

Morphology, anatomy and morphogenesis of pollen cones of *Cupressus vietnamensis* (Cupressaceae)

Abstract

The morphology, anatomy and morphogenesis of pollen cones of *Cupressus vietnamensis* (Farjon & T.H. Nguyễn) Silba were studied in the light of pollen cone evolution. Within the genus *Cupressus*, *Cupressus vietnamensis* is one of the most basal taxa. The study showed that the pollen cones are amongst the smallest within extant *Cupressus* species, compared with those of the more derived taxa being much larger. Apart from the pollen cone size, the number of microsporangioophores and microsporangia is also much higher in derived taxa. Thus, from basal to derived *Cupressus* species, a tendency to increase the pollen cone size and the number of microsporangia per cone is observable. This leads to a high variable number of microsporangioophores and microsporangia per microsporangioophore in pollen cones of strongly derived *Cupressus* species, while the situation in basal taxa is nearly more or less constant. This fits well to earlier studies on the female cones of *Cupressus vietnamensis*, which describe an increase of the seed cones size, the number of cone scales per cone and also the number of ovules per cone scale in derived *Cupressus* species. The morphogenesis of microsporangioophores in *Cupressus vietnamensis* is similar to those of microsporangioophores of *Cephalotaxus* and *Podocarpus*.

Keywords: *Cupressus vietnamensis*, pollen cone, morphology, anatomy, morphogenesis, microsporangia, microsporangioophores, pollen.

1 Introduction

In 1999 a new Cupressaceae was found in Vietnam by local botanists, and in 2002 first placed in a newly described genus *Xanthocyparis* Farjon & T.H. Nguyễn as *X. vietnamensis* Farjon & T.H. Nguyễn. They also transferred the North American species *Cupressus nootkatensis* D. Don to this genus (Farjon *et al.* 2002). In 2005, Silba merged *Xanthocyparis* into *Cupressus* L., which was supported by several later studies (e.g. Xiang & Li 2005; Rushforth 2007; Eckenwalder 2009; Christenhusz *et al.* 2011; Jagel & Dörken 2015; Dörken *et al.* 2017). It is suggested that *C. vietnamensis* and *C. nootkatensis* represent the most basal taxa, reflecting the primitive condition in the genus *Cupressus* (Jagel 2002; Jagel & Stützel 2001; Dörken *et al.* 2017), which makes both taxa important for suggesting evolutionary scenarios within the genus *Cupressus*.

While *C. nootkatensis* is frequently planted in suitable climates as an ornamental tree in parks and gardens, *C. vietnamensis* is still rare in cultivation and living material – especially fertile material – is hardly available. Thus, it is not surprising that no detailed study of the male reproductive structures exists until now. However, in 2017, the plant in the living collection of Hubertus Nimsch (Bollschweil, Germany) produced a large crop of pollen cones, so that the morphology and anatomy of pollen cones as well as their morphogenesis and pollen could be studied with paraffin technique and SEM.

This study pursues different goals. First, gaps in the general knowledge about the morphology and anatomy of *C. vietnamensis* pollen cones should be assessed. Second, the data gained should be compared to other living *Cupressus* species, to check if the features found in pollen cones of *C. vietnamensis* are unique to this taxon or typical of other *Cupressus* species. With *C. vietnamensis* belonging to the most basal *Cupressus* species, especially the comparison with the more derived, modern *Cupressus* species seems to be very promising to gain new insights into the evolutionary pathway and the evolutionary trends of male reproductive structures within *Cupressus*. Third, morphogenetic studies on the pollen cones – focused on their microsporangioophores and

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microsporangia – were performed, and the comparison to other gymnosperm pollen cones used to gain new insights into the morphogenetic differences and similarities existing with closely and distantly related taxa.

2 Material and methods

2.1 Material

147 pollen cones were collected from the plant cultivated in the living collection of Hubertus Nimsch, Bollschweil, St. Ulrich (Germany). This plant was received from the Botanical Garden Bonn (Germany), where it was propagated from a cutting.

2.2 Methods

Freshly collected material was photographed and then fixed in FAA (100 ml FAA = 90 ml 70% ethanol + 5 ml acetic acid 96% + 5 ml formaldehyde solution 37%) before being stored in 70% ethanol. The cone anatomy was studied from sections using the classical paraffin technique and subsequent astrablue/safranin staining (Gerlach 1984). For SEM-analysis the FAA-material was dehydrated in formaldehyde dimethyl acetal (FDA) for at least 24 hours (Gersterberger & Leins 1978) and critical point dried. Sputter coating was done with a sputter coater SCD 50 BAL-TEC (BALZERS). The specimens were examined with an AURIGA ZEISS TM. Macrophotography was accomplished using a digital camera (CANON POWERSHOT IS2) and microphotography with a digital microscope (KEYENCE VHX 500F) equipped with a high-precision VH mounting stand with X-Y stage and bright field illumination (KEYENCE VH-S5).

2.3 Special terms

Most authors regard the microsporangia (= pollen sacs) bearing structure in conifers as “microsporophylls”. We avoid using the term “sporophyll” or “microsporophyll”. Otherwise we would introduce *a priori* a homology that is applied to it. Thus we are calling them microsporangiohores.

3 Results

Cupressus vietnamensis is monoecious; male and female cones develop on the same tree and even on the same branch. Pollen cones are developed exclusively on lateral, scale-leaved branches (Fig. 1). The pollen cones are always developed terminally on 0.5–3 cm long lateral branchlets (Fig. 1B). During the development of the pollen cones, these branchlets become more and more downward orientated, so that finally mature pollen cones are plagiotropic or pointing downward (Fig. 1A). The surface of the pollen cone is covered with a whitish cuticle. Mature pollen cones are 3–4 mm long and 2–2.5 mm wide (Figs 1A & 2B). They are unbranched, uniaxial. Bracts within the pollen cones do not exist. 97 (= 66 %) of the 146 investigated pollen cones had 6 and 49 (= 34 %) pollen cones had 8 decussate, hyposporangiate microsporangiohores (Tab. 1 & Figs 2B, 3, 4). Each microsporangiohore has a central stalk and a terminal, adaxial, brownish-green scutellum with a strongly serrate margin (Fig. 2B). The microsporangiohores are supplied with a single collateral vascular bundle strand with xylem located towards the adaxial and phloem towards the abaxial side. Below the vascular bundle strand a resin duct is developed. Abaxially two microsporangia are developed (Figs 2B & 4). Only the microsporangiohores in the distal pair are exceptionally carrying only a single sporangium. The terminal scutellum of the basal microsporangiohores is more or less roundish, with a strongly serrated margin (Figs 2B & 4F). The scutellum of the more distal microsporangiohores is significantly smaller (Fig. 2B). In some of the most distal microsporangiohores a scutellum is completely lacking so that those microsporangiohores consist only of a short central stalk and two abaxial microsporangia (Fig. 2C), rarely of a single microsporangium. The microsporangia are globose and 0.3–0.7 mm in diameter (Fig. 2B). They are exclusively attached to the central stalk and not fused to each other or to the scutellum (Figs 2E, 3I, 4B). Each microsporangium produces a large number of pollen grains (Fig. 3I) of a whitish or creamy-white colour. Before anthesis, the scutellum of the microsporangiohores covers the microsporangia of the subsequent distal microsporangiohores.

Thus, in young pollen cones only the scutella are visible from outside at first (Figs 2A & 4C). At anthesis the cone axis is significantly elongated so that the microsporangia are freely exposed to the airflow (Fig. 2B). Mature microsporangia open along a longitudinal preformed line. It reaches from the insertion point to the tip of the sporangium (Fig. 2F). The released pollen grains are collected first on the scutellum of the lower microsporangioophores. From here the pollen grains are transported by the wind. After releasing the pollen, the cones dry out and are abscised.

The microsporangioophores develop laterally at the pollen cone apex as small cylindrical primordia (Fig. 3A). They are inserted directly at the cone axis, and not axillary in the axil of a bract. Within a pollen cone the differentiation of the microsporangioophores take place in an ascending direction from basal to distal. In the earliest developmental stages the microsporangioophores develop a dorsiventral structure, with a fertile abaxial side carrying the microsporangia and a sterile adaxial side developing the scutellum (Figs 3C-I). The primordia of the microsporangioophores show several marginal initial cells at its tip (Fig. 3B). These cells show an anticline cell division. The pericline cell division in the tissue located towards the lower side of the primordium marks the beginning of development and differentiation of the archesporium (Fig. 3B). The development of the microsporangia runs ahead of the development of the scutella (Figs. 3D-F, 4A). The microsporangia are developed as lateral structures at the base of the abaxial side (Figs. 4C-E). The sporangium does not incurve. The sporogenous tissue within the microsporangia is developed at an early stage (Fig. 3B-D). The wall of the microsporangia originates from epidermal cells, which shows an intensified anticline cell division in the area of the young microsporangia so that finally an abaxial sac-like structure is formed (Figs. 4B-E). At nearly the same time, when the differentiation of the microsporangia takes places, the stalk of the microsporangioophore starts its differentiation (Fig. 3E). After the differentiation of the microsporangia and the stalk, the adaxial scutellum starts with an intensive growth (Figs 3F-I, 4B-F), so that it finally forms a shield-like, terminal, adaxial structure at the tip of the stalk (Fig. 4F). The microsporangia and the scutella are not fused to each other. The elongation of the stalk by intercalary growth represents the last step in the morphogenesis of each microsporangioophore (Figs 3H-I). It takes place when the pollen mother cells in the microsporangia (Fig. 3H) starts meiosis to produce 4 pollen grains each. The mature microsporangium contains a large number of uniform pollen grains (Fig. 3I) which are surrounded by the wall of the sporangium and two inner layers of tapetal tissue (Fig. 2D).

4 Discussion

4.1 Comparison of pollen cones among *Cupressus*

Typical cupressaceous pollen cones are unbranched, consisting of several microsporangioophores, each with a terminal adaxial scutellum and abaxial microsporangia at the end of the stalk (e.g. Pilger 1926; Dallimore & Jackson 1966; Krüssmann 1955, 1983; Page 1990; Eckenwalder 2009; Farjon 2005, 2009, 2010). Exceptionally the pollen cones of *Juniperus drupacea* Labill. differ from that by representing a branched structure in the sense of an inflorescence (e.g. Lemoine-Sebastian 1967; Farjon 2005; Eckenwalder 2009; Jagel & Dörken 2015; Dörken & Nimsch 2016). Thus, all typical features of cupressaceous pollen cones are found in *Cupressus vietnamensis*. In comparison to pollen cones of other Cupressaceae and to nearly all other *Cupressus* species, the situation in *C. vietnamensis*, with 6-8 microsporangioophores per cone and two microsporangia per microsporangioophore, is very uniform with less variability. Among *Cupressus* and most other Cupressaceae, the number of microsporangioophores and the number of the microsporangia per microsporangioophore vary inter- and even intraspecifically for a single pollen cone.

Based on their morpho-anatomical and morphogenetic studies on seed cones of *C. vietnamensis* and *C. nootkatensis*, Jagel & Dörken (2015) and Dörken *et al.* (2017) suggested that these taxa represent the most basal *Cupressus* species. This is also supported by molecular cladistics studies (Yang *et al.* 2012). The seed cones of the basal taxa *C. vietnamensis* and *C. nootkatensis* are conspicuously small and consist of only one or two (very rarely, three) fertile pairs of cone scales, bearing a low number of ovules which are mostly arranged in one row per cone scale. In strongly derived taxa the seed cones are large, consisting of several pairs of cone scales with a high number

of ovules per cone scales, arranged in up to four rows per cone scale. Thus, within seed cones of *Cupressus*, an increase in the number of cone scales and ovules per cone scale takes place which finally leads to larger seed cones (Jagel & Dörken 2015).

Within pollen cones developed among the derived *Cupressus* species, the number of microsporangiophores and of microsporangia per microsporangiophore varies (Table 1). Within *Cupressus*, a clear tendency to increase the pollen cone size, the number of microsporangiophores, and also the number of microsporangia per microsporangiophore, is observable from basal towards derived species. The pollen cones of most derived taxa are the largest ones, developing the highest number of microsporangiophores and microsporangia per microsporangiophore (Tables 1 and 2).

Table 1: Morphometric data for microsporangiophores in pollen cones of some basal (*C. vietnamensis* and *C. nootkatensis*) and derived *Cupressus* species (*C. sempervirens* L., *C. macrocarpa* Hartw. & *C. arizonica* Greene). Data for *C. sempervirens*, *C. macrocarpa* & *C. arizonica* from Lemoine-Sebastian 1972; data for *C. nootkatensis*: Dörken unpublished).

taxon	Sample size [# cones]	# of microsporangia by microsporangiophore									Avg # of microsporangia per cone
		1	2	3	4	5	6	7	8	Total	
<i>C. vietnamensis</i>	147	49 2.5%	1913 97.5%	-	-	-	-	-	-	1962 100.0%	13.2
<i>C. nootkatensis</i>	80	26 1,3%	1920 97,8%	6 0,3%	-	-	-	-	-	1964 100.0%	13.3
<i>C. sempervirens</i>	20	17 5.0%	56 16.6%	98 29.1%	137 40.7%	25 7.4%	4 1.2%	-	-	337 100.0%	16.9
<i>C. macrocarpa</i>	40	78 14.4%	118 21.8%	118 21.8%	126 23.2%	76 14.0%	22 4.1%	3 0.6%	1 0.2%	542 100.0%	13.6
<i>C. arizonica</i>	30	35 8.3%	95 22.5%	165 39.0%	119 28.1%	9 2.1%	-	-	-	423 100.0%	14.1

Table 2: Morphometric data for microsporangia in pollen cones of some basal (*C. vietnamensis* and *C. nootkatensis*) and derived *Cupressus* species (*C. sempervirens*, *C. macrocarpa* & *C. arizonica*). Data for *C. sempervirens*, *C. macrocarpa* & *C. arizonica* from Lemoine-Sebastian 1972; data for *C. nootkatensis*: Dörken unpublished.

taxon	Sample size [# cones]	# pairs of microsporangiophores per cone				
		5	6	7	8	9
<i>C. vietnamensis</i>	147	-	97 65.9%	1 0.7%	49 33.3%	-
<i>C. nootkatensis</i>	80	-	46 57,5%	-	34 42,5 %	-
<i>C. sempervirens</i>	20	-	-	1 5.0%	8 40.0%	11 55.0%
<i>C. macrocarpa</i>	40	2 5.0%	13 32.5%	15 37.5%	10 25.0%	-
<i>C. arizonica</i>	30	-	-	24 80.0%	6 20.0%	-

The basal position of *C. vietnamensis* leads to the hypothesis that its small pollen cones, which are similar to those of the closely related *C. nootkatensis*, still show several primitive features as was shown for the seed cones in earlier studies. Due to the fact that the size of the microsporangia within different *Cupressus* species is more or less the same, microsporangiophores in e.g. *C. sempervirens* with a majority of 3-4 microsporangia per microsporangiophore (Table 1) can produce significantly more pollen than the microsporangiophores of *C. vietnamensis*, with only two microsporangia per microsporangiophore. The increased amount of pollen produced in pollen cones of derived *Cupressus* species lead to a more efficient and successful pollination. The hypothesis can

be proposed that the increased number of ovules per seed cone and the increase of the pollen production per pollen cone evolved in parallel to each other. However, to support this hypothesis further research is needed. Here not only the number of microsporangiophores per pollen cone or the number of microsporangia per microsporangiophore play an important role, but also the total number of pollen cones per tree.

4.2 Comparison of microsporangiophores of *Cupressus vietnamensis* to those of other conifers

The hyposporangiate type of microsporangiophores of *C. vietnamensis* represents the most common type among extant conifers and can be found among all coniferous families. Only in Taxaceae, where apart from hyposporangiate microsporangiophores, perisporangiate ones are also developed (Wordsell 1901; Dupler 1919; Dluhosch 1937; Thomson 1940; Wilde 1975; Mundry & Mundry 2001; Dörken *et al.* 2011), although this represents a fusion product of several hyposporangiate ones (Dörken & Nimsch 2016). At first glance within conifers, mature hyposporangiate microsporangiophores are very similar to each other, especially those developing a low number of microsporangia per microsporangiophore. The microsporangiophores of Araucariaceae differ significantly from the rest of the conifers in producing 6-20 microsporangia per microsporangiophore (Farjon 2010). The dimension of the adaxial scutellum varies between the different genera from strongly reduced to strongly elongated. The number of two microsporangia per microsporangiophore in *C. vietnamensis* belongs to the lowest number among extant conifers, and is also typical for taxa in Pinaceae, Podocarpaceae and Sciadopityaceae. With 2-4 microsporangia per microsporangiophore, the number is also low in Taxaceae (Farjon 2010 [as Cephalotaxaceae]). Despite the fact that the microsporangiophores developed in these taxa are quite similar to each other in mature stages, they differ however in their morphogenesis, especially in the point of time when the dorsiventrality of the microsporangiophores occurs first and also in the way how the microsporangia are inserted and developed (Mundry 2000). The microsporangiophores of e.g. *Podocarpus macrophyllus* (Thunb.) Sweet already have a dorsiventral structure at the initiation of the microsporangia (Mundry 2000). Also the microsporangia of *Cephalotaxus harringtonii* (Knight ex J.Forbes) Koch show a comparable early dorsiventrality of the microsporangiophores (Mundry 2000), as is also the case for the investigated *Cupressus vietnamensis*. The microsporangiophores of *Pinus sylvestris* L. and *Picea asperata* Masters differ significantly from the taxa above because the dorsiventrality of mature microsporangiophores is developed secondarily, obviously caused by the available space within the maturing pollen cone (Mundry 2000). In *Cupressus vietnamensis* the microsporangia are developed as sac-like evaginations in the basal parts on the lower dorsal side of the microsporangiophores, caused by an intensified anticline cell division of epidermal cells in this region; this is as Mundry (2000) demonstrated for *Podocarpus macrophyllus* and *Cephalotaxus harringtonii*. In *Cupressus vietnamensis* and *Cephalotaxus harringtonii* the microsporangia remain in this position throughout the complete morphogenesis of the microsporangiophore. In *Podocarpus macrophyllus* they become strongly incurved so that they are finally orientated parallel to the stalk of the microsporangiophore. A pronounced elongation of the stalk ensures the required space within the maturing pollen cone. Thus mature microsporangiophores of *Podocarpus macrophyllus* appear quite similar to those of *Pinus sylvestris*. However, in *Pinus sylvestris* the microsporangia show a completely different morphogenesis. The microsporangia are already developed parallel to the stalk and they remain in this position during their complete morphogenesis (Mundry 2000). Due to the fact that within *Cupressus vietnamensis* the microsporangia are developed in a more or less vertical downward position and remain in this position throughout the whole morphogenesis of the microsporangiophore, an immediate elongation of the stalk (as needed in *Pinus sylvestris* or *Podocarpus macrophyllus*) is not necessary. Despite that, mature microsporangiophores of *P. macrophyllus*, *Cephalotaxus harringtonii* and *Cupressus vietnamensis* have different appearance. Their early morphogenesis is, however, nearly identical. This is an important fact that can be used to regard the microsporangiophores of all three taxa as homologous structures. Microsporangia of *Podocarpus* differ from *Cephalotaxus* and *Cupressus* by developing bisaccate pollen grains (e.g. Sporne 1965; Pocknall 1981; Bortenschlager 1990; Tomlinson 1994; Tomlinson *et al.* 1991;

Tomlinson *et al.* 1997; Owens *et al.* 1998; Gelbart & von Aderkas 2002; Salter *et al.* 2002; Tomlinson & Takaso 2002; Schwendemann *et al.* 2007; Fernando *et al.* 2010; Leslie 2010; Dörken & Jagel 2014; Dörken & Nimsch 2015), which is however, caused by significant differences in the structure of female cones leading to differences in the pollination mechanisms.

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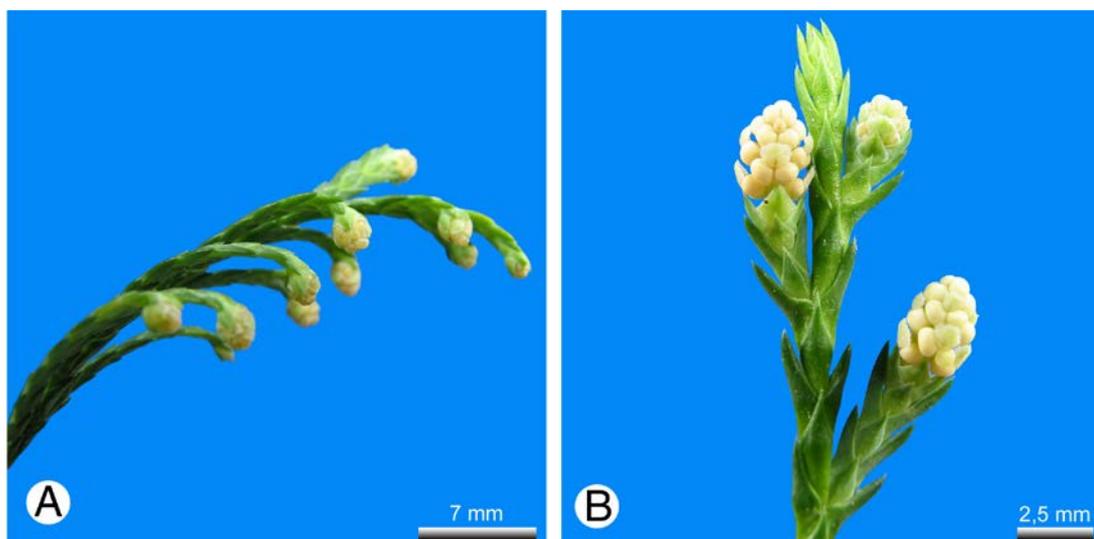


Fig. 1: *Cupressus vietnamensis*, position of pollen cones at a lateral branch.

A: lateral branch in its original position with several pollen cones; pollen cones plagiotropic or downward pointing; **B:** Detail of A; pollen cones terminal on small lateral, scale leaved branchlets.

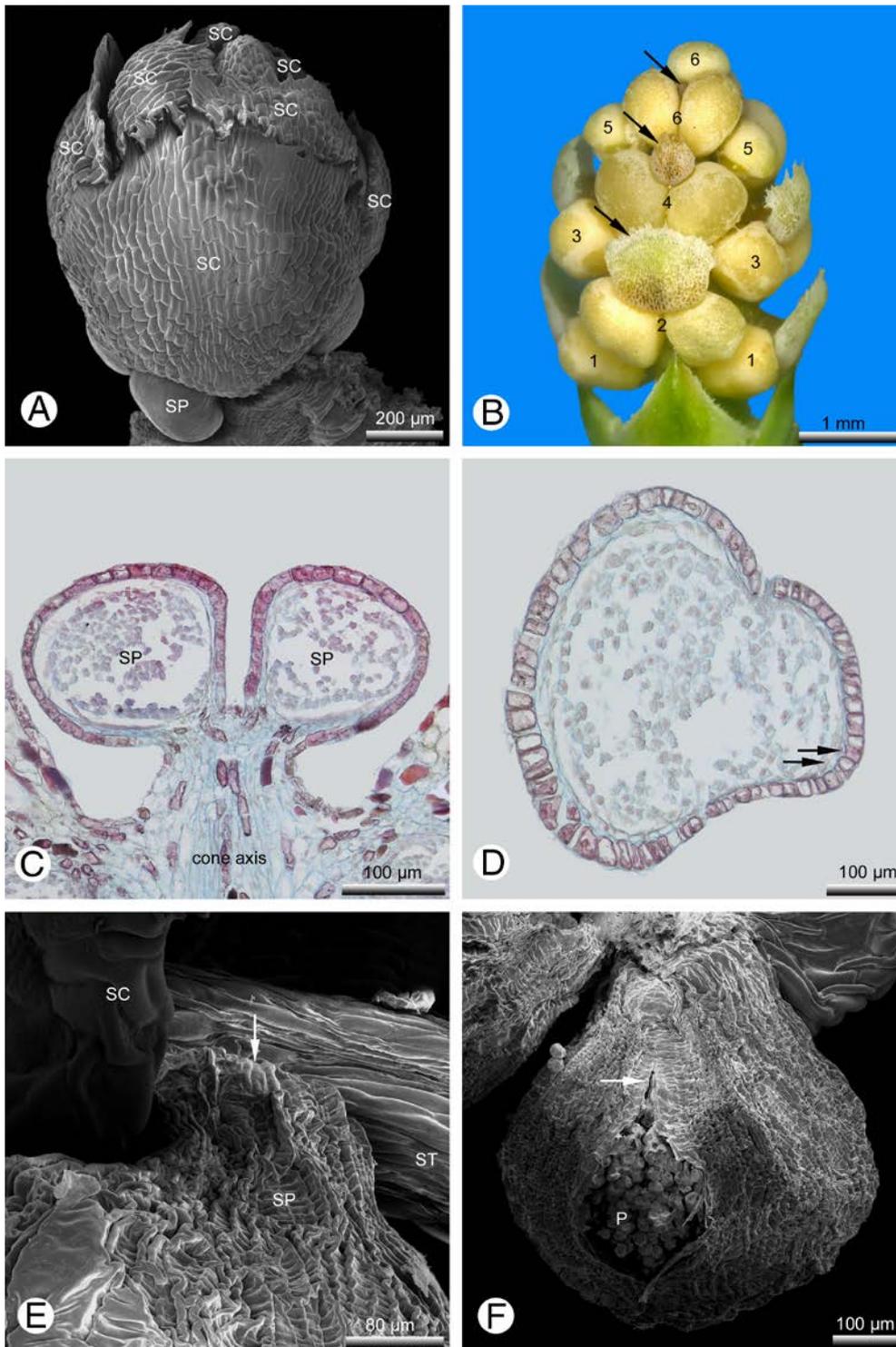


Fig. 2: *Cupressus vietnamensis*, morphology of pollen cones.

A: Young pollen cone long before anthesis; the scutella (SC) of the microsporangioophores are covering each other and protect the microsporangia (SP); **B:** Pollen cone with 6 pairs of microsporangioophores (numbers indicate each pair) shortly before anthesis; microsporangioophores are spreading distinctly from each other and microsporangia are exposed; scutella (arrows) of distal microsporangioophores distinctly smaller than of the basal microsporangioophores; **C:** Longitudinal section of two distal microsporangioophores, each with only a single microsporangium, the scutellum is missing; **D:** Cross section of a microsporangium; two layers of tapetal cells (arrows) covering the inner wall of the microsporangium; **E:** Detail of the insertion point of a microsporangium at the stalk (ST) of the microsporangioophore (arrow); **F:** Each microsporangium opens along a longitudinal preformed line (arrow) to release the pollen (P).

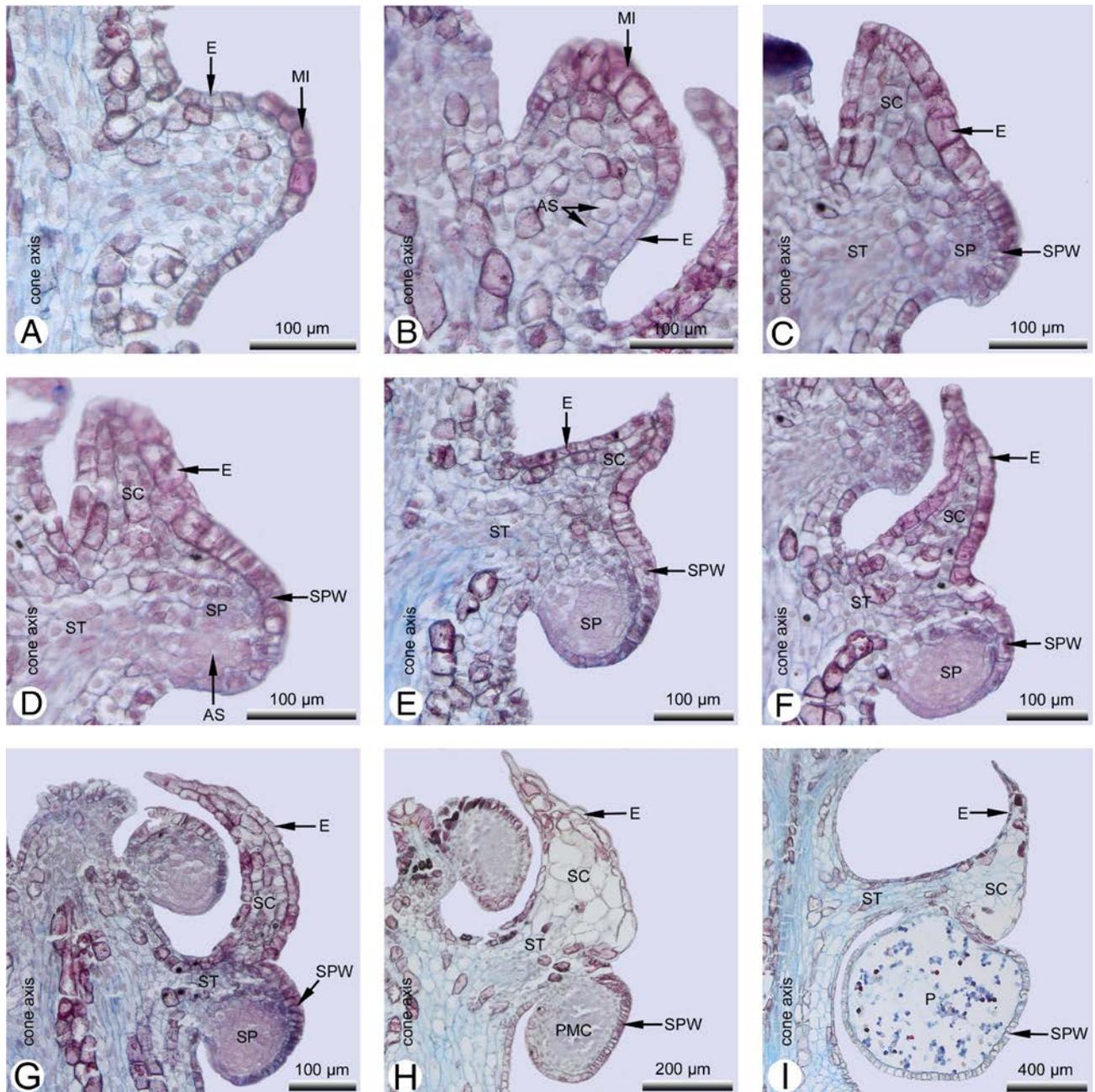


Fig. 3: *Cupressus vietnamensis*, morphogenesis of microsporangioophores (microtome longitudinal sections).

A: Young bulge-like primordia lateral at the cone axis; marginal initial cells (MI) larger than epidermal cells (E); **B:** Young primordia with terminal marginal initial cells; two pericline divided cells marking the beginning of the archesporium (AS) development; **C:** Beginning of the differentiation of the microsporangium (SP) towards the lower side and the scutellum (SC) towards the upper side of the bulge; the microsporangium wall (SPW) originated from epidermal cells; the stalk (ST) is still short; **D-G:** Differentiation of the microsporangia and the scutellum; the stalk (ST) of the microsporangioophore still rudimentary; **H-I:** Elongation of the central stalk (ST) by intercalary growth as latest steps in the morphogenesis of the microsporangioophore; **H:** Microsporangium with several pollen mother cells (PMC); **I:** Mature microsporangium filled with pollen (P).

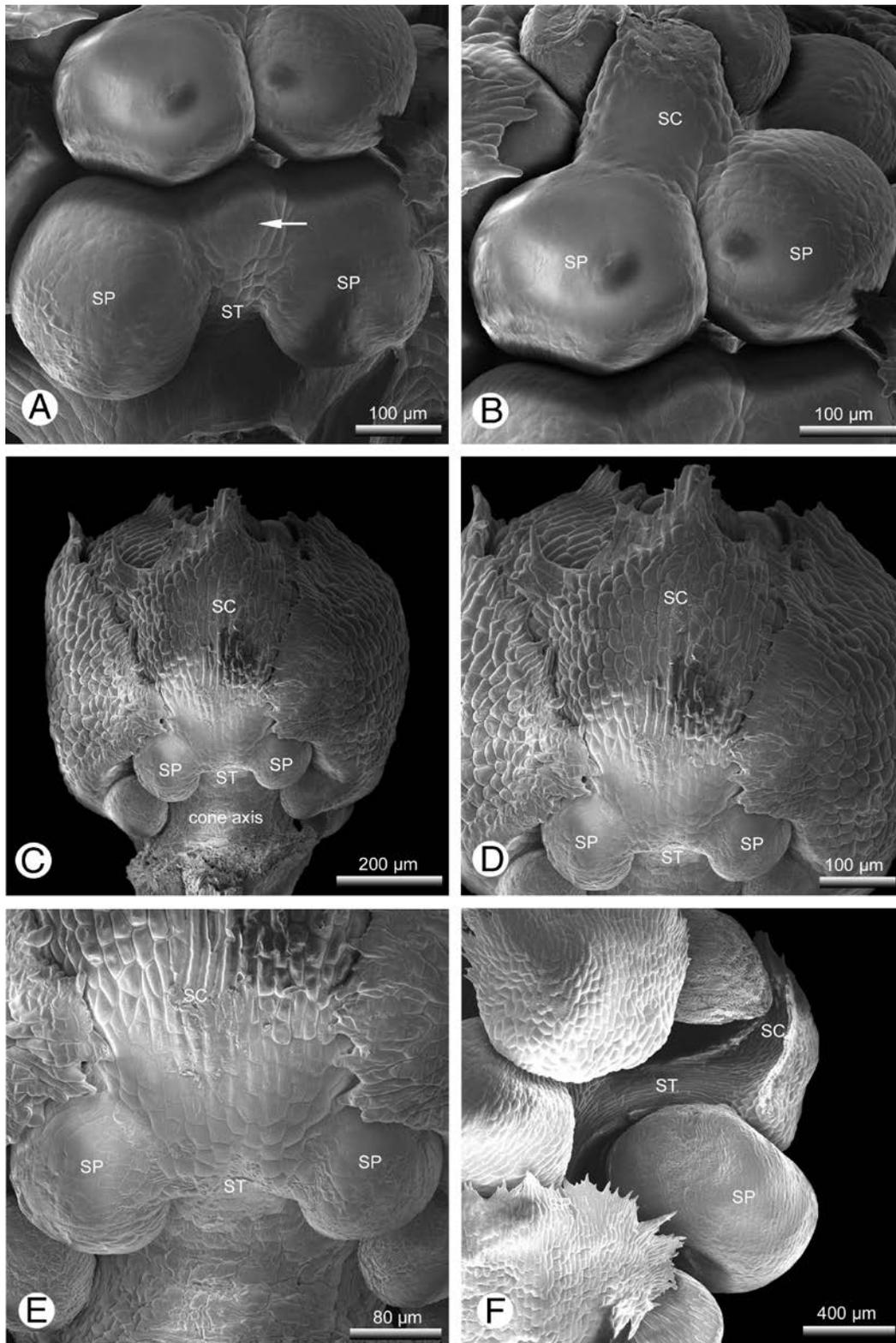


Fig. 4: *Cupressus vietnamensis*, morphogenesis of the microsporangioophores (SEM).

A: Two microsporangia (SP) are developed on a short stalk (ST); the scutellum just starts to develop (arrow); **B-C:** Scutellum (SC) develops rapidly and covers each other and also the microsporangia of the more distal microsporangioophore; the stalk of the microsporangioophore is still quite short; **D-E:** Details of C; **F:** Mature microsporangioophore with strongly elongated stalk and freely exposed microsporangia; microsporangia and scutellum not fused to each other.