterns and morphological divergences.

The results confirm a highly conserved developmental pathway where both species exhibited spore germination approximately 6 days after propagation and reached maturity as cordate (heart-shaped) prothalli within 30 to 40 days. However, distinct morphological differences in early development were captured. While *C. falcatum* followed a linear growth pattern, *Nephrolepis* sp**.** displayed a tendency for spore clustering, interpreted as a potential reflection of the genus's strategy for colonization of habitats by vegetative stolons seen in the sporophytic generation.

Furthermore, the thesis addresses the phenomenon of apomixis (specifically apogamy), the absence of documented antheridia and archegonia suggests a potential shift toward asexual sporophyte development triggered by *in vitro* conditions. These findings provide a detailed morphological foundation for these taxa and underscore how microscopic traits may mirror broader ecological adaptations, offering a basis for future research into apomictic mechanisms.

1. Zusammenfassung

Farne (Pteridophyta) stellen ein wichtiges evolutionäres Bindeglied zwischen Moosen und Samenpflanzen dar und zeichnen sich durch einen unabhängigen Generationswechsel aus. Während der Sporophyt die dominante Phase bildet, ist der haploide Gametophyt mit seiner durch morphologische Ereignisse geprägten Entwicklung ein essentielles Modell für die Untersuchung von Zelldifferenzierung und Pflanzenentwicklung. Die vorliegende Arbeit dokumentiert anhand einer fotografischen Serie die aufeinanderfolgenden Entwicklungsstadien der Gametophyten zweier homosporer Farnarten, *Cyrtomium falcatum* und *Nephrolepis* sp., um sowohl konservierte Muster als auch morphologische Divergenzen zu identifizieren.

Die Ergebnisse bestätigen einen hochgradig konservierten Entwicklungsweg: Bei beiden Arten setzte die Sporenkeimung etwa 6 Tage nach der Aussaat ein, und die Reife als cordates (herzförmiges) Prothallium wurde innerhalb von 30 bis 40 Tagen erreicht. Dennoch wurden deutliche morphologische Unterschiede in der frühen Entwicklung festgestellt. Während *C. falcatum* einem linearen Wachstumsmodell folgte, zeigte *Nephrolepis* sp. eine Tendenz zur Clusterbildung der Sporen. Dies wird als potenzielle mikroskopische Spiegelung der ökologischen Strategie der Gattung zur dichten Kolonisation interpretiert, die parallel zu der in der Sporophyten-Generation beobachteten vegetativen Ausbreitung durch Stolonen verläuft.

Darüber hinaus befasst sich die Arbeit mit dem Phänomen der Apomixis (insbesondere der Apogamie). Das Fehlen dokumentierter Antheridien und Archegonien deutet auf eine potenzielle Verschiebung hin zu einer asexuellen Sporophytenentwicklung hin, die durch *In-vitro*-Bedingungen ausgelöst wurde. Diese Erkenntnisse liefern eine detaillierte morphologische Grundlage für diese Taxa und unterstreichen, wie mikroskopische Merkmale breitere ökologische Anpassungen widerspiegeln können, was eine Basis für zukünftige Forschungen zu apomiktischen Mechanismen bietet.

2. Introduction

2.1. Ferns in general

Ferns, traditionally grouped as Pteridophytes, represent one of the most ancient and successful lineages of vascular plants on Earth, being the largest group of vascular plants after the angiosperms and comprising more than 300 genera and around 10 000 to 12 000 different species (PPG I, 2016; Smith et al., 2006). They can be found in almost all climates and environments as pioneers in the terrestrial life which helped shape the planet's ecosystem (Kessler, 2010). Their fossil records date back 380 million years, meaning they originated all the way back in the Devonian Period. Plants present during this time period started to spread out in different ecosystems and use up the CO_2, which is an atmospheric gas responsible for high atmospheric temperatures at the time. Since plants started to use it up, the CO_2 no longer made up the majority of the atmosphere, and so the planet started to cool down which allowed plants to develop larger forms without the consequence of overheating (Algeo et al., 1995). However, in order to maintain these larger growth forms, plants had to develop a system that would help them transport water and nutrients throughout their whole body. This was achieved with the vascular system and ferns are among the earliest plants to develop it, allowing an efficient option for transport of water and nutrients. The vascular system is also considered an anatomical adaptation for the support of the larger plant body since it's made out of a central vascular strand or stele (Friedman and Cook, 2000). For smaller plants, this was done by turgor which supported them in an upright position; however, in an evolutionary view, the vascular system is considered more successful. The stele is made out of xylem-conducting cells called tracheids. Their walls have lignin thickenings for mechanical support in transporting water up the stem. Along the xylem, another important component of stele is the phloem, consisting of cells without thickened cell walls, whose main function is transporting photosynthetic products, e.g. sugars. This efficient water and nutrient transport resulted in the development of larger growth forms of plants, including ferns (Duriex et al., 2021; Stein et al., 2007).

Another adaptation to the land life included an outer cuticle protecting the plant from dehydration and pathogen attacks. However, plants having to uptake atmospheric gases (CO_2 and O_2) for photosynthesis and respiration as well as for water vapour release, resulted in the development of stomata. These are pores surrounded by guard cells which control their opening and closing (Edwards et al., 1998). However, not only the leafy sporophytes of ferns developed adaptations to land life. Their spores are also protected from dehydration and high temperatures by a durable outer layer known as sporopollenin, which is also present in pollen grains (Wellman, 2004). These adaptations enabled ferns to take on a pioneering role in colonizing and shaping the terrestrial environment as we know it today.

Actually, the most ancient fernlike group of plants, the Cladoxylopsida, also called preferns, appeared in the middle Devonian. Some species of this group even had a treelike form with a vascular system made out of protosteles, which is a simpler version of stele than in present day ferns. Later on, from the Devonian to Permian period, the Coenopterid ferns evolved (Stein et al. 2007). Meanwhile, the leptosporangiate ferns (Polypodiopsida) which we know today, also referred to as "true" ferns, evolved through three key periods of diversification:

Paleozoic radiation, followed by the Late Permian to Early Mesozoic radiation, and then Late Cretaceous to Paleogene radiation which resulted in the diversity that is seen today (Schneider et al., 2004).

Phylogenetically, ferns hold an interesting position. While the traditional Pteridophyta is recognized as a paraphyletic group for all seedless vascular plants, modern phylogenetic analysis place ferns to be the sister group to seed plants (Spermatophytes) (Pryer et al., 2001). On the other hand, Lycophytes which are also a major lineage of seedless vascular plants, are considered to be the sister group to all other vascular plants, so along with ferns and seed plants they are grouped under the clade Euphyllophytes (Pryer et al., 2004). Because of this evolutionary relationship, ferns are important in understanding the diversification of plants. Since they have the characteristics of both major groups, studying them allows the understanding of transition from seedless, spore producing plants to seed plants. Based on molecular and morphological data, the broader clade of ferns is considered monophyletic (Pryer et al., 2001). However, some 'internal' relationships, such as the Equisetidae placement, still remain subject of the debate. (Christenhusz and Chase, 2014).

Therefore, the evolutionary history of ferns shows a progression of adaptations to terrestrial life resulting in the development of certain features such as vascular tissue and megaphylls. Their phylogenetic placement as the sister group to seed plants makes them important in understanding the history of all vascular plants in general (Pryer et al., 2001). This evolutionary journey is represented nicely in the fern life cycle.

2.2. Fern life cycle

Just like all plants, ferns exhibit an alternation of generations, illustrated in Figure 1. This life cycle is characterized by two distinct, multicellular stages: the diploid sporophyte and the haploid gametophyte. These two stages are morphologically and, most of the time, chromosomally different. Therefore, ferns' life involves constant cyclical shifts between sexual reproduction in gametophytes and asexual reproduction in sporophytes, which are linked by meiosis and fertilization (Banks, 1999; Geng et al., 2022). However, what actually distinguishes ferns from the seed plants is the independence of the sporophyte and the gametophyte generations . This sets ferns apart from other lineages. While bryophytes, for example, also reproduce by spores, their life cycle is characterized by the dominant gametophytic phase, while the sporophyte generation is reduced and physically and nutritionally dependent on the gametophyte (Gilbert, 2000). On the contrary, seed plants actually have reduced gametophytes entirely dependent on their sporophytes. Therefore, ferns represent an evolutionary bridge between these two lineages. They show a life cycle balanced between these two, the gametophyte-dominant one as seen in bryophytes and sporophyte dominant one in seed plants. In ferns, both of the generations become physiologically independent at maturity (Krieg and Chambers, 2022). Initially, the young sporophyte is nutritionally dependent on the gametophyte from which it's growing and developing (Racusen, 2002). Eventually, however, both generations mature into free-living individuals. This physiological independence at maturity is a defining feature that distinguishes them from bryophytes and allows the two different morphological forms of ferns to explore and settle in different environments, which is especially highlighted by the fern's production of minute spores that can travel far from the parent plant (Krieg and Chambers, 2022). However, it has been observed that sporophytes do share the same environmental requirements as their gametophytes. Essentially, they will succeed in a certain environment only if the haploid phase is able to endure long enough in a certain niche (Pinson et al., 2017). Ferns type of life cycle also allows for certain reproductive processes such as apogamy and apospory, where one of the generations skips meiosis and develops without the other (Grusz, 2016). This developmental adaptability of ferns highlights why they are so important in understanding the diversification of plants as a whole.

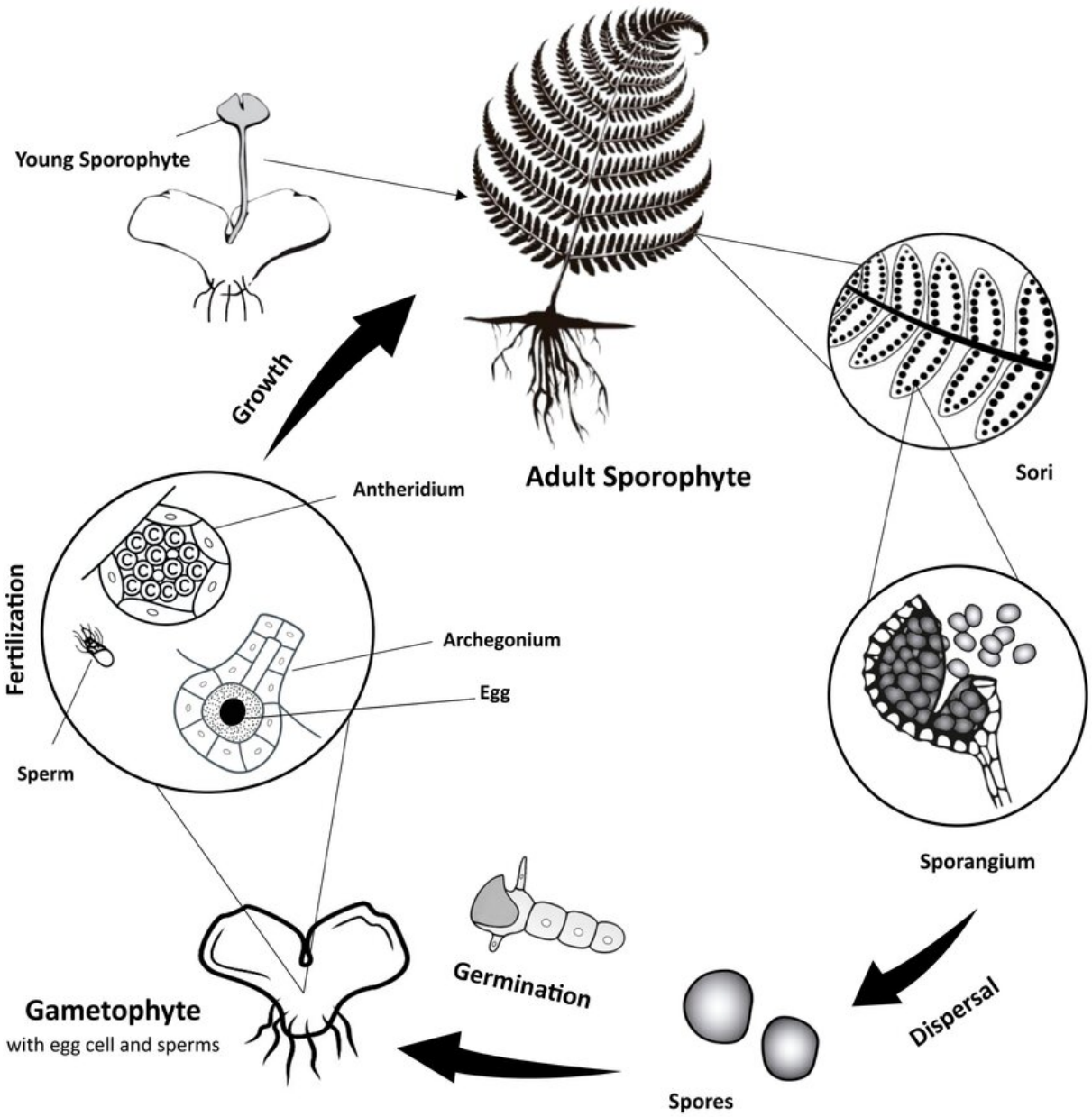

Figure 1. Illustration of the fern life cycle alternating between the two generations, sporophyte and gametophyte. Reprinted from "Survival below zero: Overlooked aspects of freezing-tolerance in photosynthetic fern tissues" by Firoozi et al. 2025, *Plant Ecophysiology*, *1*(1).

The familiar and conspicuous leafy fern plant represents the sporophyte generation. This diploid and generally longer lived stage of the ferns fascinating life cycle consists of true roots, a stem which is often an underground or creeping rhizome, and leaves called fronds (a ferns specific term) that make up the most noticeable part of the fern plant. Fern roots are usually thin but structurally considered "true" roots similar to the ones in seed plants. The rhizomes can be long and creeping, climbing or erect and are often used by the plant for vegetative or asexual reproduction allowing to spread horizontally while forming extensive colonies. Ferns have highly divided "true" leaves or macrophylls. A frond, as visible in Figure 2, is comprised of a stalk also called a petiol or stipe and the photosynthetic blade or lamina. The lamina is usually divided into primary segments called pinnae which are arranged along the central axis or rachis. These pinnae can then be further divided into smaller pinnules and sometimes even into tertiary and quaternary segments.

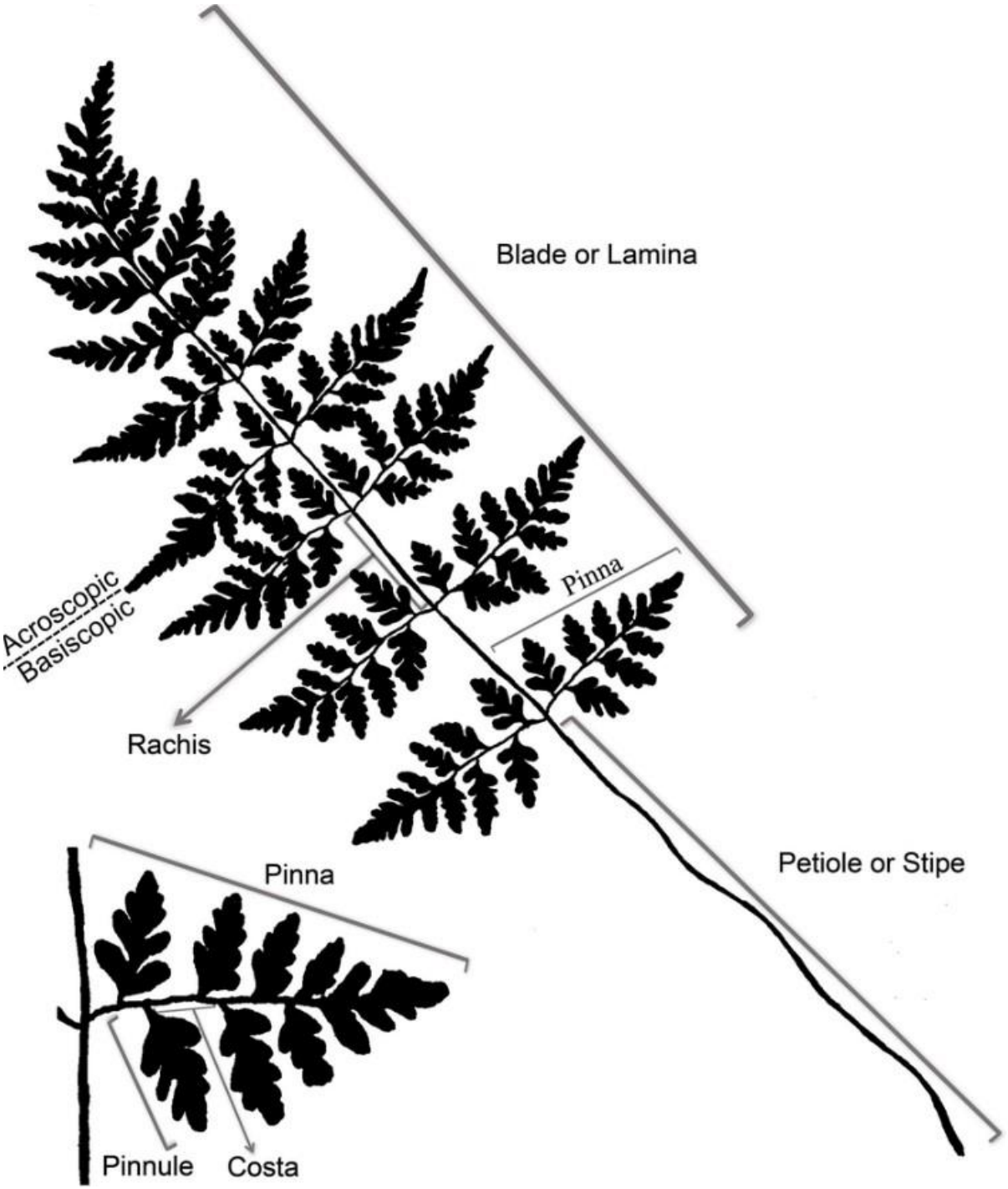

Figure 2: Structure of a typical fern macrophyll or frond. Reprinted from "The evolution, morphology, and development of fern leaves" by Vasco et al. 2013. *Frontiers in Plant Science, 4*.

In many fern species, fronds can be morphologically differentiated into fertile or spore producing and sterile or non-spore producing. Depending on this differentiation, they may exhibit some differences in their form. The fertile fronds bear sori which are just clusters of spore producing sporangia. Their positioning, whether marginal, along the veins (medial) or abaxial, is an important taxonomic character (Moran, 2004). Usually ocated on the abaxial or underside of pinnae, these sori are often protected by a membranous or scale-like outgrowth of the frond tissue shielding them from desiccation and mechanical damage, known as indusium. The presence, shape and structure of the indusium are common features used in fern classification. Other than the presence or absence of an indusium, along

with its specific shape and attachment which can be reniform, peltate, etc. is a key diagnostic feature in fern classification. Furhtermore, distinct sori shapes and sizes such as lines, dots or bean shaped clusters, as well as the previously mentioned pattern of frond division, are also considered important identifiers for distinguishing in between and classifying fern families (Chrustenhusz and Chase, 2014).

Fundamental classification of ferns is actually based on their mode of sporangial development, dividing them into two major evolutionary groups: eusporangiate and leptosporangiate ferns. This distinction is based on significant differences noticed in the anatomy, ecology and evolutionary history of certain ferns. Eusporangiate ferns, which include ancient lineages such as Ophioglossales (e.g. Adder's tongue ferns) and Marattiales (e.g. Giant ferns), are characterized by sporangia originating from multiple epidermal initial cells. These cells are present on the surface of leaves or stems, hence the name epidermal initials. The cells divide periclinally (parallel) and anticlincally (perpendicular) to the spore surface. Divisions then produce a multi-layered sporangial wall and a large number of sporogenous cells (Raghavan, 1989). As a result, their mature sporangia are relatively large and capable of producing hundreds to thousands of spores. Because these sporangia lack specialized structures for active dehiscence, their relay on passive spore release aided by wind or by the opening of the sporangium upon drying (Pryer et al., 2004). In contrast, leptosporangiate ferns, which comprise the majority of extant ferns (over 95%), develop their sporangia from just one initial cell, which undergoes a series of divisions and gives rise to an entire sporangium. This includes a thin, one cell layer thick capsule held up on a slender stalk. A key innovation in leptosporangiate ferns is the presence of an annulus, a ring of specialized hollow mechanosensitive cells with unevenly thickened walls. The cells in the annulus are considered mechanosensitive because they are filled with water, but since they are also highly permeable, this water can easily evaporate as a response to dry weather. This water loss initiates the core physical mechanism for dispersal, which is the rapid buildup of cohesive tension in the remaining water within the annulus cells. (Schuettpelz and Pryer, 2009). As water continues to evaporate, the force generated by the surface tension of the water column causes the thin outer cell wall to collapse, while slowly pulling the annulus open and peeling the sporangium back. Once the tension reaches a critical point, the water column within the annulus cells rapidly breaks, and so the annulus explosively recoils forward. This specialized dehiscence mechanism acts as a microscopic catapult, launching the tiny spores into the air. This type of dispersal is mediated by the wind, and it's the reason why ferns usually produce such high numbers of spores, ranging from several ten thousands to over a million, depending on the species. Due to their tiny size, once they are exposed to the wind, they can be dispersed to great distances travelling even hundreds or thousands of kilometres. This high amount of spores produced by ferns, actually compensates for the possibility that many might actually land in an unsuitable environment. Furthermore, their thick walls allow them to remain in a state of dormancy, surviving long periods in the atmosphere or soil until they find suitable conditions for germination. This adaptation allows for successful and effective reproduction even with the production of smaller numbers of spores, typically a power of 2 (e.g. 16, 32, 64, or 128) per sporangium (Noblin et al., 2012).

Another distinguishing characteristic between these two groups which reflects the evolutionary divergence very clearly is the sorus structure. In eusporangiate ferns, sporangia are usually solitary, fused into multi-chambered structures called synangia (as seen in Psilotaceae or Marattiacae), or embedded in strobili. The difference between synangia and sori traditionally seen in leptosporangiate ferns, is that in synangia sporangia are united or coherent during development and forms a single structure which has multiple internal compartments due to the partial or complete fusion of sporangia walls while in sori the sporangia is unfused and each sporangium is its own unit with its own dehiscence mechanism such as annulus. As previously mentioned, in leptosporangiate ferns, sporangia are clustered into sori, highly organized and usually covered by an already described protective tissue, the

indusium. These developmental and morphological differences highlight the evolutionary split and diversification caused by it, within the fern lineage (Smith et al., 2006).

Another key feature, which separates ferns from seed plants, is that fertilization happens after the spore has left the parent sporophyte. This isn't the case in angiosperms, where pollination, fertilization and then fruit development occur while the gametophyte is attached and nourished by the parent plant. Meanwhile, in ferns, once the spore lands in a moist and nutrient-rich environment, it germinates.

Most ferns are homosporous, meaning they produce spores of the same or similar size and shape. These spores then develop into an exosporic gametophyte, which grows outside of the spore wall. This gametophyte is usually small and of a heart-shaped structure, also called a prothallus. The prothallus is a free-living, small in size, photosynthetic organism anchored to the substrate by rhizoids (Pryer et al, 2004.). On its underside, two types of sexual organs develop, as seen in Figure 1: antheridia and archegonia. Antheridia produce numerous flagellated sperms, which need a film of water, such as rain or dew, to swim to the egg producing archegonia. This characteristic of ferns being dependent on water to reproduce limits their distribution to moist environments (Page, 2002.). Homospory is prevalent, however, some ferns exhibit heterospory. A less common but evolutionary significant trait where the spores are dimorphic, differing in shape and size into microspores and megaspores. Eventually this translates into heterosporous ferns producing different types of gametophytes, male ones from the microspores and female ones from the megaspores. Unlike homosporous ferns, the gametophytes of heterosporous ferns are endosporic, meaning they develop within the confines of the spore wall. Consequently, these gametophytes are not photosynthetic but instead rely on stored food reserves from the original spore. This shift represents an evolutionary step towards the reduced and dependent gametophytes seen in seed plants (Bateman and DiMichele, 1994).

After fertilization, in ferns with either of those types of spores, the diploid zygote develops eventually into a new sporophyte. At first, the young sporophyte is attached and dependent on the gametophyte for nutrition. Through its development, as the sporophyte becomes larger and independent, the gametophyte withers and dies. This signifies the completion of the alteration of generations and so this cycle can now start again (Gilbert SF, 2000).

Still, the diploid sporophyte is considered to be the dominant stage of the life cycle, mostly because it's larger in size, has more morphological complexity and a longer lifespan. Other than the difference in the ploidy level, so if the chromosomes are in the diploid or haploid state, the two generations also differ morphologically and ecologically. Gametophytes are smaller in size, measuring only around 1 cm, and not as complex as sporophytes. Because of this they're difficult to taxonomically identify and are also known as a "morphologically cryptic" stage of ferns life cycle. The gametophytes lack certain characteristics important for surviving harsher environmental conditions, such as waxy cuticles covering their surface, stomata for water and gas exchange, as well as complex vascular structures Instead they got adapted to living in shady places, absorbing water and nutrients directly from the soil by rhizoids and have the ability to quickly recover from unsuitable conditions, such as fluctuations in water availability. The fern gametophyte has its own photosynthetic machinery. This independence from the sporophyte extends to the distribution in the natural environment so that gametophyte populations can develop and thrive far away from their sporophytic counterparts. Meaning that these haploid stages of fern life cycle create gametophyte only populations which exist from a few meters to hundreds of kilometres away from sporophytes. These gametophyte-only populations become self-sustaining, reproducing asexually usually through gemmae, small multicellular propagules which can detach and grow into new gametophytes (Krieg and Chambers, 2022).

2.3. Gametophyte development

The gametophyte generation has so far been under-investigated in many species. While fern gametophytes themselves generally have little economic value, their simple morphology, and rapid development actually make it an interesting subject for scientific research. Because they can easily be propagated *in vitro,* researchers can manipulate environmental cues and observe fundamental developmental processes. Due to this simplicity they are also useful model systems for understanding certain biological processes such as cell differentiation and multicellular development which in the end offers insight that can then be applied to more complex plants (Kinosian and Wolf, 2022).

Spores are haploid, single-celled products of meiosis formed within the sporangia of the mature sporophyte. They exhibit distinct surface markings that are critical for the viability and germination, and these markings are actually scars left over from their formation in the sporangium. Based on the type of their markings, fern spores can be broadly classified into trilete or monolete types (Ščevková et al., 2022). As it can be seen in Figure 3, trilete spores have a faintly visible Y-shaped marking indicating they were formed in a tetrahedral tetrad, while monolete spores have a linear marking due to their formation in an isobilateral tetrad. These markings are actually an aperture which serves as the primary site for rupture during germination, through which the gametophyte then emerges (Raghavan, V. 1989).

The process of gametophyte development consists of a series of morphological and cytological events, with spore germination marking the first step towards a sexually mature gametophyte. After germination, this process also includes the formation of a germ filament, the establishment of the apical cell, transition to a two-dimensional prothallus phase, formation of the meristematic plate and finally, gametangia development. Unlike the highly reduced and sporophyte-dependent gametophyte in seed plants, which lacks the pluripotent meristem, fern gametophytes complete their life cycle as free-living, mainly due to the capacity to develop and maintain an indeterminate meristematic region. This meristem continuously divides and renews itself which drives the growth and enables differentiation of cells into various components of the prothallus and gametangia. The process of meristem development, maintenance and cell differentiation has so far been thoroughly researched within a homosporous fern *Ceratopteris richardii* (Cook et al., 1995). This plant serves as a model organism in ferns due to its rapid life cycle and an easily altered genome. However, recently, research has expanded to other species as well, helping to discover these conserved and divergent developmental pathways (Xie et al, 2025).

The most important part of germination begins with breaking of spore dormancy which is initiated by favourable environmental conditions, primarily moisture and light uptake. Some species also require a specific temperatures or the presence of certain chemical cues such as antheridiogens released from other gametophytes (Banks, J.A. 1999). The first cell division within the spore is asymmetric, resulting in two daughter cells proceeding on different developmental paths. In most ferns, the smaller of these cell gives rise to the first rhizoid, a root hair lookalike that pokes through the spore coat. Its appearance is one of the first visible signs of germination, serving to anchor the developing gametophyte to the substrate and initiate nutrient and water absorption. The bigger cell divides as well and forms a protonemal initial, a parent cell of the photosynthetic prothallus (Campbell, 1885).

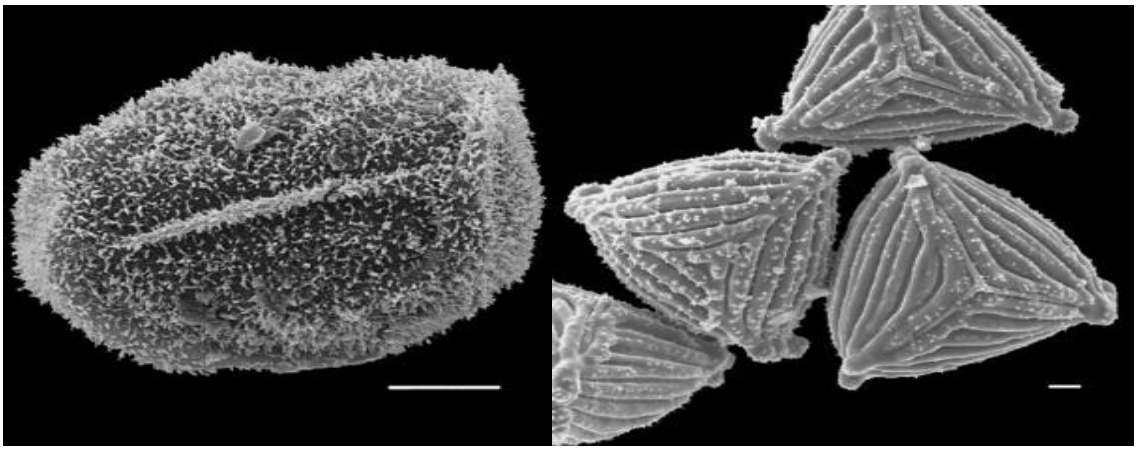

Figure 3: Monolate spore of the *Lastreopsis amplissima* fern species with a single line across its centre, and trilete spores of *Anemia asper* fern species with Y-shaped markings. (scale bar: 10 μm). R.Morran. 2013. *Interesting Plant Stories.*

The protonemal initial continues with multiple transverse divisions and elongations, parallel to the spore surface, eventually forming a linear, one-dimensional germ filament. As the name indicates, the germ filament is a filamentous structure usually composed of three to ten chlorophyllous cells. Although the exact number can vary from anywhere between five to twenty cells, depending on the species and external factors such as light availability and intensity, e.g. higher light leads to shorter filaments. Each cell in this filament contributes to the developmental process and photosynthetic capacity of the gametophyte, but the most important developmental switch happens at the terminal cell. This cell divides obliquely and forms three new cells. One of these turns into a wedge shaped cell, which then becomes a specialized triangular apical cell. This differentiation of the wedge cell into the apical cell is a key moment in indicating the transition in the growth of the gametophyte, from filamentous or one dimensional to flat, plate-like or two dimensional (Tor, 1970). The transition is also affected by environmental cues such as light quality. For example, blue light has an inducing impact in ferns, causing cell division planes to reorient from transverse to oblique. However, after its establishment, the apical cell drives the rest of the development and signifies the beginning of the prothallia differentiation phase (Miller, J.H. 1968).

Due to the continuous activity and divisions of the apical cell, the prothallus begins to slowly expand laterally, contributing to its growth in width and the formation of a spatulate mass of cells. The form of prothallus is characterized by a narrow base from which rhizoids emerge, and a broad, rounded top region. As the cell continues dividing and the lateral expansions proceed, an apical notch is formed. The prothallus matures into its adult, cordate or heart shape form and is organized into three main regions: (1) a central, thickened 'midrib' region consisting of several cell layers and spreading out from the base towards the apical notch. Here, the future archegonia will be located and eventually the new sporophyte will start to develop. (2) Extending outwards from the midrib are the lateral wings or lobes. These extensions are primarily photosynthetically active but thinner and consisting of only one cell layer. (3) The region with rhizoids developing from the bottom of the prothallus (Testo and Watkins, 2011).

Shortly after the cordate shape formation, the initial apical cell is replaced by a broader meristematic plate. This is a cluster of actively dividing cells at the base of the apical notch controlling the prothallus growth while also contributing to its size and bilateral symmetry. However, gametophytes can exhibit a variety of shapes across different taxa, diverging from the classic heart shape and resulting in asymmetric cordate, strap-like structures and filamentous structures or even undifferentiated thalloid forms.

The final stage of gametophyte development is the formation of reproductive structures, the gametangia. A common characteristic in almost all homosporous ferns in the chronological differentiation of gametangia, starting with antheridia (male reproductive organs), followed by archegonia (female reproductive organs). Antheridia are usually located at the basal region of the prothallus near the rhizoids while archegonia are located close to the meristematic plate or apical notch. The separation of the reproductive organs, both spatial and temporal, can promote outcrossing and genetic diversity within a population (Schneller, 2008).

Antheridia are formed from a single, superficial initial cell on the underside of the gametophyte, typically in older parts of it such as the area among rhizoids. Its developmental pathway varies in the complexity of their cell wall, among primitive and advanced fern groups. While in primitive fern groups, such as Osmundaceae, antheridia have simple walls composed of three to four cells, in advanced or more derived fern lineages, such as Dryopteridaceae and Pteridaceae for example, the antheridia are larger with more complex cell walls. The multi-layered cell wall of the antheridium is formed through series of cell divisions. The antheridial superficial undergoes divisions forming an outer jacket of sterile wall cells that enclose an inner mass of spermatogenous cells. These inner cells then undergo repeated mitotic divisions producing numerous spermatocytes, each eventually differentiating into a motile flagellated sperm. Simultaneously, specialized cap cells differentiate at the apex of antheridium forming an operculum. The operculum acts as a lid. When the sperm is mature and it comes into contact with water, it swells and ruptures allowing for the explosive release of the sperm (Raghavan, 1989). The multicellular wall actually helps maintaining the integrity of antheridia until the sperm is mature and ready for dispersal.

Unlike the antheridium development, the formation of an archegonium is more complex and uniform across homosporous fern species. Archegonia are flask-shaped structures which are almost always located in the thick midrib region, on the underside of the gametophyte close to the apical notch. Their placement on the gametophyte is important because it ensures proximity to the gametophytes growing tip allowing for its nutrient supply. The archegonium originates from a single, superficial archegonial cell which undergoes periclinal divisions, separating it into an outer jacket cell and inner central cell. Further development of these cells is due to a series of mitotic divisions. Continuous transverse and anticlinal divisions of the outer jacket cells result in the formation of the archegonium neck which protrudes from the gametophyte surface. The inner, central cell also undergoes a transverse division splitting into an upper primary neck canal cell and lower primary ventral cell. The primary neck canal cell usually divides further and forms one or more neck canal cells. In more derived ferns, the neck canal then has two nuclei while in primitive ferns it is mononucleate. The primary ventral cell divides once more to give rise to a smaller ventral canal cell positioned above the egg cell which is singular, large and non-motile hidden in the basal region of archegonium, which is deeply embedded in the gametophyte tissue. After maturation of the gametophyte, before fertilization, the neck and ventral canal cells undergo programmed cell death (Dyer, 1979). The breakdown of these cells creates a mucilaginous substance which swells upon contact with water forcing open the apex of the neck. This then provides a path for the motile sperm, guided by chemical attractants, to swim down to the egg (Conway and Di Stilio, 2020).

2.4. Apomixis

Among all vascular plants, ferns have the biggest tendency towards asexual reproduction with almost 10% of ferns exhibiting apogamous reproduction (Liu et al., 2012). In other vascular plants, such as flowering plants, this number is only around 1% (Becks and Alavi, 2015). Apomixis includes apospory, apogamy and parthenogenesis. In an apogamous alternation of generations, the unreduced spores end up being diploid bypassing the halving of genetic material typical for mitosis, the gametophyte is therefore somatic and doesn't produce male and female gametes so then the new sporophyte develops without the fusion of the sperm with an egg cells. The unreduced spores develop through sporogenesis, just like in sexual lineages. However, in the case of apomictic processes, the spores go through one of the two different pathways which result in the production of unreduced spores or diplospores with unreduced number of chromosomes. When these spores germinate, the prothallium is able to generate the sporophyte from somatic cells, in this case called the apogamous bud, located at the apical notch. The two differing pathways that the spores can go through during apomitic life cycle, the premeiotic endomitosis (PE) and meiotic first division restitution (MFDR), both start with three continuous mitotic divisions. From then on, spores following the PE pathway, go through the final mitotic division which ends up being unsuccessful and the spore mother cell develops from it, with twice the amount of chromosome complements. This spore mother cell then continues with the reductive meiosis and produces only half the number of spores per sporangium than the spores following full sexual reproduction and each of these spores ends up having the same number of chromosomes as the parent sporophyte plant. In the MFDR pathway, the last cell division in meiosis is successful but the first division is actually the unsuccessful and incomplete one since here, chromosomes fail to pair. Out of the two, the PE pathway is more common (Braithwaite, 1964, Grusz, 2016).

2.5. *Cyrtomium falcatum*

The genus *Cyrtomium* sp. belongs to the family Dryopteridaceae, a vast and widespread group with approximately 35 to 45 genera and 1700 species spread out worldwide. Plants within this diverse family exhibit a wide range of growth habits, including terrestrial (growing in soil), epipetric (growing on rocks or rocky surfaces) as well as hemiepiphytic (rooted in soil at the beginning of life cycle then later becoming epiphytic and vice-versa) and true epiphytic (spending their entire life unrooted often living on tree trunks or branches) (Yatskievych, 2008). The genus *Cyrtomium* sp., is often taxonomically divided into various series, such as the Falcata or Fortuna series, with their distinction depending on the frond texture, degree of undulation and whether the frond margins are serrate or entire.

Cyrtomium falcatum (L.f) C. Presl, widely recognized under common names Japanese Holly Fern or just Holly Fern, also has historically known names, such as *Asplenium falactum*, *Dryopteris falcata*, *Polypodium japonicum*. This homosporous fern is native to East Asia, and countries such as Japan, South China, Korea, Indochina and Taiwan (Imai et al., 2021). From its native range, it spread out globally to Australia, New Zealand, North America and various parts of Europe, countries such as Italy and Croatia, where it is now considered to be a locally established alien species. This species is easy to distinguish from *C. fortune* species from the Fortuna series, based on its thicker, leathery lamina and smooth margins of fronds as well as wider pinnae.

The sporophytic form of *Cyrtomium falcatum* is herbaceous just like the majority of ferns. Its broad nature allows it to flourish in diverse habitats but preferentially settling in moist, humus-rich substrate found in shady locations such as forests with well-drained soil. It can also be frequently found colonizing coastal cliffs, rocky slopes and stream banks, showcasing its ability for adaptation even to rocky substrates and maritime conditions. The rhizome is erect, covered with brown scales. The plant

of this species is found having usually pinnate fronds reaching 60 to 70 cm in length, bearing 4 to 15 pinnae or leaflets each being on a short stalk. The pinnae are ovate or oblong shape with a leathery texture. The margins of their leaflets contribute to the species evergreen nature since they don't shrivel at maturity. The sporangia are spread out on the underside of leaflets and are organized in numerous, large clusters of sori being brown and uniformly scattered on the underside of pinnae. Each sorus is covered and protected by a peltate (round and centrally attached) indusium which exhibits an irregular or wavy edge. The spores of the species are bilateral and usually oval or bean-shaped, with a monolete aperture. Young spores are light green and gradually darken as they mature (Farooq Shah, A. and Kumarasamy, D. 2021).

The gametophyte development in this species follows the common homosporous pattern and stages of development, such as spore germination and rhizoid emergence, then protonemal filament eventually transitioning into a spatulate prothallus while the apical cell is formed. After series of divisions, a cordate gametophyte is established. Since this is a homosporous species, its gametophyte is photosynthetic and free living. Antheridia and archegonia are developed on the same prothallus, making them monoecious. As in majority of ferns, the development of antheridia in the basal region takes place before the development of archegonia in the apical region. Yet another developmental pattern noticeable in this species is the tendency for apogamy. This tendency is highlighted in nutrient poor media as well as growth media with high sucrose concentration, also in the absence of water and during high light intensity. Based on the type of reproduction, either sexual or asexual, there are two cytotypes of this species, the sexual diploid and apogamous triploid (Matsumoto S., 2003). While observations on its gametophytic form in natural habitats are less extensive than for its sporophyte, some studies such as the one by (Sato and Sakai, 1981), suggest that *C. falcatum* gametophytes may show a preference for dryer microhabitats compared to the sporophyte, with the number of developing gametophytes appearing almost equal to the number of nearby sporophytes. However, despite such studies and observations, the morphology and development of *C. falcatum* gametophytes still remains less researched than those of its sporophyte or of model fern species.

2.6. *Nephrolepis* sp.

The genus *Nephrolepis* sp., known as Sword Ferns, comprises approximately 30 recognized species. Historically it was often classified under Lomariopsidaceae or Davalliaceae families. However, through molecular studies it was placed within its own unique evolutionary lineage representing the sole genus within its family, Nephrolepidaceae (Hovenkamp and Miyamoto, 2005). These plants are adaptable, inhabiting many habitats and environments, from tropical and subtropical regions worldwide to warmer temperate zones. Their sporophytic forms are mostly terrestrial but can also be epiphytic, utilizing their specialized aerial roots for anchorage and nutrient uptake.

Their evergreen or semi-evergreen sporophytes maintain their fronds throughout the entire year or most of it. A robust rhizome system, which can be either creeping or erect, bears a dense tuft of fronds. A distinctive and ecologically significant feature of *Nephrolepis* sp. is the presence of lateral branches, runners or stolons, producing roots and eventually giving rise to new plantlets allowing for vegetative propagation (Richards et al., 1983). This stoloniferous habit makes *Nephrolepis* sp. an exceptionally effective colonizer because it allows it to quickly occupy space and dominate the understory while forming dense, monocultural stands that outcompete other vegetation (Quiroz, 2015). Protective scales are also present on the rhizome and lower parts of frond stalks or stipes. The fronds themselves are pinnate meaning they are divided into numerous individual pinnae or leaflets arranged along a central rachis. The overall blade is once pinnate but the margins of pinnae can vary from entirely complete to subtly serrate. Just like in all ferns, reproduction occurs via spores with spore producing sporangia clustered into sori, again on the abaxial side of pinnae, protected by a reniform (kidney

shaped) or round indusium. The spore aperture is monolete while the spores themselves are elongate and brown when mature (Silverio, 2015). The ease of their propagation via stolons as well as an attractive foliage of many *Nephrolepis* species such as *Nephrolepis exaltata* and its cultivars, have made them popular ornamental plants (Quiroz, 2015).

Gametophyte development in *Nephrolepis* species, particularly in its widely studied representative *Nephrolepis exaltata,* also follows a homosporous pattern from spore germination and germ filament formation to the transition into a spatulate and then mature, cordate prothallus. Just as in *Cyrtomium falcatum*, the gametophytes are also free living and photosynthetically active, using rhizoids for water and nutrient uptake. A characteristic present in this fern, often also observed in *Ceratopteris richardii* and some other model ferns, is the sensitivity to antheridiogens. These compounds are released by older surrounding gametophytes, causing changes in sex expression and timing of formation or maturation. In settings when a hermaphrodite gametophyte is developed, the antheridia can again be found near the base and developing first, while archegonia are found near the apical notch but developing last. When antheridiogens are present, these compounds can trigger premature development of antheridia while simultaneously suppressing archegonial development. This promotes outcrossing by ensuring a mix of female, male and hermaphroditic gametophytes and therefore has a positive influence on genetic diversity (Banks, 1997).

3. Aims of this thesis

The primary objective of this thesis is to document and characterize the developmental stages of gametophytes in two fern species: *Cyrtomium falcatum* and *Nephrolepis* sp. Through a comprehensive photographic record, this thesis seeks to provide a comparative analysis of the morphological transitions from spore germination to the mature prothallus phase.

3.1 Specific objectives

There are three specific objectives of this thesis. Primary one includes capturing the chronology of development from the emergence of rhizoids and formation of the germ filament (protonema), to the transition from one-dimensional to two-dimensional, cordate growth. Secondary being the identification and description of morphological nuances in the development of the apical notch and the midrib 'cushion' area between the two species. And lastly, to observe possible unique growth patterns, such as spore clustering or solitary development, in a controlled laboratory environment.

3.2. Hypotheses

Based on the already existing botanical literature on this topic, this thesis is guided by the following hypotheses:

1. Conserved developmental pathway: It is hypothesized that both *C. falcatum* and *Nephrolepis* sp. will follow the classic homosporous developmental pattern typical of leptosporangiate ferns, sharing a similar timeline for initial germination and the transition to a cordate structure (Pryer et al., 2001; Dyer, 1979).

2. Morphological divergence: Despite shared milestones, it is expected that the species will show clear differences in the morphology of their early prothallia. Specifically in the thickness of the wings and the density of the initial germinating population, which would reflect their differing ecological strategies, since *C. falcatum* is an epipteric plant while *Nephroelpis* sp. is a stoloniferous colonizer (Yatskievych, 2008).

3. Potential for apomictic development**:** Given that both species are recognized for their high tendency toward apogamy, especially when cultured in vitro, it is hypothesized that the sporophytic generation may develop directly from somatic gametophyte tissue without the requirement of fertilization through gametic fusion (Manton, 1950; Schneller, 2008).

4. Materials and methods

The fern fronds with spores used in this experiment were collected from actively growing *Cyrtomium falcatum* and *Nephrolepis* sp. plants, cultivated in the Botanical Garden of the University of Vienna.

4.1. Medium preparation

At first, Benecke medium (Benecke, 1903; as cited in Dyer, 1979) a nutrient solution, was prepared using four distinct stock solutions. Specific concentrations of these stock solutions, prepared per 100 mL of distilled water, are detailed in table 1.

Table 1. Concentration of 4 stock solutions

Stock solution	Per 100 ml
NH_4NO_3	2g
KH_2PO_4	4g
$MgSO_4x7H_2O$	1g
$CaCl_2 \cdot xH_2O$	1g

To prepare a volume of 1 L of the Benecke medium, 10 ml of each of the four stock solutions was required. The pre-prepared stock solutions were pipetted into a measuring glass cylinder (volume 1000 mL (1L) using a 1000 µl pipette. Each stock solution was pipetted five times, totalling 5ml per stock solution. The cylinder was then filled with distilled water up to the 500 mL mark. This entire process was repeated once more, in order to achieve the final desired volume of 1 L. The prepared liquid medium was transferred into two 0.5 L heat resistant glass bottles. For solidification, 6 grams of agar were added to each bottle, resulting in a 0.6% w/v concentration. To ensure thorough homogenization of the medium and proper dissolution of the agar, rotary magnets were inserted into each bottle. The bottles were then placed on the IKA RH basic 2 shaker, set to level 3, and agitated for 2 minutes.

During the mixing process, the pH of the medium was determined using pHenomenal IS 2100L pH meter and, if necessary, adjusted. The electrode arm was carefully placed into the medium. The pH was then precisely adjusted to 5.8 by adding a few drops of 1% KOH solution which is an optimal level for most of the fern species. After the pH adjustment, the glass bottles containing the medium were tightly closed and labelled with autoclave tape. The medium then had to be sterilized by autoclaving overnight. This is typically done at 121 °C for 20 to 30 minutes. Following autoclaving, the sterile medium bottles were stored in a refrigerator at approximately 4 °C. The prepared medium can be used for up to 3 months, however, to maintain sterility and ensure that agar remains pliable, the medium must be autoclaved again before each subsequent use.

4.2. Agar pouring

The process of pouring sterile agar medium in Petri dishes was conducted under highly controlled aseptic conditions within the laminar flow hood. Before the start of the process, the laminar flow was disinfected by exposing its interior to 30 minutes of UV light. In the meantime, the bottle with the Benecke medium was taken from the refrigerator and warmed up in the microwave to melt the solidified agar, transforming its consistency from thick, cloudy gel to a transparent liquid. To prevent overheating and to allow the steam to freely evaporate, the lid of the glass bottle was slightly loosened during heating. The medium was heated using a ''Bevarage'' setting on a SHARP microwave. Heating was performed for one and a half minute, split into 30 second intervals in order to check on the liquid so that it doesn't overheat. This process was typically done twice, until the liquid reached a gentle boil and became completely transparent.

Once the UV light disinfection was complete, the interior of the laminar flow was further disinfected by wiping all surfaces thoroughly with 70% ethanol. The heated, liquid medium, still contained in a glass bottle along with two packs of Greiner Bio 60x15 mm Petri dishes, were placed inside the laminar flow hood. The Petri dishes were arranged in two neat rows. Under these sterile conditions, each dish was filled approximately 10 to 15 mL of the liquid Benecke medium, ensuring an even layer. They were then left under the laminar flow hood to cool down and solidify for 20 to 25 minutes. Once solidified, the Petri dishes were labelled and immediately transferred to a 4°C refrigerator for storage. These prepared dishes, containing the sterile Benecke medium can be used for spore propagation within the next several weeks, ideally within 2 to 4 weeks.

4.3. Spore sterilization and propagation

Fronds containing spores, of the two chosen species, *Cyrtomium falcatum* and *Nephrolepis* sp., were dried at room temperature in sheets of paper for several days. The spores then naturally started to fall off; in some fronds, aided by gentle tapping. For each fern species, spores were transferred into separate sterile Eppendorf tubes. The exact quantity of spores in each tube was not measured however, an adequate amount was used (a quarter of a tube was filled with spores) to ensure enough material for propagation. To minimize contamination during the germination on the sterile agar medium, the collected spores were sterilized using freshly prepared 2% solution of NaClO, by pipetting 20 µL of 6-14% active chlorine bleach in 1000 µL (1 mL) of sterile distilled water (SDW). The solution was then briefly centrifuged to help with the mixing. The prepared 2% NaClO solution was added to each tube with the spores. The spores were then gently mixed with the solution, afterwards they were left to settle to the bottom of the tubes for 15 minutes. This duration has shown to be effective in surface sterilization without harming spore viability.

After the allotted time of surface sterilization, the NaClO solution was carefully removed as thoroughly as possible and the spores were pipetted into new eppendorf tubes containing only sterilized distilled water (SDW). A single wash of the spores with SDW was done to remove residual bleach which could possibly inhibit germination. The entire process was performed in the previously disinfected laminar flow to maintain sterility.

The Petri dishes containing the medium were marked with either '*Nephrolepis*' or '*Cyrtomium*' according to the species of the spores they'll contain. A total of six Petri dishes were used in this experiment, three replicants for each species. The SDW from the Eppendorf tubes was pipetted out, and using a sterilized pipette with the sterile micropipette tip, the spores were spread out over the medium in the Petri dishes. After this inoculation, the Petri dishes were closed and sealed around the edges with Parafilm® to minimize drying out of the spores and future gametophytes, as well as to prevent possible contamination. The inoculated plates were then transferred to a Conviron growth chamber and were maintained and grown at the constant temperature of 20 °C under continuous 16/8 day/night period .

4.4. Light microscopy

The developing spore samples, showing different stages of their gametophyte development, were continuously observed using Nikon Eclipse N light microscope. Images were captured using 4x and 10x objective lenses, depending on the size of indidivual prothallia and prothallia clusters documented. This was done every 3 to 4 days. Images of germinating spores developing into prothallia were captured with a high-resolution Nikon Camera DS-Ri2. The acquired images were afterwards saved and processed using NIS-Element BR 5.11.03 (64 bit) computer software. Microscopy was performed at the Core Facility Cell Imaging and Ultrastructure Research, University of Vienna - member of the Vienna Life-Science Instruments (VLSI).

5. Results

The development of a gametophyte, from spore germination to mature prothallus, was observed and documented for the two fern species through a series of photographs. The results are presented in this chapter.

5.1. Developmental stages of *Cyrtomium falcatum*

Several developmental stages of *Cyrtomium falcatum* were captured and are shown in Figures 1 to 4. The primary stage of its development, the spore germination (Figure 4), was first observed approximately 6 days after spore propagation. The second stage of its development, the one-dimensional filament, took around 14 days, while the mature heart-shaped prothallus took 30 days. In Figure 1, the germinating spore is visible and can be recognized by the spore coat opening at the very top, through which the faintly visible, colourless rhizoid is emerging. Inside the spore coat, the initial photosynthetic cell is visible as well.

Figure 4: Spore germination in *Cyrtomium falcatum*. A light microscope image showing the initial emergence of the rhizoid and the protonema. (magnification: 10x, scale bar: 250 µm).

The next stage in the gametophyte development is shown in Figure 5, where the spore coat of the germinated spore is still visible but this time it's surrounded by several germ filament cells.

Figure 5: Germ filament of *Cyrtomium falcatum*. A light microscope image showing a germinated spore with its developed germ filaments. (magnification: 10x, scale bar: 250 µm).

A more detailed view of the germ filament is seen on Figure 6. And a fully developed prothallus of *C.falcatum* 35 days after propagation, as the last stage of the fern gametophyte development can be seen on Figure 7. This image shows the characteristic heart shape of the mature gametophyte with a developed apical notch at the top between the two lobed wings as well as the faintly seen rhizoids at the bottom of it structure.

Figure 6: Germ filament of *Cyrtomium falcatum*. (magnification: 10x, scale bar: 250 µm).

Figure 7: Microscopic image of fully developed prothallus of *Cyrtomium falcatum*. A microscopic image showing the heart shape and the structure of the fully matured gametophyte. (magnification: 4x, scale bar: 500 µm).

5.2 Developmental stages of *Nephrolepis* sp.

In the other species, *Nephrolepis* sp., spore germination was also first observed after 6 days from propagation. The development of the filament stage took 14 days while the mature prothallus was reached 40 days from propagation. Figure 8, shows a cluster of spores on the growth medium, many of which have germinated and with the protonema initials also now present.

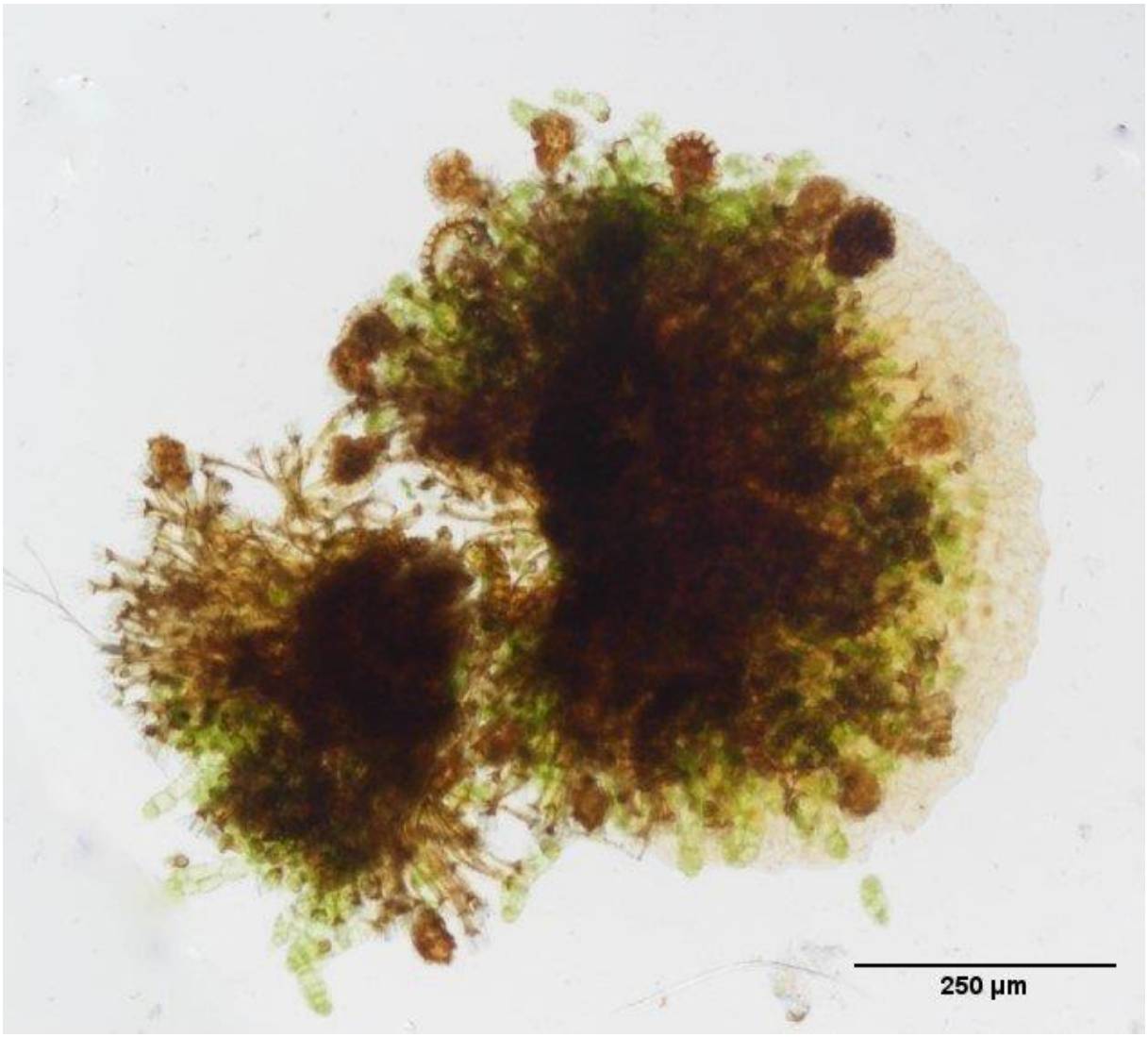

Figure 8: Cluster of germinating spores of *Nephrolepis* sp. The image shows the initial developmental stage within a group of spores. (magnification: 10x, scale bar: 250 µm)

Figure 9 and 10 show various clusters of germinating spores and developing germ filaments of *Nephrolepis* sp. The spores are visible as brown structures while the green photosynthetic cells are shown emerging from them.

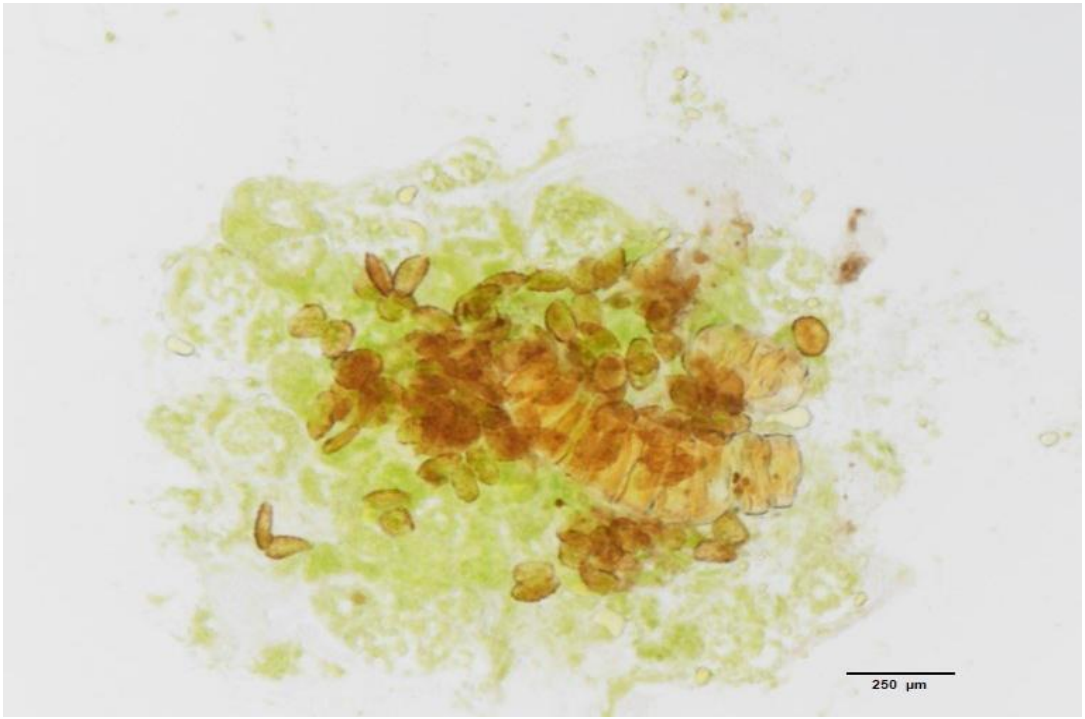

Figure 9: Clusters of developing gametophytes of *Nephrolepis* sp. (magnification: 10x, scale bar: 250 µm).

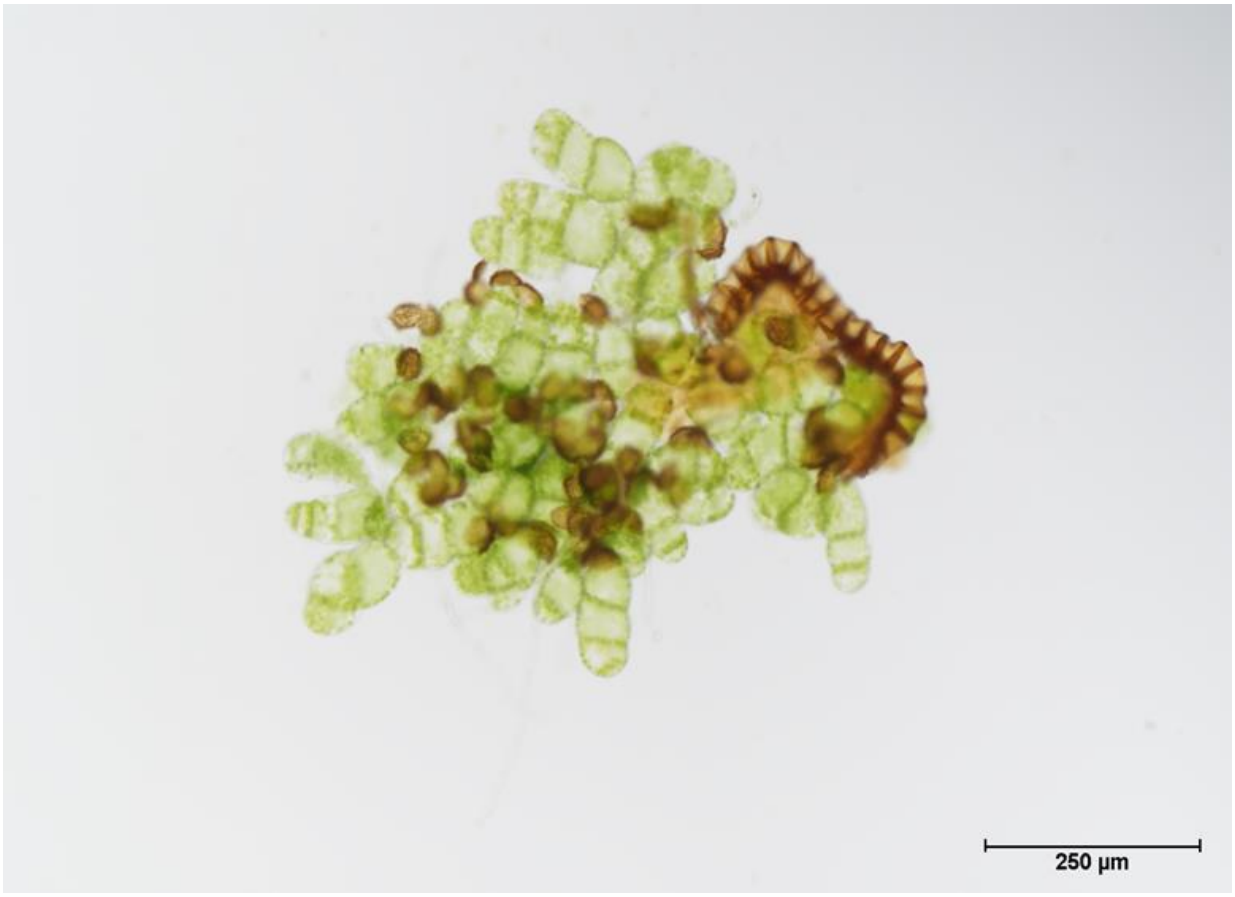

Figure 10: Germinating spore and filaments of *Nephrolepis* sp. (magnification: 10x, scale bar: 250 µm).

Figures 11 and 12 show the early stages of the two-dimensional development of the gametophyte. This can be observed from the widening of the filaments and the formation of a flattened plate of cells. Also visible at the bottom are the colourless rhizoids. In Figure 12, the developed apical notch and the pair of wings around it show that the gametophyte has almost reached its fully mature form. Figure 13, as the final captured image, shows a mature gametophyte of *Nephrolepis* sp, 40 days after propagation. The distinct shape of the fully developed prothallus can be seen.

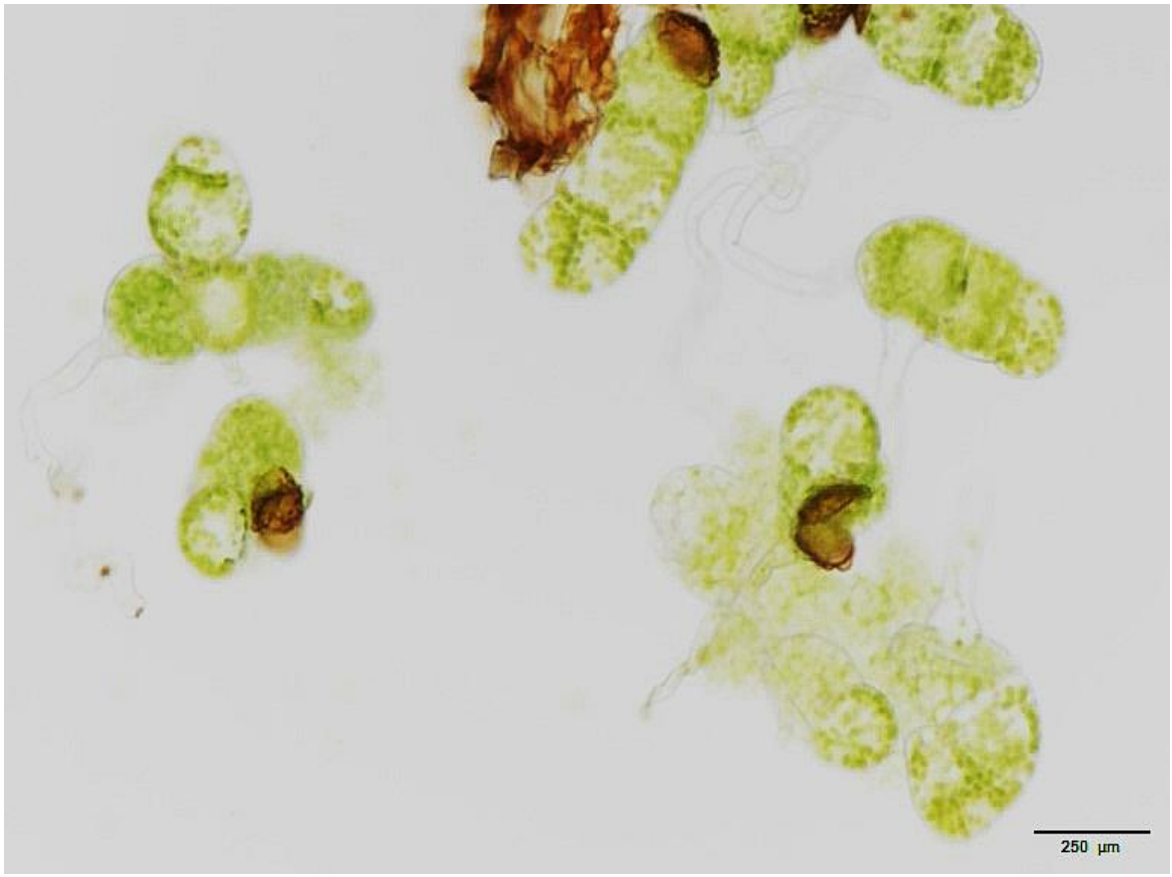

Figure 11: Early two-dimensional development of *Nephrolepis* sp. (magnification: 10x, scale bar: 250 µm).

Figure 12: Developing gametophyte of *Nephrolepis* sp. nearing the final stage of development, seen by the present apical notch and the two wings on each side of it. (magnification: 4x, scale bar: 250 µm).

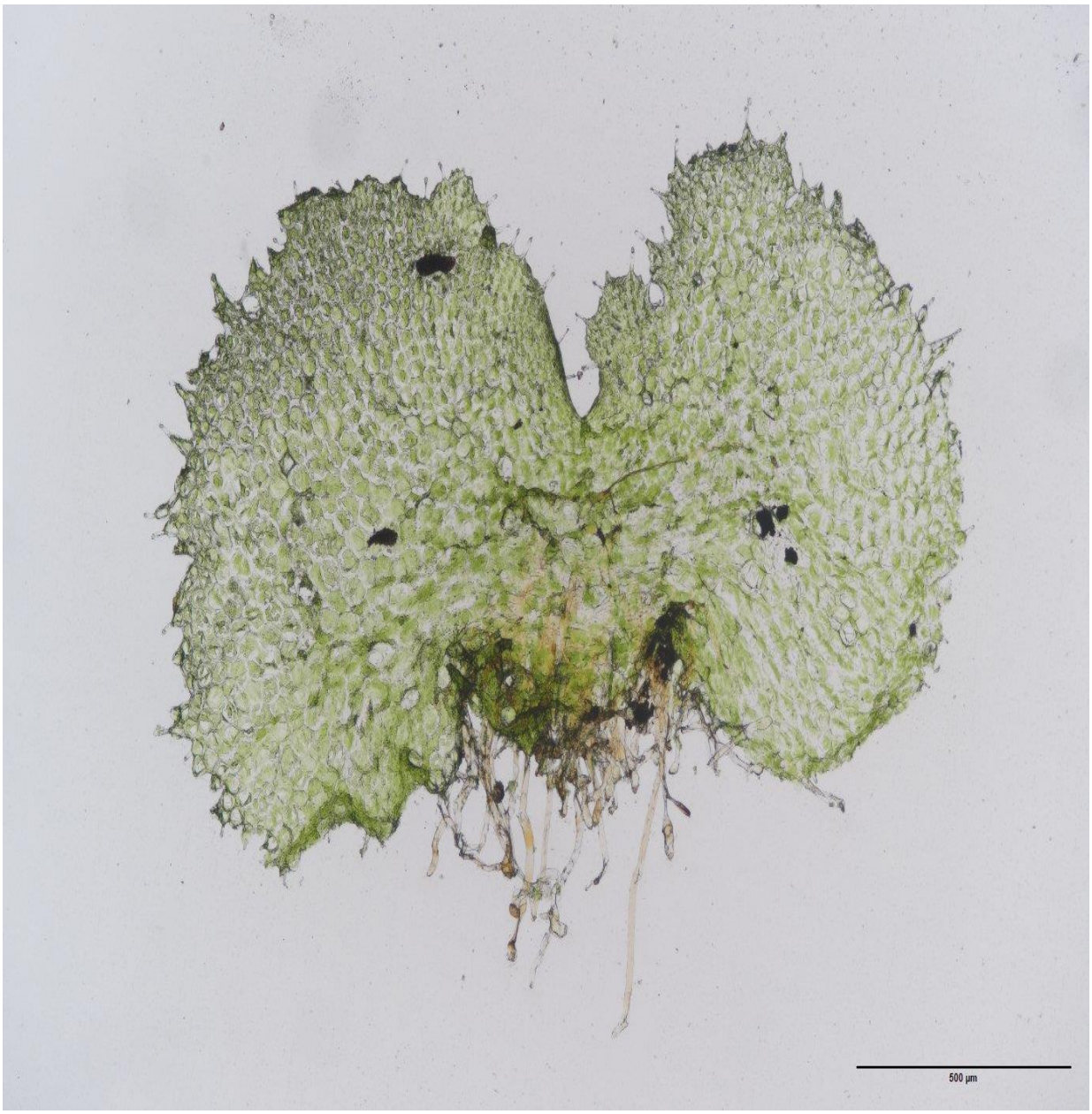

Figure 13: Mature prothallus of *Nephrolepis* sp. A fully developed gametophyte with noticeable characteristics of the two-dimensional growth stage. (magnification: 4x, scale bar: 500 µm).

6. Discussion

The objective of this study was to document and describe successive developmental stages of gametophytes in two fern species, *Cyrtomium falcatum* and *Nephrolepis* sp., as well as to observe and identify any possible morphological differential similarities in their early development. The results successfully captured the progression from spore germination to the mature gametophyte, confirming that the developmental milestones previously described in the Literature review chapter exist in the haploid generation of these two species.

6.1. Capturing developmental stages

Developmental stages captured and observed in *C.falcatum* align with the pattern of development known in homosporous ferns. The first step in this process, as previously described, is breaking of the spore dormancy, noticeable by the emergence of a colourless rhizoid initial as one of the first and best known visible signs of germination, and appearance of a protonema initial (Figure 4). This is the beginning of forming a free-living, photosynthetic gametophyte and it clearly reflects the polar division of the spore in Leptosporangiatae ferns (Raghavan, 1989). This is followed by the growth of a one dimensional, filamentous protonema (Figures 5 and 6). The successive transition from one dimensional protonema to two dimensional growth marks the shift in the place of the cell dvision which resulted in the formation of a fully developed, heart-shaped prothallus (Figure 7), signifying the maturation of the gametophyte and its preparation for sexual reproduction (Schneller, 2008).

6.2. Differences and similarities in the selected species

An important observation was the distinct difference in the developmental morphology of early stages between the two species. While *C. falcatum* appeared to progress from a single spore to a single filament and then to a mature prothallus, the *Nephrolepis* sp. images showed a tendency for germinating spores to form dense clusters (Figures 8 to 13). This growth pattern of *Nephrolepis* sp. could possibly be influenced by environmental factors such as spore density on the growth medium, which can affect light exposure and nutrient availability to individual spores. However, *Nephrolepis* species are also known for being highly adaptable and inhabiting a wide range of habitats from tropics to temperate zones, and as already mentioned, they can form dense populations in nature through vegetative reproduction (Quiroz, 2015). The clustered spores formed during this study may also reflect a natural adaptation present in already in the gametophytic phase and tendency to colonize dense habitats in vivo. Another possible explanation of the clusters could be the release of growth-promoting substances into the substrate or soil, which was observed in cases of high spore density (Weinberg and Voeller, 1969). In contrast, *Cyrtomium falcatum* thrives in diverse habitats but is most frequently found on coastal cliffs, rocky slopes, and stream banks, meaning it can adapt to substrates other than soil, while it also has a tendency and the ability to grown in humus rich soils, which could suggest a less crowded growth pattern in nature (Farooq Shah, A. and Kumarasamy, D. 2021). This observation of a more single spore, linear development in the lab could be parallel with the fact that this species doesn't naturally form dense, cultured populations as *Nephrolepis* sp.

Beyond the clustering of germinating spores, some other subtle morphological differences could be noted between the two species, such as the thickness of the wings in the mature prothalli as well as the structure of the apical notch. And even though both species take on the cordate structure in their mature form, the shape itself slightly differs.

Despite some of the observed differences in the early growth of these species, they do also share key developmental similarities. Both species followed a classic homosporous developmental pathway, from spore germination through one dimensional to two dimensional heart shaped gametophyte.

Furthermore, both followed a similar timeline for development in the lab with spore germination and the occurrence of the first rhizoid within 5 to 6 days from spore propagation and the maturing of the prothallus 30 to 40 days from propagation. This tie of development is consistent with the previous in vitro studies (Dyer, 1979). These shared characteristics highlight the conserved nature of fern life cycle even across different genera and species (Pryer et al., 2001).

6.3. Investigating apomixis

Another key aspect of fern biology to consider, which could be relevant for these observations, is apomixis. As it was mentioned in the introductory literature review, ferns have the highest tendency among all vascular plants to reproduce asexually via apogamy, where a new sporophyte develops without fertilization, meaning the spores produced are unreduced (Liu et al., 2012). They germinate into prothallia that generate a new sporophyte from a somatic cell. Both, *C. falcatum* and *Nephrolepis* sp. are species in which this tendency for apogamy has been observed before. The tendency for apomixes is highlighted in stressful conditions such as, nutrient poor media, absence of water and high light intensity (Schneller, 2008). The absence of observed fertilization in the provided images suggests the possibility that apomixis is a potential developmental pathway for these species. Future research would be required to determine if they utilized apomixis as part of their life cycle under these experimental conditions. Several methods could be used to do so, with flow cytometry being the most efficient one. Measuring the DNA content of the nuclei from the parent sporophyte and the resulting gametophyte, would give a clear insight weather the unreduced or diploid state of the gametophyte has been sustained (Grusz, 2016). Spore counting is another method which could be used for apomixes determination. Since the apomitic ferns following the premeiotic endomitosis (PE) pathway typically produce only 32 spores per sporangium, unlike the sexual ones which produce 64 (Manton, 1950). And lastly, just observing the apogamous bud and the place of growth of the sporophyte, since in case of apomixes the sporophyte will grow near the apical notch without the presence of an embryo.

6.4. Antheridia and Archegonia

A key limitation in this study is the lack of documentation of the fertilization process and the following emergence of the new sporophyte, as well as documentation of antheridia and archegonia in the mature gametophytes. In this study, on all the prothalli analysed, we were not able to observe archegonia nor antheridia, although the prothalli were observed until full development based on their shape. This could have been due to the dense midrib 'cushion' and rhizoids which can conceal these structures from being easily observed (Nayar and Kaur, 1971). Furthermore, the conditions required for fertilization, primarily the presence of water, were not maintained to complete the life cycle. Gametophytes are also sensitive to chemical cues such as antheridiogens released by other gametophytes, which can trigger premature development of antheridia while suppressing archegonial development. This then promotes outcrossing and influences the sexual expression of the gametophytes (Conway and Di Stilio, 2020). Future studies could focus on capturing this final stage by employing histological staining for better visualization of these microscopic organs as well as investigate the potential for apomixes in different environmental conditions to be able to better understand its role in the life cycle of these two species.

In summary, this thesis provides a comprehensive photographic record of gametophyte development in two fern species. The findings confirm the fundamental similarities in their life cycle while also suggesting certain subtle differences in their early morphology, which may be connected to their broader ecological and reproductive strategies in nature. This thesis contributes to the limited knowledge available on the gametophyte generation and underscores the importance of studying this often over looked stage of the ferns life cycle.

7. Conclusion

This thesis proves the establishment of a successful experimental setup for fern propagation in vitro, including surface sterilization of spores. Furthermore, it does provide visual evidence and photographic documentation of the distinct developmental stages of the fern gametophyte in *C. falcatum* and *Nephrolepis* sp. The differences observed in early developmental stages may reflect underlying ecological adaptations related to their natural environments, with *Nephrolepis* sp. showing a clustered growth habit that could be a parallel to its dense populations in nature. Both species follow the fundamental developmental stages from spore to a mature, heart-shaped prothallus, clearly demonstrating the important role that the haploid generation of ferns has in their life cycle.

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