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Male Gametophyte Development and Evolution in Extant Gymnosperms

Danilo D. Fernando[1*] • Christina R. Quinn[1] • Eric D. Brenner[2] • John N. Owens[3]

[1] Department of Environmental and Forest Biology, State University of New York College of Environmental Science and Forestry, 1 Forestry Drive, Syracuse, NY 13210, USA
[2] The New York Botanical Garden, 200th St. and Kazimiroff, Bronx, NY 10458, USA
[3] 69/35 Sukhumvit Rd., Dtumbon Na Jomtein, Amphur Sattahip, Chonburi 20250, Thailand

Corresponding author: * **dfernando@esf.edu**

ABSTRACT

The male gametophytes of gymnosperms are characterized by the diversities in pollen morphology, cellular composition and pattern of cell division, pollen tube morphology, sperm delivery, growth pattern through the ovule and nucellus, and pollen tube wall composition both within and among the four living orders, i.e., Cycadales, Ginkgoales, Coniferales, and Gnetales. At dehiscence, gymnosperm pollen grains contain a variable number of cells yet none have sperm at this stage. Pollen germination in the ovule usually occurs within a few hours or days in gnetophytes, about a week or so in conifers and *Ginkgo*, or after several months in cycads. Complete development of the male gametophytes typically involves two to five mitotic divisions. Evolution of the male gametophyte appears to have involved a reduction of its component cells with prothallial cells being among those reduced or eliminated. There is a shift in the site of sperm discharge from a proximal position in pollen grains of cycads and *Ginkgo* to distal in conifers and gnetophytes. Two methods of sperm delivery occur in gymnosperms: zooidogamy, defined by pollen tubes with motile sperm as exhibited in cycads and *Ginkgo*, and siphonogamy, defined by pollen tubes with non-motile sperm which are directly delivered into the egg as exhibited in conifers and gnetophytes. Different pollen tube morphologies occur in the nucellus, i.e., branched and haustorial in cycads and *Ginkgo*, and unbranched and non-haustorial in conifers and gnetophytes. Pollen tubes form heterotrophic relationships with the nucellus, but it is only in cycads that intracellular penetration results in significant destruction of the nucellus. Pollen tube walls of gymnosperms contain cellulose and arabinogalactan proteins; however, pectins are prevalent in cycads and mixed β-glucan in *Ginkgo*. A standard terminology to describe the cellular composition of the male gametophytes in gymnosperms is proposed.

Keywords: pollen grain, pollen tube, siphonogamy, sperm, sexual reproduction, zooidogamy

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INTRODUCTION

The appearance of pollen grains is a key innovation in seed plant evolution. Before pollen grains, fertilization was restricted to a wet environment that was required for the sperm to swim to the archegonia containing the egg cell. This mode of fertilization still occurs in land plants such as bryophytes, lycophytes and pteridophytes. This innovation of the male gametophyte increased the success of fertilization in seed plants by eliminating the chances and hazards associated with the aquatic transfer of sperm (Gifford and Foster 1989). Within the gymnosperms, and seed plants in general, two methods of sperm delivery occur: zooidogamy and siphonogamy. In zooidogamous seed plants such as the cycads and *Ginkgo*, the pollen tubes function as haustoria, i.e., multiple branches emerge at their distal end that penetrate the nucellus to absorb water and nutrients for the development of the sperm. At their proximal end, the pollen tube releases flagellated sperm into a specialized chamber in the ovule from which the sperm subsequently swim to the egg cell (Friedman 1993; Poort *et al*. 1996; Rudall and Bateman 2007). The fossil record also provides several examples of zooidogamy in extinct gymnosperms (Poort *et al*. 1996; Nishida *et al*. 2003, 2004; Leslie 2008). The discovery of the zooidogamous mode of fertilization in *Ginkgo biloba* (Hirase 1896) and *Cycas revoluta* (Ikeno 1896) linked gymnosperms with pteridophytes, which is considered as one of the seminal biological discoveries in the 19th century. In conifers and gnetophytes, pollen tubes elongate from the nucellus to an archegonium (or female gametophyte in *Gnetum* and *Welwitschia)* and deliver nonflagellated sperm directly into the egg (siphonogamy). The shift in the posi-

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Invited Review

tion of sperm release and loss of sperm locomotion are among the key traits in the transition towards siphonogamy (Poort *et al.* 1996; Rudall and Bateman 2007), the predominant mode of fertilization in seed plants. Phylogenetic analyses show that siphonogamy may have evolved at least twice in gymnosperms, once within the conifers and once in the gnetophytes (Doyle and Donoghue 1992; Friedman 1993; Rudall and Batemen 2007). Siphonogamy occurs in both gymnosperms and angiosperms, but the development and cellular organization of their pollen grains and tubes are considerably different (Gifford and Foster 1989; Fernando *et al*. 2005a). There is also a marked difference in the rates of pollen tube growth and fertilization intervals between gymnosperms and angiosperms (Williams 2008).

The process of male gamete formation has been reviewed for higher plants, but has focused on angiosperms, with no inclusion of gymnosperms (see Boavida *et al*. 2005). Where gymnosperms were included, as reviewed by Moitra and Bhatnagar (1982), description of the male gametophyte concentrated on pollen grain and gamete development, with minor mention of pollen tube development. A comparison of the male gametophytes among seed plants has been carried out but focused on the evolution of pollen grain polarity and siphonogamy (see Rudall and Bateman 2007). In gymnosperms, a review on pollen tube growth and development is available (see Fernando *et al*. 2005a), but it is limited to the conifers. Therefore, further discussion is needed on the nature of pollen grains and tubes in the extant gymnosperms.

This review will describe male gametophyte development focusing on morphology, polarity and cellular composition of the pollen grain, pollen germination and tube growth, sperm formation, growth of the pollen tubes in the ovules, chemical composition of the intine and pollen tube wall, and evolution in the four living orders of gymnosperms: Cycadales, Ginkgoales, Coniferales, and Gnetales. A standard terminology to describe the cellular composition of male gametophytes in gymnosperms, which is consistent with that of the angiosperms, is proposed. Immature or developing male gametophyte refers to all stages starting from the formation of the microspores, except sperm formation. Formation of the sperm indicates that the male gametophyte is mature. The concept of the nature of sperm in gymnosperms is confusing, i.e., whether they are composed of cells or nuclei. Therefore, this review will clarify which extant gymnosperms have sperm cells and which have sperm nuclei. By studying the development and evolution of the male gametophytes of the living gymnosperms, this review will help further our knowledge of the reproductive processes in the more primitive seed plants and increase our awareness of the sexual reproductive diversity in seed plants.

EVOLUTION AND CLASSIFICATION OF GYMNOSPERMS

Stratigraphic evidence places the origin of Coniferales in the Upper Carboniferous (Rothwell *et al*. 1997; Hernandez-Castillo *et al.* 2009), Cycadales (Mamay 1976; Gao and Thomas 1989) and Ginkgoales (Rothwell and Holt 1997; Royer *et al*. 2003) in the Permian, and Gnetales in the Triassic (Stewart and Rothwell 1993; Crane 1996; Rydin *et al.* 2006). From Permian to Late Jurassic, many seed plants became extinct including lyginopterids, medullosans, Callistophytaceae, glossopterids, Cordaitales, and Voltziales (Stewart and Rothwell 1993). The Cycadales reached their greatest abundance during the Jurassic then began to decline at the end of the Mesozoic era; however, a few families endured to give rise to the modern cycads (Mamay 1969, Gao and Thomas 1989). Similarly, the Ginkgoales reached their greatest abundance and widest distribution during the Jurassic; however, before the end of the Cretaceous, the order diminished and is now represented by a single genus, *Ginkgo* (Rothwell and Holt 1997; Royer *et al*. 2003; Zhou and Zheng 2003). The Coniferales began extensive diversification during the Mesozoic and maintains moderate diversity to this day. In spite of the extinction of many of its members, conifers are still a dominant group based on number of extant species (627 species according to Farjon 2008), number of individuals, and distribution.

Modern cycads occupy a remnant of their former range and are composed of three families, eleven genera, and about 300 species: Cycadaceae (one genus), Stangeriaceae (two genera), and Zamiaceae (eight genera) (Norstog and Nicholls 1997; Hill *et al*. 2003, 2005). These genera are sharply defined and, although all exist in tropical or subtropical areas, there is no genus found in both the Eastern and Western hemispheres. In addition, most cycads are local in distribution, except for *Zamia*, which extends from Florida to Chile, and *Cycas*, from Japan to Australia, but none form continuous natural stands (Hill *et al*. 2005). Cycads are characterized by sturdy, branched or unbranched trunks covered with persistent leaf bases and topped with a crown of large, pinnately compound leaves. All species are dioecious, i.e., individual plants bear either separate male or female sporophylls. Cycad micro- and megasporophylls are considered to be much-reduced leaves that are usually aggregated into large, laterally displaced cones (strobili). In the case of *Cycas*, the basal genus, megasporophylls are loosely arranged and not clustered into cones. At the time of pollination, pollen grains are released from the male (pollen or microsporangiate) cones and transferred to the female (seed or megasporangiate) cones by wind or insects (Choi and Friedman 1991).

Although widely distributed during the Mesozoic era, the Ginkgoales was never a large order and continued as a single line into the present, represented only by a single living species, *Ginkgo biloba* (Coulter 1909). Recognized by its tall stature and spreading branches with bilobed, fan-shaped and dichotomously veined leaves, *Ginkgo* is now presumed to be extinct in the wild, but has been distributed worldwide through cultivation (Gifford and Foster 1989; Rothwell and Holt 1997; Royer *et al*. 2003; Shen *et al.* 2005). *Ginkgo* is dioecious, ovules are typically borne in pairs on the end of short stalks, microsporangia occur in cones, and pollen grains are wind dispersed (Chamberlain 1935).

With a fossil record extending back to the Upper Carboniferous (Pennsylvanian), conifers are considered to have arisen from primitive gymnosperms with seed-bearing cupules (Miller 1977; Meyen 1984, 1997). Single or multi-seeded, the cupules were composed of modified branchlets that fused to eventually form cone-like structures containing ovules that resulted in naked seeds. The Podocarpaceae, found in the tropics and southern hemisphere, is the first conifer family recognized in the fossil record, although most of the other families are apparent at the end of the Triassic and beginning of the Jurassic (Miller 1977, 1999; Farjon 1998). The earliest fossil evidence for Pinaceae is from the Late Jurassic (Miller 1977; Farjon 1998). During these warm periods, conifers extended towards the polar regions and higher elevations. In contrast, as temperatures cooled, conifer distribution contracted to equatorial and tropical regions. These trends may have resulted in many monotypic families, genera, and isolated species (Farjon 1998).

Early conifer evolution enabled separate and distinctive compound female cones and simple male cones to appear and remain as distinct conifer traits. Evolutionary diversification of the conifers has led to a great diversity in cone morphology, male and female gametophyte development, gamete structure, fertilization, embryogenesis, and seed development (Singh 1978; Bruns and Owens 2000). This is particularly true of the male gametophyte and conifer pollination mechanisms (Singh 1978; Tomlinson 1994; Owens *et al*. 1998; Fernando *et al*. 2005a). Most conifers are monoecious, but all are wind pollinated; however, the pollen grain and sperm structures, the method by which pollen grains enter the ovules, the number of cells within the shed pollen grain, and the time and method by which pollen

tubes form have evolved along several different lineages (Owens and Bruns 2000; Fernando *et al*. 2005a).

Conifers are the largest and most diverse of all extant gymnosperms, with 627 species belonging to 70 genera, which are distributed worldwide (Farjon 2008). Several studies have shown that they are composed of seven families based on morphological cladistic analysis (Hart 1987) and molecular phylogenetic analyses (Chaw *et al*. 1993; Price *et al*. 1993; Chaw *et al*. 1995; Stefanovic *et al*. 1998). These studies also show that Pinaceae is the first lineage of conifers to diverge. Other morphological analyses consider Phyllocladaceae as separate from Podocarpaceae resulting in eight families (Tomlinson *et al*. 1997; Farjon 1998, 2008). The conifers are monophyletic and starting from the basal branch, are composed of Pinaceae, Podocarpaceae *sensu lato*, Araucariaceae, Sciadopityaceae, Taxaceae, Cephalotaxaceae, and Cupressaceae (Stefanovic *et al*. 1998). The placements of Pinaceae and Podocarpaceae in the above and many other molecular phylogenetic analyses are not consistent with the fossil record of the conifers (Stewart 1983; Farjon 1998, 2008). Therefore, the number of conifer families and their position in an evolutionary classification remain to be settled.

The gnetophytes have been grouped within the gymnosperms based on their naked ovules. The gnetophytes have also been regarded as a sister group to the angiosperms due to many shared morphological characteristics including double fertilization (Friedman 1990a, 1990b; Carmichael and Friedman 1996). However, several studies indicate otherwise (Goremykin *et al*. 1996; Chaw *et al*. 1997; Hansen *et al*. 1999). Sequence analyses of mitochondrial and nuclear small subunit rRNA (Bowe *et al*. 2000) and examination of molecular markers such as the MADS-box gene subfamilies (Winter *et al*. 1999; Chaw *et al*. 2000) have provided further evidence that gnetophytes are monophyletic with a closer affinity to the conifers than angiosperms. Moreover, sequence information from RNA polymerases I, II, and III has grouped the gnetophytes within the conifers as the sister group of the Pinaceae (Hajibabaei *et al*. 2006). The fossil record also provides some evidence for a relationship between the Coniferales and Gnetales (Hernandez-Castillo *et al*. 2001).

In spite of the discrepancies in the placement of Gnetales, it is agreed that three genera compose this order: *Ephedra*, *Gnetum* and *Welwitschia*. Although different in appearance and life history, the three genera are grouped together based upon shared morphological characteristics including vessels in the secondary wood, compound cones in both male and female plants, double-integumented ovules, long micropylar tubes formed by the inner integuments, double fertilization, embryos with two cotyledons, opposite and net-veined leaves, and lack of resin canals. Most of these features are not found in any other gymnosperms. The monophyly of these three genera are also supported by various molecular data (Goremykin *et al*. 1996; Price 1996; Bowe *et al*. 2000; Chaw *et al*. 2000; Rydin *et al*. 2006).

MORPHOLOGY AND POLARITY OF POLLEN GRAINS

Pollen grains are produced by all seed plants and generally range in size from 10-100 microns. In gymnosperms, pollen grains represent the developing male gametophytes that are surrounded by a complex wall, the pollen wall, composed of an outer layer called the exine (which is subdivided into ectexine and endexine) and the inner layer is the intine (Faegri and Iverson 1989). The ectexine is relatively thick and composed primarily of sporopollenin (which is the most decay- and chemical resistant biopolymer available in nature), while the endexine is thin, with a nonhomogenous laminate appearance, and degradable. The exine protects the developing male gametophyte from impacts and abrasion, and allows for the expansion and reduction of the pollen grain size with changing humidity. The intine is composed of various proteins and polysaccharides, and plays a role in pollen germination and extension of the pollen tube (Derksen *et al*. 1999; Yatomi *et al*. 2002; Chichiricco and Pacini 2008).

The pollen grains of cycads (**Fig. 1A**), which probably represent the nearest living equivalent to the earliest gymnosperm pollen grain (Chaloner 1970), are smooth, spheroidal, and aperturate; the aperture occupies almost half of the pollen surface (Dehgan and Dehgan 1988; Tekleva *et al*. 2007). Pollen grains range in size from those with small volume, such as in *Encephalartos* where the volume is approximately 7 μm^3 (Pacini *et al*. 1999), to those with large volumes, such as in *Macrozamia* and *Microcycas* with volumes of approximately 31 μm^3 (Pacini *et al*. 1999). Polarity is manifested in the thickness of the outer wall of the pollen grain. The exine covers the entire surface of the pollen grain; however, it is thickest at the proximal area, moderate on the sides, and thin at the distal area. Proximal refers to the surface where the microspores join in the tetrad following meiosis and distal refers to the outer surfaces of the four microspores when in the tetrad and after they separate. The intine is a single, thin, continuous layer (although it is thicker on the sides in some species) composed of cellulose, callose, and pectins (Downie 1928; Pettitt 1982; Pacini *et al*. 1999; Yatomi *et al*. 2002).

Pollen grains of *Ginkgo biloba* (**Fig. 1B**) are asymmetrically spheroidal, i.e., with a convex proximal side and flat distal end; the latter bears the aperture that also occupies almost half of the pollen grain (Tekleva *et al*. 2007). They are smooth, monosulcate, moderate in volume at about 13 μm^3 (Pacini *et al*. 1999; Tekleva *et al.* 2007) and polarized cytologically, with the position of the prothallial cell denoting the proximal end and the large tube cell the distal end (Friedman 1987). An exine of intermediate thickness covers the proximal side of the pollen grain, while a much thinner exine layer occurs on the distal side (Tekleva *et al*. 2007). A continuous layer of sterified pectin, cellulose, and mixed β-glucans make up the intine (Pacini *et al*. 1999; Yatomi *et al*. 2002).

The features of conifer pollen grains vary among families (**Figs. 1C-F**); they may be saccate or non-saccate, with smooth, orbiculate, or highly sculptured walls (Fernando *et al*. 2005a). The most detailed ultrastructural description yet made of the formation of all layers of the conifer pollen wall, including for each layer the structure, terminology, and the sequence of deposition has been made for *Tsuga canadensis* by Kurmann (1990). Therefore, readers are referred to this and other articles by this author for details on pollen wall structure in other conifers (Kurmann 1994), as well as comparisons among extant gymnosperms (Kurmann 1992), and between extant and fossil gymnosperms (Kurmann and Zavada 1994). In most Pinaceae, pollen grains are saccate and strongly polarized by the presence of distal sacci (**Fig. 1C**) (Owens and Simpson 1986). The exine is thicker at the proximal end and thinner but continuous in the distal region. The cellulose-rich intine contains the prothallial cells at the proximal end. In other Pinaceae (e.g., *Pseudotsuga* and *Larix*), pollen grains are non-saccate, are very finely sculptured (**Fig. 1D**), and lack external polarity but have internal polarity in the presence of prothallial cells embedded within the intine. In the Cupressaceae, pollen grains are spherical, smooth, non-saccate and the exine surface bears many tiny spherical orbicules (**Fig. 1E**) deposited from the tapetum. In *Tsuga* (Pinaceae), the pollen grains are also non-saccate but the exine forms spines (**Fig. 1F**). Their spherical pollen grains become irregularly indented when dry and are internally polarized (Owens and Simpson 1986). The cellulose-rich inner layer of the intine is uniform in thickness, while the outer layer, which is rich in pectins, is irregular in thickness (Chichiricco and Pacini 2008). In contrast, pollen grains of the Taxaceae lack internal polarity. Their exine is continuously thick as is the intine, which is made up of a thick inner layer rich in cellulose and a thinner outer layer containing pectin (Anderson and Owens 2000; Fernando *et al*. 2005a). Their pollen is non-saccate and also covered with orbicules. Saccate pollen grains are also found

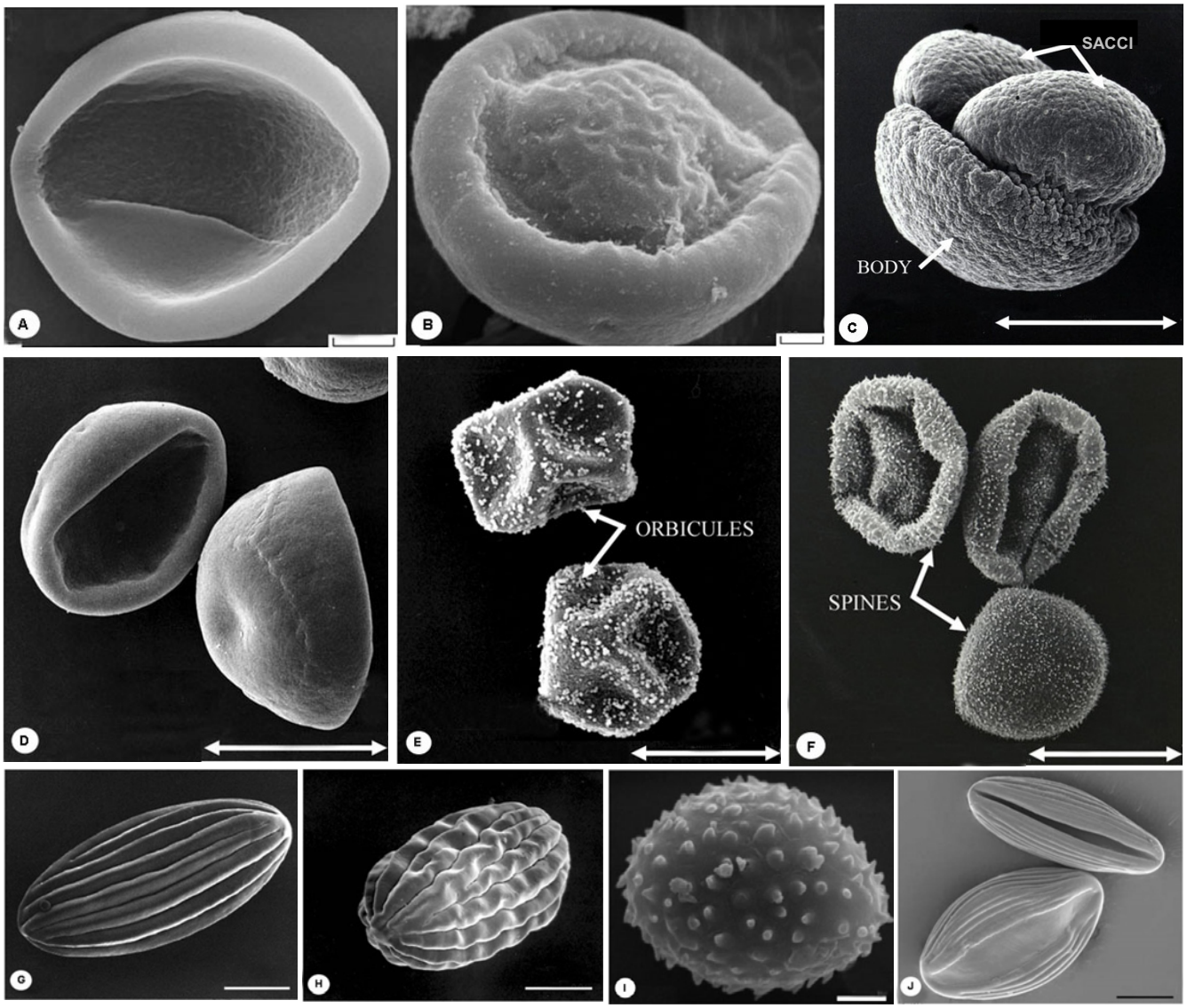


Fig. 1 A-J Scanning electron micrographs of the basic types of pollen grains in gymnosperms. (**A**) *Cycas micholitzii* Dyer (Cycadaceae) pollen grain exhibiting the development of a loop-like pattern on the distal face. Scale bar = 3 µm (from Tekleva *et al.* 2007). (**B**) *Ginkgo biloba* L. (Ginkgoaceae) pollen grain, hydrated, with a protruding aperture and slightly sunken margin. Scale bar = 3 µm (from Tekleva *et al*. 2007). (**C**) *Pinus ponderosa* (Pinaceae) pollen showing sacci and body (corpus) Scale bar = 20 µm (from Fernando *et al.* 2005a). (**D**) *Pseudotsuga menziesii* (Pinaceae) pollen with indentation caused by normal dehydration before shedding. Scale bar = 20 µm (from Fernando *et al.* 2005a). (**E**) *Chamaecyparis nootkatnesis* (Cupressaceae) pollen showing many small orbicules characteristic of the family. Scale bar = 20 µm (from Fernando *et al.* 2005a). (**F**) *Tsuga heterophylla* (Pinaceae) pollen showing spines on exine. Scale bar = 20 µm (from Fernando *et al.* 2005a). (**G-H**) *Ephedra americana* (Ephedraceae) pollen grain showing straight furrows (**G**) and undulated furrows (**H**), both morphologies can occur within the same pollen sac. Scale bar = 10 µm (from Doores *et al.* 2007). (**I**) *Gnetum macrostachyum* Hook. (Gnetaceae) pollen grain showing spinulose sculpture. Scale bar = 3 µm (from Tekleva and Krassilov 2009). (**J**) *Welwitschia mirabilis* Hook. (Welwitschiaceae) pollen grain showing a distinct sulcus. Scale bar = 15 µm (from Rydin and Friis 2005).

in most Podocarpaceae (Salter *et al*. 2002).

The pollen grains of *Ephedra* and *Welwitschia* are ellipsoidal (El-Ghazaly *et al*. 1997; Rydin and Friis 2005), while it is generally subspherical in *Gnetum* (Kurmann 1991; Yao *et al*. 2004) (**Figs. 1G-J**). The pollen wall surface of *Ephedra* and *Welwitschia* are polyplicate, i.e., characterized by a series of longitudinal ridges, while it is spinulate in *Gnetum* (Yao *et al*. 2004). In *Ephedra*, the exine has alternating thick and thin areas, while the cellulose-containing intine is relatively thick and evenly surrounds the entire pollen grain (El-Ghazaly *et al.* 1997; Rydin and Friis 2005). The volumes of *Ephedra* pollen grains have been estimated at 18 μm^3 (Pacini *et al*. 1999) and internal polarity is manifested by the placement of the prothallial cell at the proximal end (El-Ghazaly *et al*. 1997). The pollen grains are inaperturate in *Ephedra* (El-Ghazaly *et al*. 1997) and *Gnetum* (Yao *et al*. 2004), while monoaperturate in *Welwitschia* (Rydin and Friis 2005).

CELLULAR COMPOSITION OF POLLEN GRAINS

In seed plants, development of male gametophytes begins with haploid microspores, which are products of meiosis by microspore mother cells that occur within microsporangia. For general descriptions of the initiation and development of the microsporangium and microsporogenesis in various gymnosperms, readers are referred to Singh (1978) and Gifford and Foster (1989). In gymnosperms, pollen grains are dispersed at the one- to five-cell stage (Fernando *et al*. 2005a), except in podocarps where the prothallial cells may proliferate and so their pollen grains may be shed with as many as 40 cells or nuclei (Chamberlain 1935; Sterling 1963; Quinn 1964; Gifford and Foster 1989; Wilson and Owens 1999).

Terminologies used to describe cellular compositions both within the pollen grain and tubes have been inconsistent among various accounts of male gametophyte development for gymnosperms over the last many decades. This has led to confusion and misinterpretations by authors in

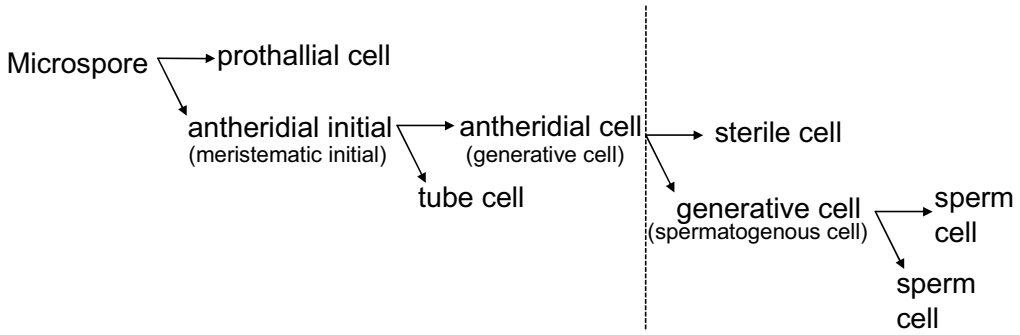


Fig. 2 Male gametophyte development in cycads.

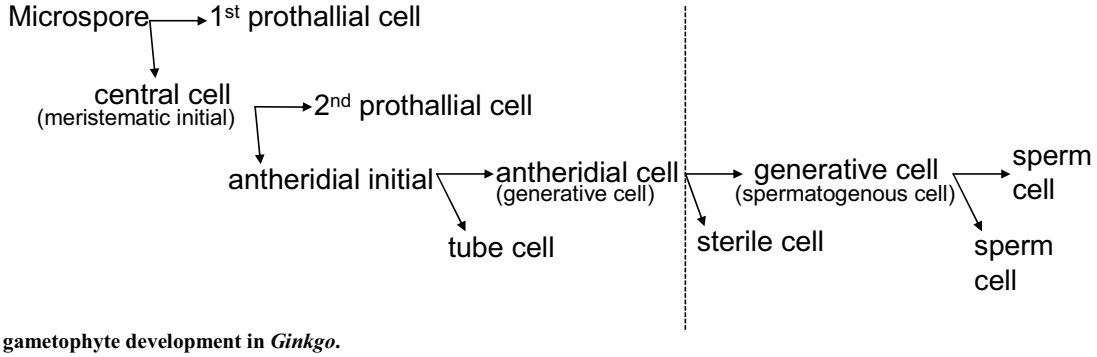


Fig. 3 Male gametophyte development in *Ginkgo*.

many articles over the years (for a complete review, see Singh 1978; Gifford and Foster 1989; Owens and Bruns 2000). In spite of the efforts by many authors (Chamberlain 1935; Sterling 1963; Singh 1978; Gifford and Foster 1989; Owens and Bruns 2000; Fernando *et al*. 2005a) to standardize the terminology, terms used are still not consistent among some authors and even with the same author when describing a different gymnosperm. We believe that terminologies can be standardized and applied to all gymnosperms. Consistent use of terms will facilitate comparison of the whole process of male gametophyte development, not only within the gymnosperms, but also between gymnosperms and angiosperms.

The rationale behind our choice of terminologies is based on the assumption by Friedman and Gifford (1988), that the evolution of the male gametophyte and its sexual organ (antheridium) involved a significant reduction and simplification. In gymnosperms, prothallial cells are among those that have been reduced in number or eliminated. In fact, these cells have been completely eliminated in the male gametophytes of angiosperms. The discussion that follows will show that there has been a further reduction in the number of cells that constitute the male gametophytes of gymnosperms, particularly in some conifers and gnetophytes.

In this review, we present our recommended terminologies for the cells and nuclei involved in the sequence of mitotic divisions during the development of the male gametophytes in gymnosperms (**Figs. 2-10**). The following descriptions incorporate the recommended terms, followed by the previously used terms in parentheses, and where possible the rationale behind the changes. What is being proposed here is not the final solution to the problems described, but if it stimulates discussion on this subject, then this has achieved its goal.

A. In cycads (**Fig. 2**), the microspore undergoes an asymmetric cell division to form a small prothallial cell and a large antheridial initial (meristematic initial). The antheridial initial also divides asymmetrically to form a large tube cell and a small antheridial (generative) cell. Pollen grains are released with the three cells axially aligned with the flattened prothallial cell proximally situated, the antheridial cell centrally placed, and the large tube cell distally positioned (Singh 1978; Gifford and Foster 1989; Norstog 1990; Ouyang *et al*. 2004).

We support the use of the term antheridial initial by Singh (1978) instead of "meristematic initial or cell" by Gifford and Foster (1989). The cell under discussion does not give rise to any prothallial cell and is, therefore, the very first cell of the sperm lineage (or antheridium) and referring to it as the "antheridial initial" is appropriate. Gifford and Foster (1989) used the term "meristematic initial or cell" for the cell under discussion, but they also used it to describe the cell giving rise to the antheridial initial and second prothallial cell, resulting in confusion. Furthermore, their term conflicts with the concept of a meristem, as used to describe the origin of roots, shoots, secondary vascular tissues, and secondary protective tissues. Therefore, to avoid any confusion, we do not recommend the use of the term "meristematic initial or cell."

We also support the term "antheridial cell" by Singh (1978) instead of "generative cell" by Gifford and Foster (1989) to refer to one of the cells (in addition to the tube cell) produced by the division of the antheridial initial. We believe that the term "generative cell" should only be used to describe the cell that gives rise to the sperm.

B. In *Ginkgo* (**Fig. 3**), the microspore undergoes uneven cell division to produce a small and flattened first prothallial cell and a large central cell (meristematic initial). Unequal division of the central cell forms a second small prothallial cell and a large antheridial initial. The latter subsequently divides forming a tube cell and an antheridial (generative) cell, which is in contact with the second prothallial cell. The pollen grains are shed at the four-cell stage, composed of two prothallial cells, an antheridial cell, and a tube cell in an axial row (Singh 1978; Gifford and Foster 1989; Friedman 1987).

We support the use of the term "central cell" as used by Singh (1978) instead of "meristematic initial" according to Gifford and Foster (1989) because the former is descriptive of the position of the cell under discussion, whereas the latter is problematic as described earlier. Singh (1978) used the term "central cell" to replace "embryonal cell" (as used previously by Sterling 1963), which he considered unsuitable since it implies a relationship with the embryo, and we agree.

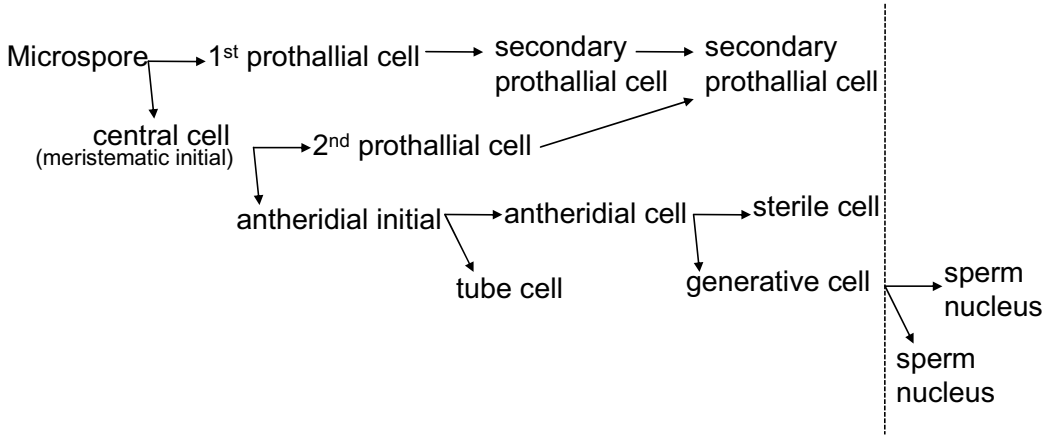


Fig. 4 Male gametophyte development in Podocarpaceae and Araucariaceae.

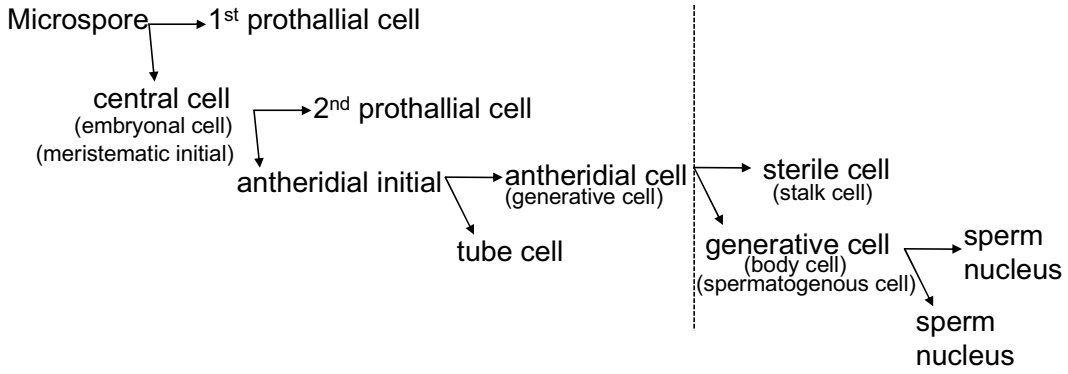


Fig. 5 Male gametophyte development in Pinaceae.

Four patterns of cell division are recognized in conifer pollen grains (Owens and Bruns 2000; Fernando *et al*. 2005a), with some families containing different numbers of cells at dehiscence (**Figs. 4-7**). The following sequence of conifer families is based in the complexity of cell divisions starting from those families with the greatest number of cell divisions prior to dehiscence and sperm formation. This sequence of conifer families will be used from this point on.

C. In Podocarpaceae and Araucariaceae (**Fig. 4**), the sequence of cell divisions is essentially similar to *Ginkgo* except that the first and second prothallial cells may undergo further divisions resulting in a variable number of prothallial cells or nuclei, which are appropriately referred to as primary and secondary prothallial cells, respectively. The term "primary prothallial cells" is used to differentiate these cells from those that they produce, i.e., the "secondary prothallial cells".

In *Araucaria*, prothallial cells form a tissue-like tier of cells and subsequently their cell walls and membranes break down resulting in many free prothallial nuclei in the tube cell cytoplasm. In *Podocarpus*, six to eight prothallial cells are formed (Quinn 1964; Wilson and Owens 1999), while as many as 40 cells or nuclei are formed in *Agathis* (Chamberlain 1935; Sterling 1963; Gifford and Foster 1989). According to Owens *et al*. (1995), most prothallial cells in *Agathis* are bound by cell membranes and not cell walls, and appear almost as free nuclei. The unequal division of the antheridial initial then forms a large tube cell and a smaller antheridial cell. The antheridial cell divides equally to form a sterile cell and a generative cell. Therefore, the pollen grains are typically shed with five cells, in addition to a variable number of prothallial cells or nuclei (Singh 1978; Gifford and Foster 1989; Owens *et al*. 1995; Wilson and Owens 1999).

D. In Pinaceae (**Fig. 5**), the sequence of cell divisions is similar to Podocarpaceae and Araucariaceae except for the lack of secondary prothallial cells. The microspore divides unequally to form a small first prothallial cell and a large central cell (embryonal cell, meristematic initial). The central cell then divides unequally to form a small second prothallial cell and a larger antheridial initial. The second prothallial cell is stacked on top of the first prothallial cell and these two lens-shaped prothallial cells are pushed to the proximal side of the pollen grain where they both become enclosed within the intine. They do not undergo further division. The antheridial initial then divides unequally to form a large tube cell and a small antheridial (generative) cell. The antheridial cell divides to form a sterile (stalk) cell and a generative (body or spermatogenous) cell. The pollen grains are shed at the four- (Singh 1978; Gifford and Foster 1989) or five-cell stage (Pettitt 1985; Said 1989; Owens and Bruns 2000; Fernando *et al*. 2005a).

Singh (1978) referred to the stalk cell in Pinaceae as such because of its position between the second prothallial cell and the generative cell. However, its position relative to the generative cell is reversed in the cypress *Athrotaxis* (Brennan and Doyle 1956). Gifford and Foster (1989) believed that the term suggests an unproved homology with the stalk of the antheridium of bryophytes and some leptosporangiate ferns. On the other hand, since the "stalk cell" in some gymnosperms is nonfunctional and therefore, sterile, it was called a "sterile cell" by Sterling (1963) and Gifford and Foster (1989), which we also consider as more appropriate. In addition, similar sterile cells occur in other conifers where they have no consistent position and may be free within the pollen tube cell cytoplasm. An exception to the concept of a sterile cell occurs in the cycad *Microcycas*, where the cell under discussion divides repeatedly and eventually giving rise to many sperm (Downie 1928). The term "secondary prothallial cell" has also been suggested by Singh (1978) to describe the cell under discussion, but this term implies that it is derived from other prothallial cells, which is incorrect. The term "sterile cell" presents fewer problems and was preferred by Owens and Bruns (2000) and Fernando *et al*. (2005a), and therefore, we suggest continuing the use of this term.

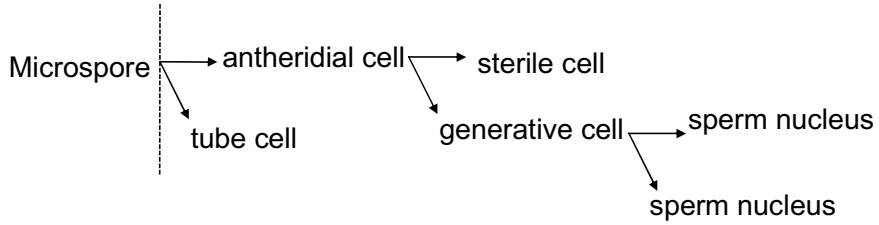


Fig. 6 Male gametophyte development in Taxaceae and Cephalotaxaceae.

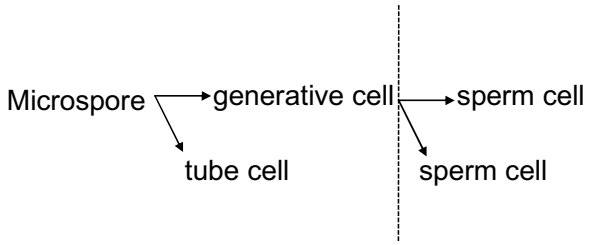


Fig. 7 Male gametophyte development in Cupressaceae.

In early gymnosperm literature as discussed by Singh (1978), the term "body cell" was used for the cell that formed two sperm. However, this term has no functional or positional meaning and we recommend that throughout the gymnosperms that the term "generative cell" be used for the cell that divides to form the two sperm, in consonance with angiosperms. Singh (1978) considered the terms "body cell", "spermatogenous cell", and "generative cell" as being morphologically equivalent. Rudall and Bateman (2007) also considered the terms "spermatogenous cell" and "generative cell" as equivalent. Although Gifford and Foster (1989) considered the term "spermatogenous cell" to be more explicit than generative cell in denoting the function of the cell under discussion, several authors (Southworth and Cresti 1997; Owens and Bruns 2000; Fernando *et al.* 2005a) have begun to use the term "generative cell" for the cell in gymnosperms that forms the two sperm. Since this is consistent with the terminology used for angiosperms, we recommend that this trend be continued.

E. In Taxaceae and Cephalotaxaceae (**Fig. 6**), the microspores do not divide prior to being shed. Therefore, at pollination, microspores are also regarded as pollen grains, which are shed at the one-cell stage (Anderson and Owens 2000; Wang *et al*. 2008).

F. In Cupressaceae (**Fig. 7**), the microspore divides asymmetrically to form a large tube cell and a smaller generative cell. Cell divisions involved in the formation of the central cell, antheridial initial, and antheridial cell appear to have been eliminated. Therefore, the pollen grains are shed at the two-cell stage (Singh 1978; Fernando *et al*. 2005a; Chichiricco and Pacini 2008).

G. In *Ephedra* (**Fig. 8**), the pattern of male gametophyte development is most similar to *Ginkgo* and Pinaceae. A major difference from these gymnosperms is that during the formation of the so-called second prothallial cell, no cell wall or membrane is laid down to separate it from the antheridial initial and therefore, only a prothallial nucleus is formed (Friedman 1990b). Another difference is that both the first formed prothallial cell and the prothallial nucleus begin to break down soon after their formation (Singh 1978; Gifford and Foster 1989; Friedman 1990b). Division of the antheridial initial forms a tube cell and an antheridial (generative) cell. The latter divides to form a sterile nucleus and a generative (spermatogenous) nucleus, which are not separated by a cell wall or membrane and thus, should be referred to as nuclei that share a common cytoplasm (Friedman 1990b). Therefore, at dehiscence, the pollen grains consist of two cells and three nuclei.

H. In *Gnetum* and *Welwitchia* (**Fig. 9**), the microspore divides unequally to produce a small prothallial cell and a large antheridial cell (antheridial initial). The latter divides

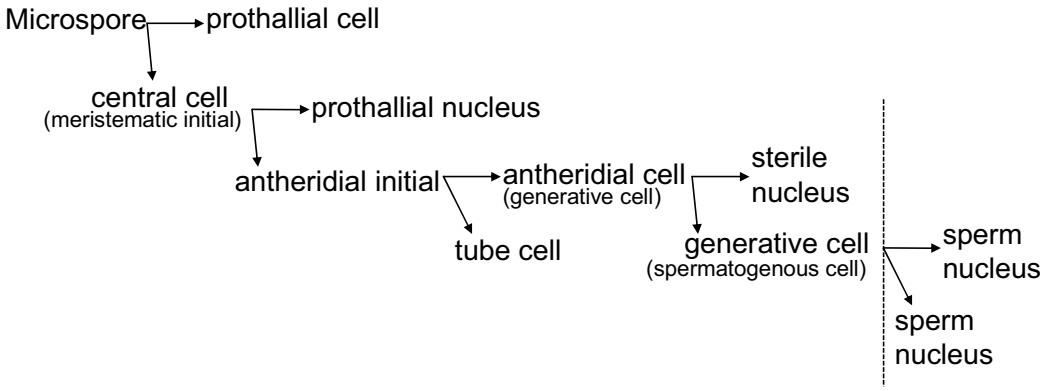


Fig. 8 Male gametophyte development in *Ephedra*.

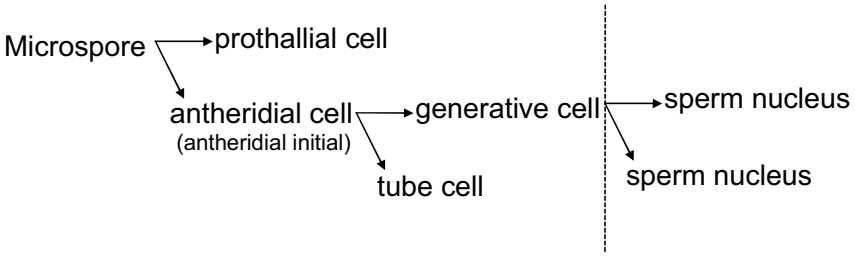
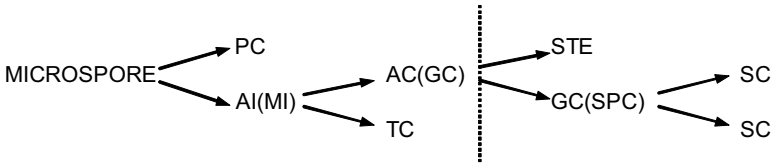
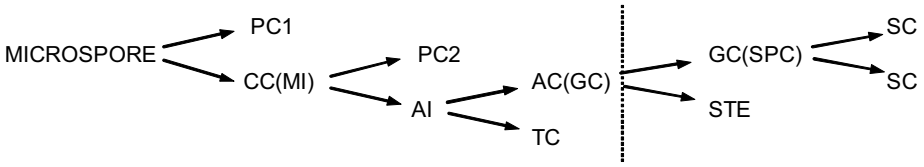


Fig. 9 Male gametophyte development in *Gnetum* and *Welwitchia*.

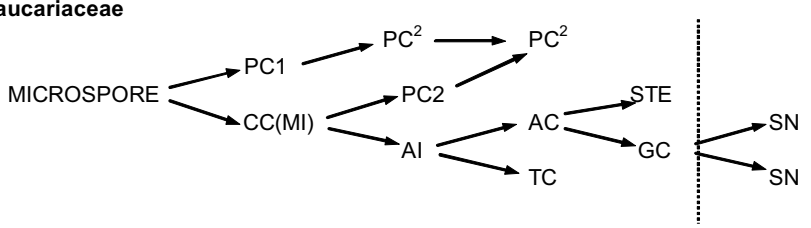
A. Cycads



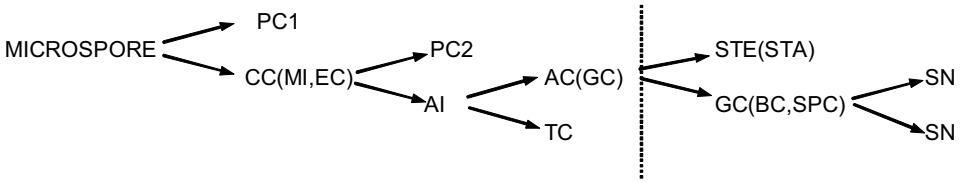
B. Ginkgo



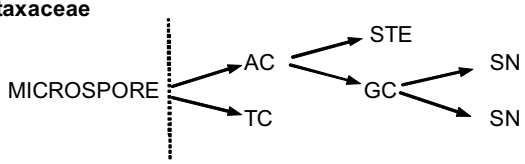
C. Podocarpaceae and Araucariaceae



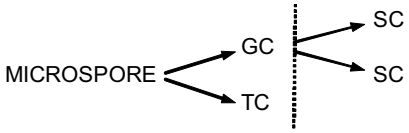
D. Pinaceae



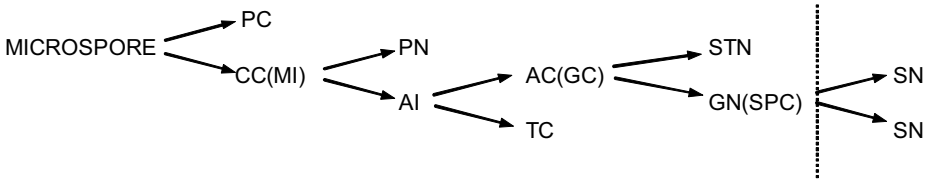
E. Taxaceae and Cephalotaxaceae



F. Cupressaceae



G. Ephedra



H. Gnetum and Welwitschia

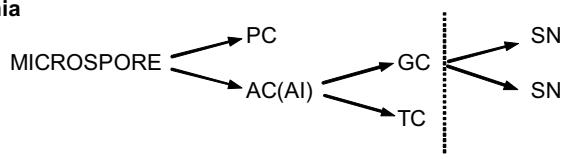


Fig. 10 Sequence of cell divisions, using proposed standard terminology, in male gametophyte development in extant gymnosperms. The previous terminology is shown in parentheses. The dashed lines indicate the stage of pollen grain dispersal. **AI**, antheridial initial; **BC**, body cell; **CC**, central cell; **EC**, embryonal cell; **GC**, generative cell; **MI**, meristematic initial; **PC1**, first prothallial cell; **PC2**, second prothallial cell; **PC^2**, secondary prothallial cell; **PN**, prothallial nucleus; **SC**, sperm cell; **SN**, sperm nucleus; **STA**, stalk cell; **SPC**, spermatogenous cell; **STE**, sterile cell; **STN**, sterile nucleus; **TC**, tube cell.

to form a tube cell and a generative cell. As compared to *Ephedra*, only the cell divisions involved in the formation of the central cell and antheridial initial appear to have been eliminated in *Gnetum* and *Welwitschia*. Therefore, the pollen grains in these genera are shed at the three-cell stage (Singh 1978; Carmichael and Friedman 1995).

Based on the above, two patterns of cell division are recognized in the pollen grains of Gnetophytes (Singh 1978; Gifford and Foster 1989), with the three genera containing different numbers of cells and nuclei at pollination.

Fig. 10 is provided to facilitate comparison of the sequences of mitotic divisions during the development of the male gametophytes in all four living phyla of gymnosperms.

POLLEN GERMINATION AND SPERM FORMATION

A major difference in development of male gametophytes in gymnosperms and angiosperms is that in the former, no pollen grains are shed with sperm already present. Therefore, as far as we are aware, sperm formation in gymnosperms occurs exclusively after pollination. To emphasize this difference, the description of the development of male gametophytes after pollination is separated from the description of the development before pollination, and therefore, provided here under a different heading. In this case, some overlaps will be noticed but are intentional.

After pollination, pollen grains germinate outside or inside the ovule through various mechanisms. For detailed description of this subject, readers are referred to Singh (1978), Tomlinson (1994), Owens *et al*. (1998) and Labandeira *et al*. (2007). Post-pollination development of the male gametophyte is manifested by the formation and elongation of the pollen tube. At this stage, prothallial and sterile cells in some genera may migrate into the elongating pollen tube; however, the information on this subject is incomplete to draw any meaningful generalizations. Most authors did not provide information on the activity of prothallial cells probably because these cells do not have any known function in gymnosperms. The discussion on these cells will be limited to a few well-established examples. There is confusion as to the nature of the sperm in gymnosperms. To facilitate our understanding of this subject, examples will be described to show which gymnosperms have sperm cells and which ones have sperm nuclei. Therefore, this section will focus on the activity of the generative cell, time of sperm formation and nature of the sperm (**Fig. 11A-I**).

A. In cycads (**Fig. 2**), as pollen grains are brought into the pollen chamber at the tip of the nucellus, they absorb water and nutrients from the pollination drop. This hydration stimulates germination within the chamber, which may not occur until months after pollination. After the pollen tube forms from the three-cell pollen grain, the antheridial cell divides to produce a sterile cell and a (spermatogenous) generative cell, after which the prothallial cell enlarges causing it to press against the sterile cell (Swamy 1948; Ouyang *et al*. 2004). Division of the generative cell results in the formation of two sperm cells (**Fig. 11A**) with approximately 40,000 flagella each formed by a cluster of blepharoplasts; the sperm cells only have cell membranes and their nuclei comprise the bulk (Norstog *et al*. 2004; Rudall and Bateman 2007).

It is interesting to note that in *Microcycas*, the so-called sterile cell may divide to form rows of generative cells that develop into at least sixteen flagellate sperm cells (Downie 1928; Norstog 1990). It has been suggested that polyspermy in *Microcycas* reflects a plesiomorphic state in male gametophyte evolution (Norstog 1990; Norstog and Nicholls 1997). However, morphological and molecular evidences have shown that *Cycas* is the basal genus (Stevenson 1992; de Laubenfels 1999; Hill *et al*. 2003) implying that polyspermy is derived. In fact, polyspermy has been regarded as atavistic reversal (Norstog *et al*. 2004; Rudall and Bateman 2007).

B. In *Ginkgo* (**Fig. 3**), about a week after pollen grains are drawn into the ovule, germination occurs (Friedman 1987). When the four-cell pollen grain germinates, the first prothallial cell aborts while the second prothallial cell remains intact. The antheridial cell divides to produce a sterile cell and a generative (spermatogenous) cell. The latter is situated between the second prothallial cell and the sterile cell. The generative cell is believed to share a common cell wall with the second prothallial cell (Lee 1955). During sperm formation, the generative cell enlarges and two blepharoplasts form, where one is included in each of the two sperm cells (**Fig. 11B**). In each of the sperm cells, the blepharoplast gives rise to the flagellar apparatus that contains approximately 1000 flagella (Gifford and Lin 1975). The two sperm cells are contained within the generative cell cytoplasm, but are eventually released (Singh 1978; Gifford and Larson 1980; Friedman 1987).

There are at least two differences between the generative cells of *Ginkgo* and cycads. In *Ginkgo*, the cytoplasm contains a lens-shaped nucleus flanked by a pair of globular or osmiophilic bodies (Gifford and Lin 1975), while in cycads, nuclei are spherical with no such bodies observed (Norstog and Nicholls 1997).

In conifers (except Taxaceae and Cephalotaxaceae) and gnetophytes, as compared to the cycads and *Ginkgo*, the only cell division that occurs during post-pollination development of the male gametophytes is the formation of the sperm from the generative cell (**Figs. 4-9**). The pollen grains in conifers germinate upon hydration followed by the shedding of the exine or penetration of the pollen tube through a thin area in the exine (Owens *et al*. 1998). Pollen germination occurs one (Fernando *et al*. 2005b) to several weeks (Owens *et al*. 1994; Takaso and Owens 1997) after pollination.

C. In Podocarpaceae and Araucariaceae (**Fig. 4**), during germination of the typical five-cell pollen grain, the nucleus of the generative cell divides to form two sperm nuclei (**Fig. 11C-D**), which remain within the generative cell cytoplasm. Two differences occur within the Araucariaceae but not in the Podocarpaceae: 1) the generative cell that enters one of the branches leading to an archegonium divides to form two equal-size sperm nuclei that are contained within the generative cell cytoplasm; and 2) during sperm formation, the two nuclei that are formed engulf the generative cell cytoplasm and its organelles forming two large complex sperm nuclei (Owens *et al*. 1995). The latter has not been reported in Podocarpaceae and that the sperm nuclei that are formed are unequal in size (Wilson and Owens 1999).

D. In Pinaceae (**Fig. 5**), after germination of the five-cell pollen grain, the nucleus of the generative cell divides to form two sperm nuclei that remain inside the generative cell until the pollen tube enters the egg cell. In most species studied, no cell wall or membrane is formed between the two sperm nuclei (**Fig. 11E**) and, therefore, they share the organelles of the generative cell (Singh 1978; Owens and Bruns 2000). In *Picea* (**Fig. 11F**), only a partial cell wall is formed between the two sperm nuclei (Dawkins and Owens 1993; Runions and Owens 1999).

E. In Taxaceae and Cephalotaxaceae (**Fig. 6**), the one-cell pollen grain upon entering the pollination drop germinates and then divides to form a large tube cell and a smaller antheridial cell. The latter then divides to form a sterile cell and a generative cell. These cells are both contained within the elongating pollen tube (Anderson and Owens 2000; Wang *et al*. 2008). In these families, the prothallial cells, central cell and antheridial initial appear to have been eliminated. During further pollen tube development, the generative nucleus divides to form two sperm nuclei (**Fig. 11G**) that remain closely associated within the cytoplasms of the generative and sterile cells (Pennel and Bell 1986; Anderson and Owens 1999, 2000; Wang *et al*. 2008). In some cases, this association occurs even after the disintegration of the cell walls and membranes of the generative and sterile cells (Anderson and Owens 2000; Wang *et al*. 2008). Therefore, the sperm in these families are also nuclei that are surrounded typically by the generative cell cyto-

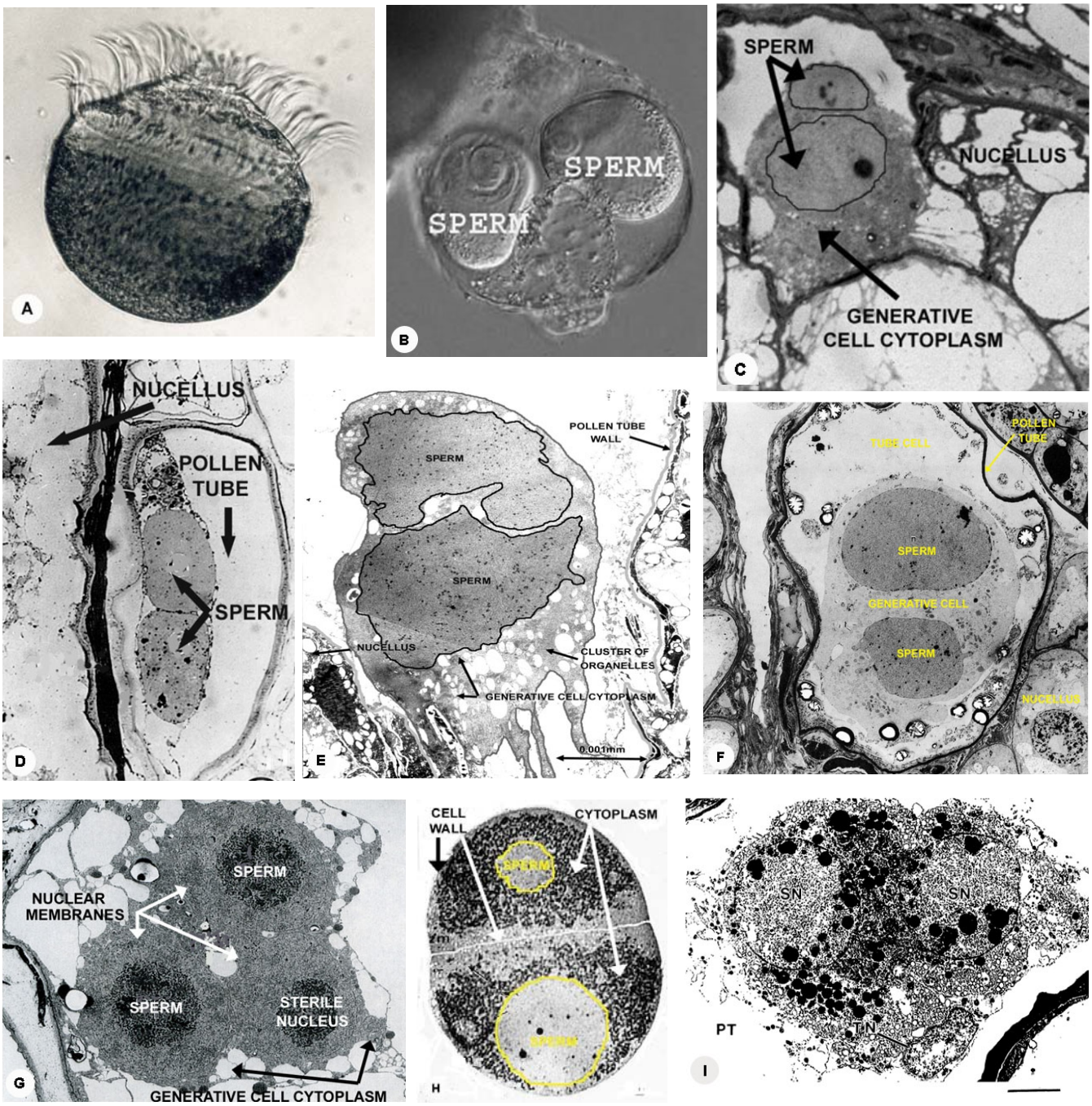


Fig 11A-I Sperm cells/nuclei of various gymnosperms (**A**) *Cycas* sperm cell showing supernumerary flagella. (**B**) *Ginkgo biloba* sperm cells showing the flagellar apparatus. (**C**) Podocarpaceae sperm nuclei, unequal in size, maintained within the generative cell cytoplasm. (**D**) Araucariaceae sperm nuclei, equal in size, engulfing the generative cell cytoplasm and its organelles. (**E**) *Pinus* sperm nuclei remain in the generative cell, as no wall forms between them. (**F**) *Picea* sperm nuclei, a partial cell wall forms between the nuclei maintained within the generative cell. (**G**) *Taxus* sperm nuclei showing a close association with the cytoplasm of the generative and sterile cells. (**H**) *Cupressus* sperm cells, note the cell wall formed between them. (**I**) *Gnetum* sperm nuclei, typical of the Gnetales, which remains in the generative cell cytoplasm (**PT**- pollen tube, **SN**- sperm nucleus, **TN**- tube nucleus) (from Carmichael and Friedman 1996).

plasm.

F. In Cupressaceae (**Fig. 7**), after the two-cell pollen grain has germinated, the generative cell divides to form two nuclei and each becomes surrounded by cell walls and membranes forming two large equal-size sperm cells (**Fig. 11H**) that contain abundant organelles (Singh 1978; Fernando *et al*. 2005a). Therefore, unlike the sperm in other conifer families, which are made up of nuclei, sperm in Cupressaceae are made up of cells.

The development of the male gametophyte in Cupressaceae parallels that of angiosperms, particularly the eudicots. Considering that this family is one of the more derived groups in conifers (Chaw *et al*. 1997; Stefanovic *et al*. 1998) and not closely related to any of the angiosperms, such similarity in the development of the male gametophytes suggests a case of convergent evolution.

In gnetophytes, the time between pollination and pollen germination is very brief, lasting only hours to a few days (Gifford and Foster 1989; Friedman 1990b; El-Ghazaly *et al*. 1997; Rydin and Friis 2005). This timeframe for pollen germination is very similar to the angiosperms.

G. In *Ephedra* (**Fig. 8**), the exine is completely shed before pollen germination (El-Ghazaly *et al*. 1998). Germination of the five-cell pollen grain is followed by the division of the generative cell nucleus to yield two sperm nuclei that are contained within the generative cell. The generative cell containing the two sperm nuclei becomes tightly appressed to the sterile cell (Friedman 1990b).

H. In *Gnetum* and *Welwitschia* (**Fig. 9**), the prothallial cell degenerates before or after germination of the three-cell pollen grain. In the pollen tube, division of the generative cell nucleus produces two sperm nuclei (**Fig. 11I**), which also remain within the generative cell cytoplasm. The generative cell containing two sperm nuclei becomes situated adjacent to the tube nucleus (Carmichael and Friedman 1995, 1996).

Based on the complete development of the male gametophytes, *Ephedra* exhibits a pattern that is distinct from those of *Gnetum* and *Welwitschia*. This trait adds further support to the position of *Ephedra* as a sister group to the *Gnetum-Welwitschia* clade (Doyle and Donoghue 1992; Goremykin *et al*. 1996; Bowe *et al*. 2000; Chaw *et al*. 2000; Rydin *et al*. 2006).

GROWTH OF POLLEN TUBES IN THE OVULE

In most gymnosperms, pollen grains are taken into the ovule, usually by a pollination drop, and sink or are drawn to the surface of the nucellus where they germinate. The pollen tube is either zooidogamous, as in cycads and *Ginkgo*, acting as a haustorial organ and not directly responsible for sperm delivery to the egg cell, or siphonogamous, as in conifers and gnetophytes, delivering the sperm directly into the egg (Poort *et al*. 1996; Rudall and Bateman 2007). The haustorial and branching nature of the pollen tubes in the cycads and *Ginkgo* may have been the result of the shift in habit of the male gametophyte from its prior free-living existence (as seen in bryophytes, lycophytes and pteridophytes) to one within the tissues of the sporophyte (Poort *et al*. 1996; Rudall and Bateman 2007). During early evolution of seed plants, it has been speculated that the "invasion" of the pollen tube into the nucellus may have elicited a host-pathogen response in the sporophyte (Choi and Friedman 1991). As a result, the pollen tube may have relied on branching and intracellular growth to gain nutrients. In the more derived groups, conifers and gnetophytes, the sporophyte may eventually have come to facilitate and assist the growth and development of the pollen tube thus, branching was reduced or lost (Choi and Friedman 1991).

A. In cycads, pollen germination involves exine rupture followed by the initiation of the pollen tube from the distal end of the pollen grain, which occurs by means of a protrusion of the intine through the germinal furrow of the monosulcate pollen grain (Pettitt 1982). The tube nucleus migrates into the elongating pollen tube, which begins to grow between degenerated nucellar cells lining the pollen chamber. As the pollen tube penetrates the nucellus, which is rich in starch, the intrusive pollen tube displaces adjacent nucellar cells and the pollen tube becomes surrounded by a single layer of crushed nucellar cells. The abundance of nutrients in the nucellus may cause a response in the pollen tube, influencing the direction of its growth away from the archegonia. Upon reaching the subepidermal layer of the nucellus, the pollen tube becomes quite broad and at some point begins to produce horizontal outgrowths that penetrate individual nucellar cells, ultimately causing necrosis of these cells. As the pollen tube elongates, it accumulates well-developed starch grains, while starch grains are nearly absent in the adjacent nucellar cells but abundant in the nucellar cells five or six cell layers away from the pollen tube. This suggests that the pollen tube absorbs substances from the surrounding sporophytic tissue to sustain its growth. This also suggests that enzymes secreted from the pollen tube outgrowths facilitate its growth and passage through the nucellus by digesting cell walls and protoplasts. Although it has not been demonstrated *in vivo*, Choi and Friedman (1991) detected polygalacturonases, cellulases, and other hydrolytic enzymes in the cycad pollen tube. Overall, the significant destruction of nucellar cells may compensate for the low surface area to volume ratio of the pollen tube, leading to greater access to nutrients (Choi and Friedman 1991). During pollen tube growth into the nucellus, the pollen chamber enlarges to form a cavity above the archegonial canal. The pollen tube at the proximal end of the pollen grain, where the prothallial and antheridial (generative) cells are located, enters this cavity and becomes swollen pushing the cells towards the pollen chamber. Here, the antheridial cell divides to ultimately produce the two multi-flagellate sperm cells. The swollen area of the pollen tube eventually ruptures and the sperm cells exit through a small orifice and proceed to swim to the egg cell (Swamy 1948). The growth of the pollen tube in the nucellus is typically intercellular. However, the pollen tube also undergoes intracellular growth by production of localized outgrowths unique to this group.

B. In *Ginkgo*, development of pollen tubes occurs in three phases (Friedman 1987; Friedman and Gifford 1997): 1) initial diffuse and roughly isodiametric growth that starts at the distal end of the pollen grain; 2) tip growth during which the originally straight pollen tube branches extensively between the nucellar cells at the apex of the nucellus; and 3) the unbranched proximal end of the pollen tube swells into a large bulbous structure. Therefore, pollen germination, as manifested by the swelling of the tube cell through the sulcus, results in the formation of a slightly bulbous projection that extends outward to form the pollen tube. The pollen tube elongates, unbranched, and grows between the nucellar cells lining the pollen chamber, causing no significant physical disruption to the adjacent nucellar cells. Upon entering the nucellus, multiaxial growth begins; the tip of the pollen tube gives rise to numerous haustorial branches that begin to ramify within the intercellular spaces of the nucellus. This growth is considered to be extremely rapid and the haustoria diameters conform to the dimensions of the intercellular space of the nucellus, and proliferate in all directions. Like in cycads, there are no nuclear or cellular divisions associated with pollen tube branching; the entire structure is continuous, formed from the single tube cell. In *Ginkgo*, the tube nucleus, however, does not migrate into any of the branches, but remains in the unbranched portion of the pollen tube just above the point of elaboration (Friedman 1987). Unlike cycads, *Ginkgo* does not appear to exhibit enzymatic activity to breakdown nucellar cells, which likely results in limited exposure to nutrients. Yet, the high surface area to volume of the branched pollen tube may compensate. Following branching, increased radial growth occurs in the pollen tube at the proximal end of the pollen grain. The swollen pollen tube develops into a large bulbous structure that fills the pollen chamber and is suspended from the nucellus just above the archegonia. This pattern of swelling is similar to the later stages of development as seen in cycad pollen tubes. The swollen portion of the pollen tube, situated just above archegonia containing the egg cells, eventually ruptures, releasing the motile sperm cells (Friedman 1993). Several pollen grains may germinate in the pollen chamber; however, once the bulbous region of one of the pollen tube ruptures and releases the sperm cells, the sperm cells within the generative cell wall of the other pollen tubes do not rupture, they instead degenerate (Lee 1955).

C. In conifers, mechanisms by which pollen grains reach the nucellus are quite diverse (Singh 1978; Tomlinson 1994; Owens *et al*. 1998; Labandeira *et al*. 2007). In the Cupressaceae, Taxaceae, Cephalotaxaceae, and some Podocarpaceae where the ovules are variably oriented, non-saccate pollen grain sinks in the pollination drop and down to the surface of the nucellus where it germinates. The pollen tube grows into the nucellus then to an archegonium (Owens *et al*. 1998; Fernando *et al*. 2005a). In contrast, in some Pinaceae (e.g., *Pinus*, *Picea*, *Cedrus*, and some *Tsuga* species) and some Podocarpaceae, in which the ovules are inverted, pendant pollination drops form and extend out of the micropyle. The saccate pollen grains float up through the micropyle and up through the micropylar canal to the tip of the nucellus (Runions *et al*. 1995; Owens *et al*. 1998). There, they germinate with their pollen tubes growing through the nucellus and into an archegonium (Owens *et al*. 1998). In *Abies*, the pollen grain germinates in the micropylar canal

and its pollen tube grows towards the nucellus as the nucellus grows towards it, with the two structures meeting about midway along the micropylar canal (Owens and Molder 1977; Chandler and Owens 2004). In *Pseudotsuga*, the pollen grain sheds its exine near the micropyle and elongates along the length of the micropylar canal. A thin pollen tube forms after the elongated pollen grain contacts or nearly contacts the nucellus, then the pollen tube grows through the nucellus and to an archegonium (Owens *et al*. 1981). Unlike the above taxa, the pollen grains in some *Tsuga* species in the Pinaceae (Colangeli and Owens 1989) and all species of Araucariaceae (Owens *et al*. 1995) germinate on the surface of a bract, scale or integument and the pollen tubes grow into the micropyle, nucellus and eventually into an archegonium. In *Agathis* (Araucariaceae), pollen grains land and germinate on the nucellar tip that grows out through the micropyle. Once within the nucellus, the pollen tube in the Araucariaceae forms many long branches; one of these branches contains the generative cell and grows into one of the several separate archegonia (Owens *et al*. 1995).

Slightly branched pollen tubes occur sporadically in various conifers. In *Pinus contorta*, many short branches (about 10 nucellar cells long) form after pollination and during the first year of cone development (de Win *et al*. 1996; Owens *et al*. 2005). The pollen tube appears to be haustorial but the tube nucleus usually enters one of the longer branches before the cone becomes dormant. Following winter dormancy, the branch containing the tube nucleus elongates more rapidly than the other branches and within a few weeks reaches an archegonium (Owens *et al*. 2005). The pollen tube branches in pines are less extensive as compared to those in cycads and *Ginkgo* and some members of the Araucariaceae (Owens *et al*. 1995). This indicates that any haustorial role in conifer pollen tubes is at best secondary (Rudall and Bateman 2007).

Pollen tubes may develop from both the proximal and distal ends of pine pollen grains, but only the pollen tube at the distal end develop further, just like in other conifers (Fernando *et al*. 2005a). Conifer pollen tubes grow through the nucellus prior to egg maturation (Takaso *et al*. 1996). Fertilization occurs as little as a few weeks after pollination in most Cupressaceae and Pinaceae (Bruns and Owens 2000), but about a year after pollination in *Pinus* and some Araucariaceae (Owens *et al*. 1995). The growth of the pollen tube appears to be intercellular within the nucellus; however, it is accompanied by modest degeneration of adjacent nucellar cells. These cells likely degenerate due to cell wall-affecting enzymes secreted by the pollen tube that enable the apex of the tube to push cells aside as it penetrates through the nucellus (Friedman 1993). The death of the nucellar cells facilitates the passage of the growing pollen tube tip and their cellular contents may be utilized by the developing pollen tube (Hiratsuka *et al*. 2002). In conifers, proteins from pollen grain walls and pollen tubes are believed to be involved in cellular degeneration (Pettitt 1985) and stress/defense responses among others (Fernando 2005). In *Pinus contorta*, each of the many short pollen tube branches contacts several nucellar cells which are believed to trigger their collapse. The short elongating pollen tube branches occupy the spaces left by the collapsed nucellar cells (Owens *et al*. 2005).

D. In *Ephedra* and *Gnetum*, after the pollen grains are brought into the ovule, they shed their exine upon germination (El-Ghazaly *et al*. 1997). In *Welwitschia*, the sulcus splits open and the pollen tube extends out of the exine, which is not shed but remains as a cap-like structure partly covering the developing pollen tube (Rydin and Friis 2005). There are fewer studies on the development of the male gametophyte in gnetophytes compared to other gymnosperms, yet it appears that their pollen tubes are simple and unbranched. The pollen tubes arise only from the distal end of the pollen grain. As the pollen tube elongates, it passes through the intercellular spaces between the nucellar cells without disrupting their cellular integrity. In *Ephedra*, when the pollen tube arrives at an archegonium, a vacuolated region (fertilization chamber) forms at the apex into which the pollen tube releases the tube nucleus, sterile cell, and the two sperm nuclei. The two sperm nuclei enter the egg cytoplasm, one fuses with the egg nucleus and the second with a sister nucleus, the ventral canal nucleus, which results in a double fertilization event unique among the gymnosperms (Friedman 1990a, 1990b). In *Gnetum* and *Welwitschia*, the nuclei within the female gametophytes do not undergo differentiation and therefore, archegonia are not produced (Singh *et al*. 1978). In all gnetophytes, multiple pollen tubes may develop and reach the archegonia or female gametophytes. In *Ephedra*, only the first pollen tube to reach an archegonium will release the sperm nuclei; the supernumerary pollen tubes do not grow further and their contents become impounded in the fertilization chamber. However, in *Gnetum*, multiple fertile nuclei are available in the female gametophyte for fertilization since no egg cell differentiates and, therefore, many or all of the pollen tubes can successfully develop and deliver sperm nuclei for fertilization (Carmichael and Friedman 1996). In *Welwitschia*, multiple fertile nuclei are also available for fertilization, but these are contained in prothallial tubes that elongate from the micropylar end of the female gametophyte to about halfway through the nucellus (Singh 1978).

The time interval between pollination and fertilization (fertilization internal) in gymnosperms ranges from 10 h to more than 12 mo. In general, pollen hydration and germination takes two or more days, and the period of active growth of the pollen tube five days or more. Except for *Gnetum* and *Ephedra*, which have a fertilization interval of 6-8 days and 10-36 h, respectively, the fertilization interval in gymnosperms is much longer as compared to angiosperms (15 min to >12 mo). In *Ephedra*, this short interval is accounted for by the shortening of the pollen tube pathway and a decrease in the pollen germination time of about 1-2 h (Williams 2008). However, like other gymnosperms, the rate of pollen tube growth, ~14 μm/h, *in vivo*, is slow compared to that observed in angiosperms, which ranges from ~80-600 μm/h. The maximum rates of pollen tube growth in other gymnosperms have been measured *in vivo*: *Zamia*, ~1 μm/h; *Ginkgo*, 2 μm/h; *Agathis*, 6 μm/h; and *Gnetum*, 5 μm/h; and *in vitro*: *Pinus*, <1 μm/h (Williams 2008).

COMPOSITION OF INTINE AND POLLEN TUBE WALL

A few reports have focused on the chemical composition of the intine and pollen tube wall in gymnosperms, and the most comprehensive of these is that of Yatomi *et al*. (2002). These authors examined 14 species belonging to eight genera and six families representing cycadophytes, ginkgophytes, and coniferophytes to localize arabinogalactan proteins (AGPs), β-glucans (cellulose and callose) and pectins (acidic and esterified) in both the intines and pollen tube walls. Their results suggest that intines and pollen tube walls of all the gymnosperms examined have AGPs and cellulose. Derksen *et al*. (1999) has reported that the cellulose in the pollen tube tip of *Pinus sylvestris* is less dense as compared to the rest of the pollen tube wall and that cellulose microfibrils are oriented parallel and transverse to the elongation axis of the pollen tube. This orientation is believed to prevent radial expansion but favor elongation. Staining for cellulose in the pollen tubes of other conifers also shows that it is distributed throughout the pollen tube walls including the tip (Lazzaro *et al*. 2003; Sheng *et al*. 2006).

The occurrence of callose appears to be variable in conifers. Yatomi *et al*. (2002) showed that callose MAb strongly labeled the outer layer of the intines of all the six pine species, *Podocarpus macrophyllus, Cryptomeria japonica* and *Chamaecyparis obtusa*; strong labeling in the pollen tube wall was only observed in *Cryptomeria japonica*, but slight labeling of the pollen tube wall was also observed in most of the conifers examined. Using aniline blue to detect callose, only the pollen tube walls of *Podocarpus*

nagi and *Chamaecyparis obtusa* showed positive reactions (Yatomi *et al*. 2002). Also based on aniline blue staining, Derksen *et al*. (1999) showed that in *P. sylvestrus*, callose is present in the pollen tube tip and younger parts of the pollen tube, but not in the older and perhaps, none elongating part of the pollen tube. In germinating *Cupressus arizonica* pollen, aniline blue staining was observed in the intine, pollen tube wall, and wall separating the generative and tube cells (Chichiricco *et al*. 2009).

Acidic and esterified pectins have been localized in the pollen tube walls and tips, respectively, in *P. sylvestris* (Derksen *et al*. 1999) and *Picea wilsonii* (Sheng *et al*. 2006). From the results of Yatomi *et al*. (2002), only *P. nagi* showed strong labeling with esterified pectin in both its intine and pollen tube wall, while in most of the other conifers, esterified pectin is more prevalent in the intines than in the pollen tube walls. Unfortunately, Yatomi *et al*. (2002) did not differentiate the localization of pectins between the pollen tube tip and the rest of the pollen tube wall.

The intines and pollen tube walls of *Cycas revoluta* and *Ginkgo biloba* differ from each other and the conifers in callose, pectin, and β-glucan compositions. In *C. revoluta*, callose has been detected in its intine, but not in its pollen tube wall, while callose staining is very weak in both the intine and pollen tube wall of *G. biloba* (Yatomi *et al*. 2002). Esterified and acidic pectins are found in both the intine and pollen tube wall of *C. revoluta*, whereas only esterified pectin is found in both the intine and pollen tube wall of *G. biloba*. No reaction was observed in both the intine and pollen tube wall of *C. revoluta* using a mixed glucan MAb (Yatomi *et al*. 2002). On the other hand, the strong reaction of a mixed glucan MAb observed in the intine and pollen tube wall of *G. biloba* suggests the presence of a β-glucan that is neither cellulose nor callose.

There is no information available on cyto- and immunochemical analyses of the intines and pollen tube walls in gnetophytes. Nevertheless, arabinogalactan proteins and cellulose are present in the pollen tube walls of the gymnosperms examined, pectin is prevalent in *C. revoluta*, and a β-(1,3)(1,4)-glucan is abundant in *G. biloba*. Callose has not been detected in the pollen tube walls of *C. revoluta* and all species of *Pinus* that have been examined, while detected in *G. biloba* and some conifers but at varying intensities. Callose plugs have not been reported in any gymnosperm and therefore, the entire pollen tube, even if it is extensively branched, remains as one continuous cell from germination through fertilization.

Maintenance of the pollen tube wall involves the formation, transport, and breakdown of many proteins. In conifers, by inhibition of certain pathways, the importance and role of some proteins has been demonstrated (Wang *et al*. 2005). Pollen tube elongation, in particular, requires the assembly of structural proteins into the walls, which are synthesized in the ER-Golgi system and transported into the apoplastic space via secretory vesicles. The cell wall components become modified if the production and transport of these vesicles is blocked. The introduction of brefeldin A into germinating pollen of *Picea meyeri* inhibits exocytosis, the final step in the secretory pathway, but stimulates endocytosis counterbalancing membrane secretion. As a result, secretory pathways are inhibited and the pollen tube cell wall is compromised, exhibiting an undulating growth pattern, increased tube diameter, and swelling of the pollen tube tip. Maintenance of pollen tube elongation in conifers is dependent on continuous protein synthesis (Fernando *et al*. 2001; Hao *et al*. 2005); therefore, a consistent balance of exocytosis and endocytosis is necessary for proper growth. Disruption of this balance causes a decrease in protein synthesis leading to the arrest of pollen tube growth.

MADS-BOX GENES AND REPRODUCTIVE DEVELOPMENT

Recent studies have given insights into the involvement of MADS-box genes in plant reproductive development. MADS-box is a conserved sequence motif found in a family of transcription factors from fungi, animals and plants (Alvarez-Buylla *et al.* 2000). While the reproductive structures of gymnosperms differ greatly from those of angiosperms, some gymnosperms have been found to possess MADS-box genes which are orthologous to angiosperm B- and C-class floral organ identity genes including *Pinus radiata – MADS*, *Pinus resinosa – MADS2*, *Picea abies – DAL1*, *2*; *Picea mariana – SAG1*; *Ginkgo biloba – GBM5*; *Cycas edentata – CyAG*; and *Gnetum gnemon – GGM1*, *2*, *3*, *9*, *13* (Rutledge *et al.* 1998; Winter *et al*. 1999; Svenson and Engström 2002; Nam *et al.* 2003; Jager *et al*. 2003; Zhang *et al.* 2004). As in angiosperms, these genes are believed to function in determining reproductive versus vegetative structures and male versus female reproductive units. Both orthologs found in *Picea* are expressed solely in the male and female reproductive tissues (Tandre *et al.* 1995; Tandre *et al.* 1998; Rutledge *et al.* 1998) Likewise in *Gnetum*, *GGM3* is not expressed in vegetative tissues, but present only in the ovule and antherophore (pollen-bearing structure) (Winter *et al.* 1999; Becker *et al.* 2003). In *Cycas*, *CyAG* is present in the ovule and megasporophyll in the female, and in the male tissues, in the central axis of the cone, microsporophyll, and microsporangium (Zhang *et al.* 2004). Unlike the other gymnosperm *AGAMOUS* homologs, the *GBM5* gene of *Ginkgo* is expressed not only in reproductive tissues, but also strongly in young leaves (Jager *et al.* 2003). In contrast to angiosperms, which are characterized by the presence of at least two genes belonging to the *AGAMOUS* (*AG*) family (C-class genes), only a single *AG* ortholog has been found in gymnosperms thus far (Jager *et al*. 2003). Further analysis of MADS-box genes including a larger number of gymnosperm representatives, may help in understanding the contribution of MADS-box genes in gymnosperm reproductive development.

Similar to angiosperms, the coupling of B gene and C gene expression is believed to control the formation of the male organs in gymnosperms (Theissen *et* al. 2000). Therefore, several studies have screened for genes containing MADS-box elements expressed in gymnosperm cones (strobili). Becker *et al.* (2003) have predicted that there is not one, but two B genes, *GGM2* and *GGM15,* in *Gnetum* that are strongly expressed specifically in male structures. Futamura *et al*. (2008) have identified twelve MADS-box genes from the cDNA library of *Cryptomeria japonica* male cones, one is a type I gene and the other eleven are $MIKC^C$-type genes. These $MIKC^C$-type genes make up five subfamilies including *DEF*/*GLO*/*GG13*- (B-class and Bsister MADS-genes), *TM8*-, *AG*-, *AGL16*-, and *TM3*-like genes. The function of the type I gene remains to be determined in *C. japonica* cones. Likewise, MADS-box genes have been identified in the cones of *Picea abies*, many of which are related to the class B and C genes in angiosperms. Tandre *et al*. (1998) showed that *DAL2*, expressed in both male and female cones, is similar in structure and function to the class C MADS-box genes in angiosperms, and therefore is suspected to act as a determinant of reproductive organ identity. Similarly, the MADS-box genes *DAL11*, *DAL12*, and *DAL13*, related to the angiosperm B-class genes, are suggested to function in specifying the identity of the pollen cones in *P. abies.* While all three are specifically expressed in the pollen cone, *DAL11* and *DAL12* are active in all tissues of the developing cone, including the meristem, while *DAL13* is only active in the peripheral zone of the pollen cone bud (Sunström and Engström 2002). In addition, Carlsbecker *et al*. (2004) have identified that the DAL1 MADS-box gene, a putative ortholog of the *Arabidopsis* genes *AGL6* and *AGL13*, may have a role in regulating the juvenile-to-adult phase transition in *P. abies*. Active in the shoots of juvenile trees at 3-5 years of age, *DAL1* expression increases with age, and is maintained at high levels in male and female cones.

There are some MADS genes from gymnosperms that cannot be grouped with any MADS gene superfamilies from angiosperms, such as *GGM7* in *Gnetum* (Becker *et al*

2000). Likewise, the MADS-box gene *DAL10* in *P. abies* shows expression specificity in developing seeds and pollen cones with no orthology to known angiosperm MADS clades (Carlesbecker *et al* 2003). Therefore, it is likely that some MADS-box genes may be ancestral and lost during the evolution of angiosperms, whereas other may have been diverged after the separation of the angiosperms and gymnosperms.

While distinct MADS-box genes have been reported to show high expression levels in pollen grains, including *AGL18* from *Arabidopsis* (Alvarez-Buylla *et al*. 2000), altogether the MADS family of genes is underrepresented in the pollen transcriptome. On the contrary, nonclassical lineages, including type I and MIKC*-type, have been found to be overrepresented in pollen grains. It is suggested that MIKC*-type genes play an essential role in late pollen development and pollen tube growth, as disruption of such genes is reported to have a negative effect on pollen maturation, competitive ability, and germination (Kofuji *et al*. 2003; Verelst *et al.* 2007a, 2007b; Adamczyk and Fernandez 2009). As these results have come mainly from angiosperms, there is a need to confirm the role of MIKC*-type genes in gymnosperm male gametophyte development. Characterization of these genes in gymnosperms may give further insight into their involvement in the evolution of male gametophytes in seed plants, as a whole.

DIRECTION OF GYMNOSPERM MALE GAMETOPHYTE RESEARCH

Studies on pollen grains and tubes in gymnosperms have been mainly confined to morphological, histological, and cytological analyses, particularly for cycads, *Ginkgo*, gnetophytes, and most conifers. There is no information available on the male gametophyte development in Sciadopityaceae and Phyllocladaceae. In spite of several decades of work on gymnosperm male gametophyte development and evolution, there is still a need to establish the morphological relationships between various cells comprising the male gametophytes within seed plants and with those of non-seed plants. A possible approach is to examine the expression of molecular markers in gymnosperms and non-seed plants that have been established in flowering plants which are specific for vegetative cells (including pollen tube) and reproductive cells such as generative and sperm cells (see Twell 1992; Mori *et al*. 2005; von Besser *et al*. 2006).

Advances in the study of gymnosperm pollen grains and tubes have come primarily from conifers, although they are very limited as compared to the publications on pollen grains and tubes of angiosperms. In gymnosperms, recent reports are all from conifers, and these deal with differential display protein analysis (Chen *et al*. 2006), regulation of Ca^{2+} uptake (Kong *et al*. 2006), cytoskeleton dynamics (Chen *et al*. 2006; Sheng *et al*. 2006; Chen *et al*. 2007; Zhang *et al*. 2007), pollen tube wall composition (Sheng *et al*. 2006; Chichiricco and Paccini 2008; Chichiricco *et al*. 2009), activities of sterile and pollen tube nuclei (Wang *et al.* 2008), and microarray and proteomic analyses (Fernando 2008). There are no studies involving both molecular biology and genetics in gymnosperm male gametophyte development, not surprising considering the long-generation time of these seed plants.

In addition to the large-scale expressed sequence tags available from various conifers including *Pinus taeda* and *Picea glauca* (http://foresttree.org/ftdb; Pavy *et al*. 2007) and *Cryptomeria japonica* (Futamura *et al*. 2008), there are also EST sequencing projects on other phyla of gymnosperms such as *Ginkgo biloba* (Brenner *et al*. 2003), *Cycas rumphii* (Brenner *et al*. 2005) and *Gnetum gnemon* (Brenner *et al*., unpublished results). However, these projects are not directly addressing pollen grain and tube development or evolution. So far, information on the genes and proteins expressed in gymnosperm pollen grains and tubes are from *Pinus strobus* (Fernando 2005), *Picea meyeri* (Chen *et al*. 2006) and *Pinus taeda* (Fernando 2008). We anticipate that as molecular data accumulate from various representatives of the four living orders of gymnosperms, evolutionary patterns regarding the development of pollen grains and tubes may emerge from comparative sequence analysis.

A comprehensive analysis of pollen grains and tubes from seed plants will facilitate our understanding of the differences between the penultimate and ultimate phyla of land plant evolution, i.e., the gymnosperms and angiosperms. To do so will require a large-scale identification of genes and proteins from the male gametophytes coupled with characterization of their expression patterns and functions at the molecular level. If initiated, such study will provide a remarkable advancement in our understanding of sexual reproduction in seed plants.

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