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**Cover photo** : Odd pollen cone of *Cupressus vietnamensis* with seven pairs of microsporangiophores. See table 2, p. 82. – © V.M. Dörken.

## 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 microsporangiophores 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 microsporangiophores and microsporangia per microsporangiophore 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 microsporangiophores in *Cupressus vietnamensis* is similar to those of microsporangiophores of *Cephalotaxus* and *Podocarpus*.

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

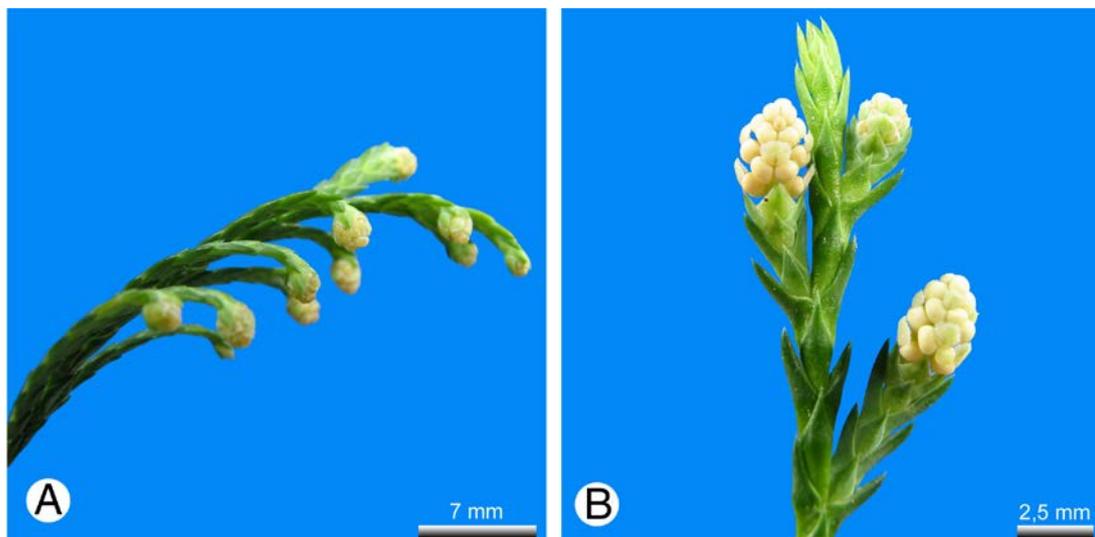
The hyposporangiate type of microsporangiophores of *C. vietnamensis* represents the most common type among extant conifers and can be found among all coniferous families. Only in Taxaceae, where apart from hyposporangiate microsporangiophores, perisporangiate ones are also developed (Wordsell 1901; 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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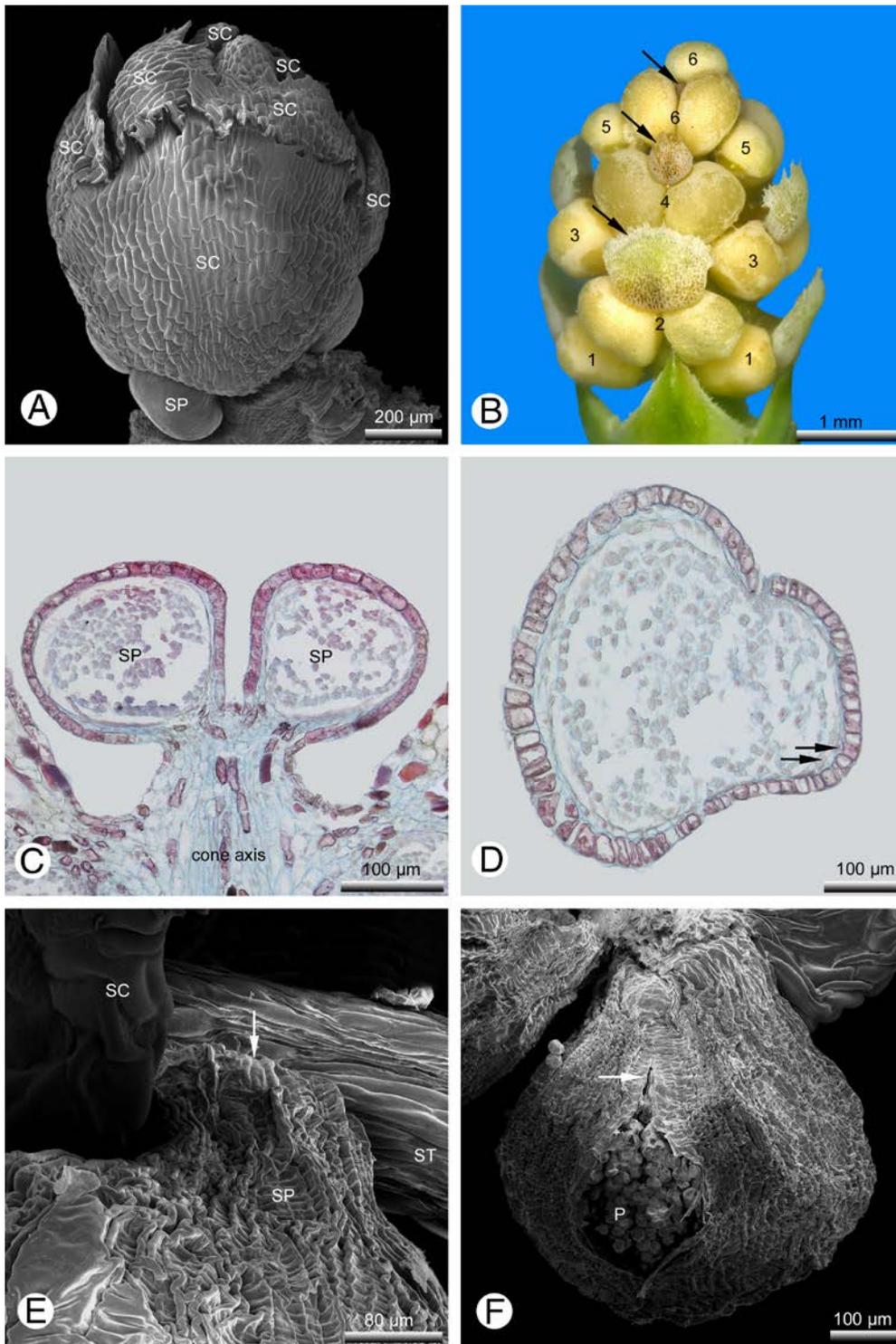
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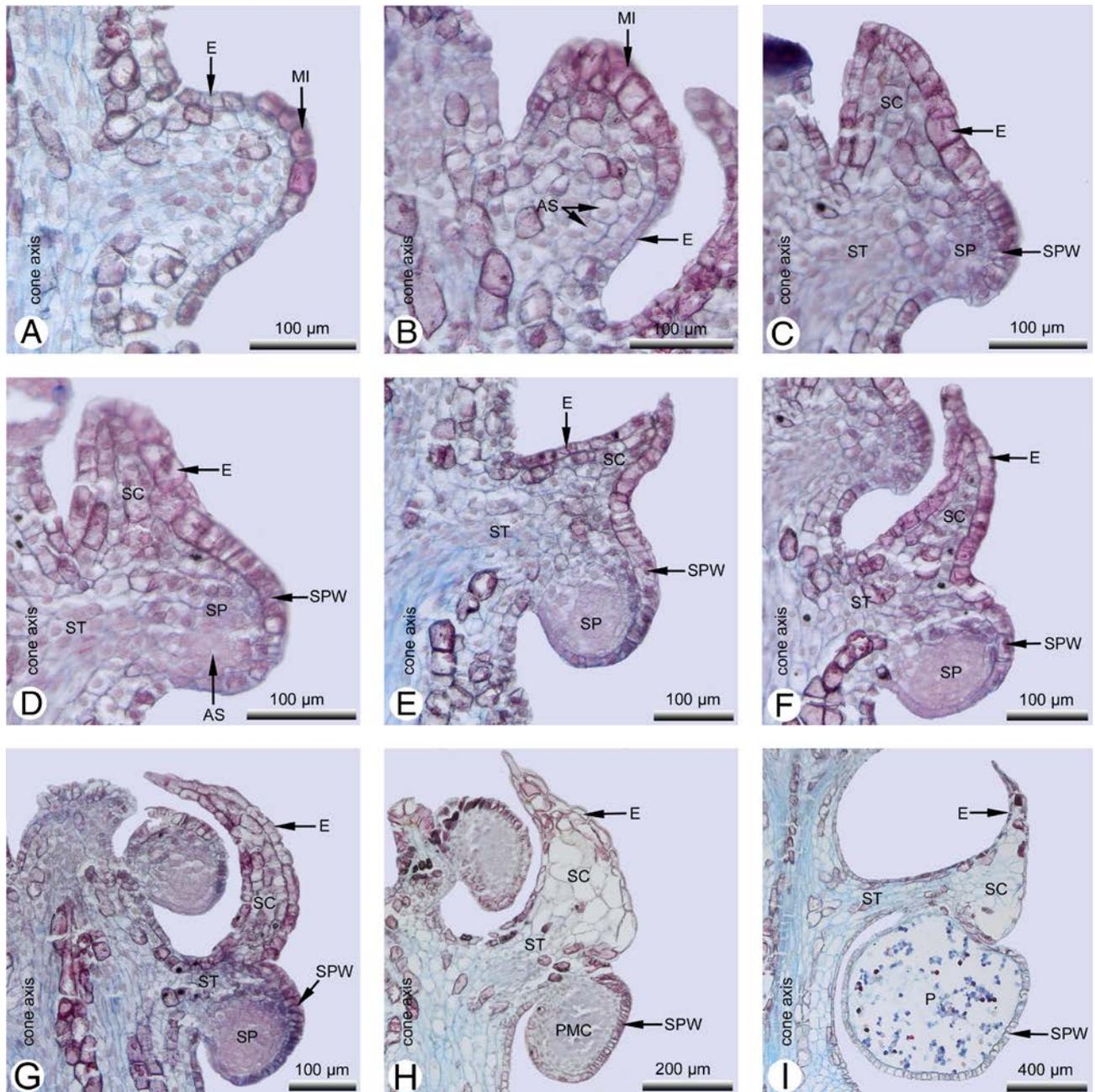
**Fig. 1:** *Cupressus vietnamensis*, position of pollen cones at a lateral branch.

**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.



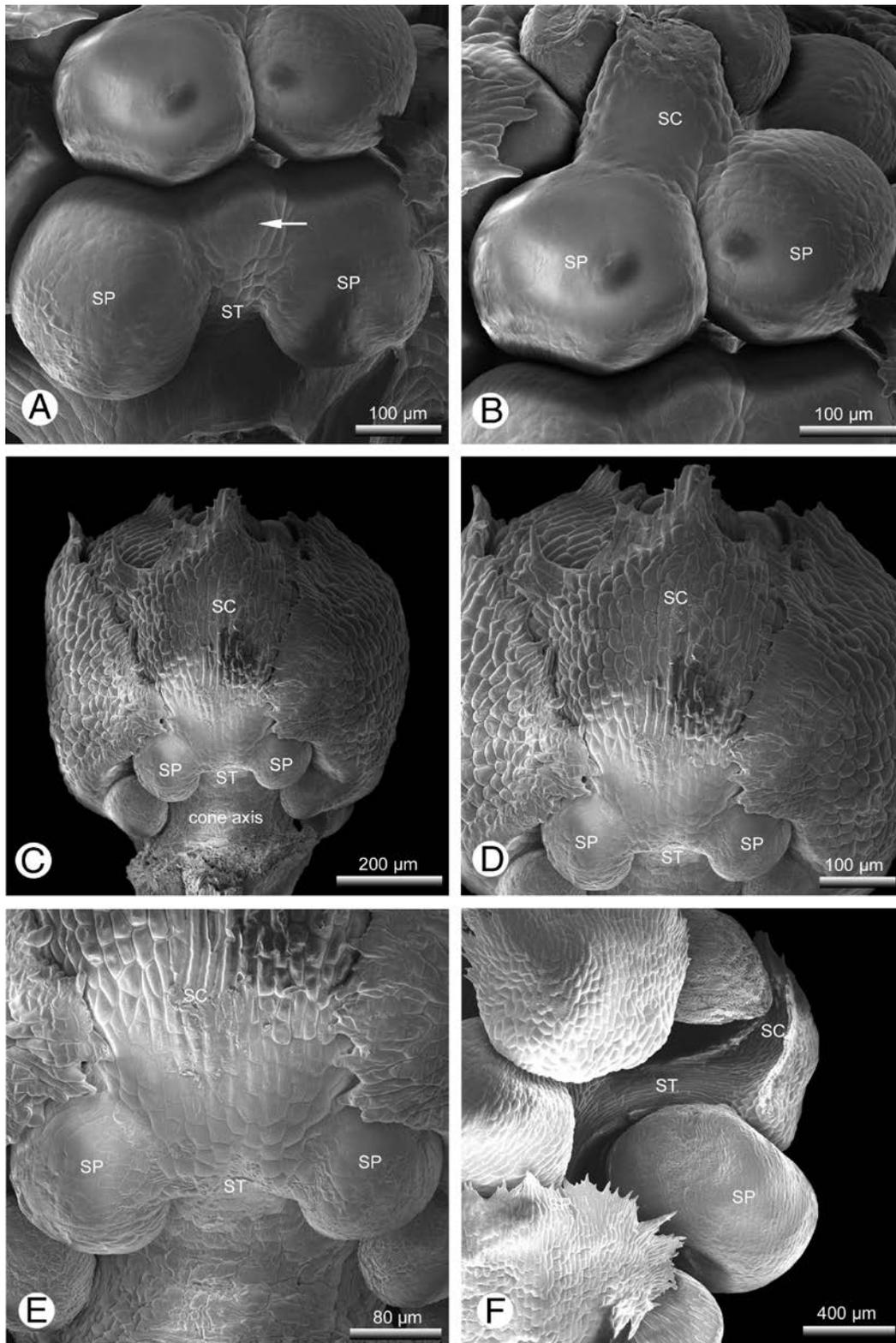
**Fig. 2:** *Cupressus vietnamensis*, morphology of pollen cones.

**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.

## Seed cone proliferation in *Cupressus vietnamensis* (Cupressaceae) and its evolutionary meaning

### Abstract

Teratological seed cones of *Cupressus vietnamensis* (Farjon & T.H. Nguyễn) Silba showing apical and axillary vegetative proliferations were investigated. This showed that the apical proliferation represents a simple elongation of the cone axis. A full transitional series from basal fertile peltate cone scales to the distal, decussate, sterile scaly trophophylls at the proliferation was documented. The investigations of the axillary proliferations show that here ovules are replaced by a homologous vegetative axillary shoot. It is suggested that the primitive condition of the Cupressaceae seed cones was an open, loose, polyaxial inflorescence, before the ovuliferous short shoot (= seed scale) was strongly reduced, so that in most derived Cupressaceae the seed scale is reduced to its ovules, which finally leads to the formation of the compact seed cone structure in living Cupressaceae.

**Keywords:** *Cupressus vietnamensis*, seed cone, morphology, anatomy, proliferation, evolution, teratology.

### 1 Introduction

Typical coniferous seed cones are characterized by the so called bract/seed scale complex. The bract scale represents a leaf which is carrying an axillary ovuliferous short shoot, the seed scale (Takaso & Tomlinson 1992; Jagel & Stützel 2001, 2003; Jagel 2002; Farjon & Ortiz-Garcia 2003; Schulz & Stützel 2007; Dörken 2011). Thus, the coniferous seed cones represent polyaxial, compound structures fulfilling the definition of an inflorescence (Schuhmann 1902; Herzfeld 1914; Pilger 1926; Florin 1951, 1954; Schweitzer 1963; Farjon 1984, 1990, 2005, 2010; Stützel & Röwekamp 1997, 1999; Mundry 2000; Farjon & Ortiz-Garcia 2003; Eckenwalder 2009). However, both types of cone scales can be distinguished so clearly from each other as two separate structures only in Pinaceae. In other coniferous groups, the bract/seed scale complex is strongly reduced and modified so that finally only one type of cone scales is developed, which represents either a fusion product of the bract and seed scale as is e.g. the case in Araucariaceae (e.g. Eckenwalder 2009; Farjon 2010), or the cone scale represents exclusively the bract scale, while the seed scale is completely reduced to its ovules as is typical for several Cupressaceae s.str. (e.g. Page 1990; Gadek *et al.* 2000; Jagel & Stützel 2001; Schulz & Stützel 2007; Jagel & Dörken 2014, 2015a, 2015b; Dörken 2011; Dörken & Jagel 2014, 2017). Among several Cupressaceae s.str. the ovules are inserted in a single or in several rows. These axillary rows of ovules are regarded as descending accessory short shoots. In the typical shaped seed cones of Cupressaceae s.str. no vegetative portions of the ovule-carrying seed scale are visible.

By chance teratological seed cones of *C. vietnamensis* showing different types of proliferations were found and their morphology and anatomy is investigated here. The structures of these proliferated seed cones are of greatest interest to gain new results helping towards a better understanding of the evolutionary pathway of seed cones in Cupressaceae and their original branching pattern.

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## 2 Material and Methods

### 2.1 Material

Teratological seed cones showing an apical vegetative proliferation were received from the Bedgebury Pinetum (UK). Seed cones showing axillary vegetative proliferations were collected in the living collection of Hubertus Nimsch (Bollschweil, Germany). In both cases, the cones were collected from trees cultivated as pot-plants.

### 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 leaf-anatomy was studied from serial sections using the classical paraffin technique and subsequent astrablue/safranin staining (Gerlach 1984). 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).

## 3 Results

### 3.1 Teratological seed cones with an apical proliferation

The basal part of these proliferated seed cones has the typical *C. vietnamensis* seed cone morphology (Figs 1A-D), with two pairs of fertile cone scales. Each cone scale carries axillary 2(-3) ovules, which are arranged in a single row. The apex of the cone axis remained vegetative and formed an apical distinct foliar proliferation (Fig. 1). The apex of the proliferated shoot axis remains vegetative. However, the duration of the proliferation is closely correlated to the life span of the seed cone. At maturity, the seed cone dries out and the cone axis and cone scales shrink to release the seeds. In consequence, also the apical proliferation dries. The apical proliferation is sterile (Figs 1A-C). The leaves at the proliferation show a full transitional series from basal peltate leaves showing the shape of typical cone scales to distal scaly, decussate trophophylls, which can be distinguished in lateral and median leaves (Fig. 1F), as is typical for the vegetative parts of *C. vietnamensis*. The basal fertile cone scales and also the cone scale like basal leaves inserted at the proliferation are peltate shaped, but do not represent peltate leaves in the classical sense. A meristematic fusion of marginal leaf tissues leading to the formation of a meristematic cross zone above the petiole lacks in both. The peltate leaf shape is caused by a strongly ventral bulge, which starts development soon after pollination to close the cone. Thus, the distal part of the cone scale becomes finally shield like, carrying the leaf tip as a more or less conspicuous central spike on its back (Figs 1A-D). While maturing the cone scales and the cone scale like leaves at the proliferation become strongly lignified. Within the leaves inserted at the proliferation, the formation of this ventral bulge is more and more reduced so that the peltate shape of the lower cone scales and the sterile cone scale like leaves gets lost so that finally the scaly trophophylls, lacking a meristematic swelling, are formed.

### 3.2 Teratological seed cones with an axillary proliferation

One seed cone was found, showing a vegetative proliferation inserted in the axil of a distal cone scale (Figs 2 & 3). Within this cone scale the axillary row of ovules is completely replaced by a sterile, scale-leaved shoot axis (Figs 3A & 3C). Each, the axillary proliferation and the cone scale are supplied by an own vascular bundle strand, which enter the vascular bundle of the cone axis in separate strands. The vascular bundle of the cone scale is collateral with xylem located towards adaxial and phloem towards abaxial. The vascular bundle of the proliferation is concentric and similar to those of the cone axis with inner xylem and outer phloem (Figs 3A & 3C). The apex of the proliferation remains vegetative and a possible further growth would be possible. However, the duration of the proliferation is closely correlated to the life span of the seed cone and is therefore limited to about max. 18 months. The leaves inserted at the axillary proliferation show the morphology and anatomy of typical trophophylls of *C. vietnamensis*: scaly, decussate, inverse

bifacial, distinguishable in lateral and median leaves (Figs 2A & 2F). Transitional leaves between the peltate shaped cone scales and the scaly trophophylls at the proliferation lack. Except for the axillary proliferation, the seed cone shows the typical conditions of *C. vietnamensis*: two decussate fertile cone scales, each carrying 2(-3) ovules, which are arranged in a single axillary row per cone scale. The ovule and the cone scale are each supplied by an own vascular bundle strand, which enter the concentric bundle of the cone axis by a separate strand. In basal parts the collateral bundle strand supplying the cone scale branches dichotomously once. One of the strands ends shortly below the tip of the cone scale, the other one supplies the strongly swollen ventral bulge (Fig. 3B).

#### 4 Discussions

Within the genus *Cupressus* the Vietnamese taxon *C. vietnamensis* and the north-western Pacific coast *C. nootkatensis* D. Don can be regarded as the most primitive taxa. Thus, it is assumed that they still represent several primitive features in their vegetative and reproductive structures as well. Within seed cones of *C. vietnamensis* their small size, the low number of cone scales and ovules per cone scale and in the entire seed cone, the arrangement of ovules in mostly merely a single axillary row and a more or less columella like seed cone apex are regarded as primitive (Dörken & Jagel 2017). Within the other most derived *Cupressus* species the seed cones are much larger, with a significantly higher number of cone scales and ovules (Jagel 2002; Eckenwalder 2009; Jagel & Dörken 2014, 2015a, 2015b). In most derived *Cupressus* species ovules are arranged in several axillary rows per cone scale. A distinct columella like apex is missing in most taxa and the apex of the cone axis is only flat or gets completely lost by forming the distal pair of cone scales. Thus, within the genus *Cupressus* a tendency towards larger seed cones developing a significantly increased number of ovules per cone scale and in the entire seed cone is observable (e.g. Jagel 2002; Jagel & Dörken 2014, 2015a, 2015b; Dörken & Jagel 2017). Due to the loss of a meristematic apex the seed cones of the most derived *Cupressus* species are of limited growth and cannot undergo a further development.

Within seed cones of several coniferous groups including basal taxa of the Cupressaceae *s.l.* sterile distal elements are developed, which form together with the apex of the cone axis a so called “terminal piece”. Within Cupressaceae a tendency to reduce such sterile terminal elements is observable, so that finally the distal cone scales become fertile, as is the case for e.g. all taxa of the genus *Cupressus* and all Callitroid Cupressaceae (e.g. Jagel 2002; Jagel & Dörken 2014, 2015a, 2015b; Dörken & Jagel 2017). The reduction of the sterile distal elements leads also to a strong reduction of the seed cone apex. In taxa showing a sterile “terminal piece”, apical proliferations are quite frequently developed as is the case for e.g. Pinaceae, Araucariaceae or Sciadopityaceae. Also within the basal Cupressaceae, especially in taxa of the former Taxodiaceae (now merged into the Cupressaceae *s.l.*) apical proliferations are not uncommon (e.g. Tosh & Powell 1986; Caron & Powell 1991; Jagel & Stützel 2001; Farjon, 2008; Owens 2008; Bateman *et al.* 2011; Dörken 2011; Rudall *et al.* 2011). In most derived Cupressaceae lacking a sterile “terminal piece” e.g. *Cupressus* and all Callitroideae such apical proliferations are unusual, and within these taxa the reduction of the distal vegetative elements including a meristematic apex of the cone axis prevents such a vegetative proliferation. Thus, it is not surprising that until now, no report of proliferated *Cupressus* seed cones existed.

The evolutionary meaning of such proliferations is, however, still highly controversial. In general, apical vegetative proliferations of seed cones are quite useless for suggesting evolutionary scenarios, because they represent only a simple vegetative elongation of the cone axis, however with a significant change in the internode length and in the size and shape of leaves. In most cases this change is quite abrupt and transitional structures leading from the basal fertile cone scales to the trophophylls inserted distally at the proliferation usually lack. The leaves inserted at the apical proliferations usually show the morphology of typical trophophylls of the respective species (Dörken 2011). Thus, the apical proliferation illustrated in Fig. 1 is special because here several intermediary scales are developed. The shape of the basal most sterile leaves inserted at the apical proliferation is quite similar to the lower fertile peltate cone scales. The subsequent leaves get more

and more similar in size and shape to the typical scaly trophophylls of *C. vietnamensis*, which can be distinguished in lateral and median leaves. It could be shown that the peltation of the cone scales and the cone scale like sterile leaves inserted at the proliferated shoot axis is not caused by a meristematic fusion of marginal leaf tissues leading to the formation of a meristematic cross zone above the petiole as is the case for peltate leaves in the classical sense (Troll 1932; Franck 1976; Natho *et al.* 1990; Gleissberg *et al.* 2005; Throm 2007; Bresinsky *et al.* 2008; Leins & Erbar 2008, 2010; Fukushima & Hasebe 2014). The peltation of cone scales in *C. vietnamensis* is caused by a strongly ventral bulge, which development starts soon after pollination to close the cone. Due to this strong swollen ventral bulge the cone scales are finally peltate-like, carrying the tip of the cone scale as a more or less conspicuous central mucro on its back (Figs 1A-D). The transitional series from basal fertile cone scales via peltate sterile cone scale-like intermediary scales to distal sterile scaly trophophylls at the apical proliferation is characterized by a continuously reduction of this ventral bulge, which is finally lacking in the distal scaly trophophylls. Thus, these leaves are scaly and not peltate. These intermediary scales demonstrate quite well all structural changes occurring between typical shaped fertile cone scales and sterile scaly trophophylls.

The monomorphic tissue of the ventral bulge differs strongly from those of the cone scale by showing larger, thin-walled cells and lacking a differentiation into palisade and spongy parenchyma (Figs 3A-B). Due to these anatomical differences and the presence of a clear boundary-layer between the tissue of the cone scale and the ventral bulge both appear as different separate structures at the first glance and the ventral bulge might be interpreted as representing the seed scale. In this case the cone scale would represent a fusion product of the bract and seed scale. However, the late point of development and the vasculature within the ventral bulge definitively exclude this. The ventral bulge is always formed later than the ovules and even after pollination. Thus, it cannot produce or carry the ovules. Furthermore, it does not have its own independent vascular strand bundle. The vascular bundle strand supplying the ventral bulge results from one basal, dichotomous branching of the collateral bundle strand supplying the cone scale. This and the collateral structure of the vascular bundle, which is typical for a leaf, exclude the shoot character of this bulge. If the ventral bulge would reflect an ovuliferous short shoot, then it would be likely that its vasculature is concentric as is typical for a shoot axis, and that it is not formed by a dichotomous branching of a collateral one. Thus, the cone scale and its secondary developed ventral bulge are not representing a fusion product of the bract and seed scale. The cone scale represents exclusively a leaf (= bract scale), which is carrying axillary the ovuliferous short shoot (= seed scale), which is however reduced to the ovules. The formation of the ventral bulge is urgently needed to close the cone soon after pollination, so that the ovules can develop well protected within the closed cone (Jagel & Dörken 2014, 2015a, 2015b).

In contrast to apical proliferations, which are representing just a simple vegetative elongation of the cone axis, axillary proliferations are, however, quite important for suggesting evolutionary scenarios and to gain new insights into the original branching pattern of the respective type of coniferous seed cones. However, such axillary proliferations are exceptionally rare. By chance one of the investigated teratological *C. vietnamensis* seed cones showed such an axillary proliferation, which was inserted exactly in the same position where normally the axillary row of ovules is developed. A comparable vegetative axillary proliferation of seed cones was described for *Metasequoia glyptostroboides* Hu & W.C.Cheng (Cupressaceae, Coniferales) (Neubauer 1976; Dörken 2011). Dörken (2011) showed that the developmental sequence of the inserted leaves at the axillary proliferated shoot axis is similar with those of the ovules which are arranged in a single axillary row. Thus, Dörken (2011) suggested that such axillary proliferations are representing a homologous replacement of a single row of ovules by a sterile axillary foliar short shoot. The leaf-like structures inserted at the proliferated shoot axis were suggested to represent sterile leaf-like shaped ovules. Furthermore, Dörken's investigations of such axillary proliferations clearly show that ovules can occur in the position of leaves and perfectly replace them in the orientation and general structure. This is an important finding, because it can explain the occurrence of non-axillary ovules as they occur in some Cupressaceae genera, e.g. *Tetraclinis* or *Juniperus* (Jagel & Dörken

2014, 2015a, 2015b). Contrasting to *Metasequoia* with leaves arranged in two parastichies, in *Cupressus vietnamensis* four parastichies are developed, which makes it quite difficult to explain the scale leaves at the proliferated shoot axis as leaf like shaped sterile ovules as is suggested for *Metasequoia*, because in *Cupressus vietnamensis* significant differences in the developmental sequence of ovules and trophophylls exist. Despite this, the axillary vegetative proliferations of *C. vietnamensis* can be regarded as an atavism, which might reflect the primitive condition in Cupressaceae seed cones with an ovuliferous short shoot showing an elongated shoot axis which was inserted in the axil of a leaf. In most derived Cupressaceae the axis of the ovuliferous shoot is strongly reduced so that the ovuliferous short shoot is reduced to its ovules, as is also suggested by e.g. Jagel (2002), Jagel & Dörken (2014, 2015a, 2015b) or Dörken & Jagel (2017). Thus, these axillary proliferations found in *C. vietnamensis* seed cones leads to the hypothesis that the original structure of cupressaceous seed cones was a more open, loose inflorescence which was strongly reduced and modified so that finally the compact seed cones of living Cupressaceae species was formed.

## 5 Conclusion

Teratological seed cones of *C. vietnamensis* were investigated showing two types of vegetative proliferations, apical and axillary. It was shown that the apical proliferation represents just a vegetative elongation of the cone axis, which maintained a longer period of growth, and was not used up by the formation of the distal cone scales. However, the apical proliferation shows a full transitional series from basal most cone scale-like sterile peltate leaves to distal most decussate scaly trophophylls which show all structural changes from a sterile trophophyll to a fertile peltate cone scale. The axillary proliferation, however, is regarded to represent an atavism and it is suggested that the primitive condition of cupressaceous seed cones was an open and loose, polyaxial inflorescence, which became strongly reduced, so that in most derived Cupressaceae species the ovuliferous axillary short shoot (= seed scale) is reduced to its ovules so that finally a compact seed cone is formed.

## Acknowledgements

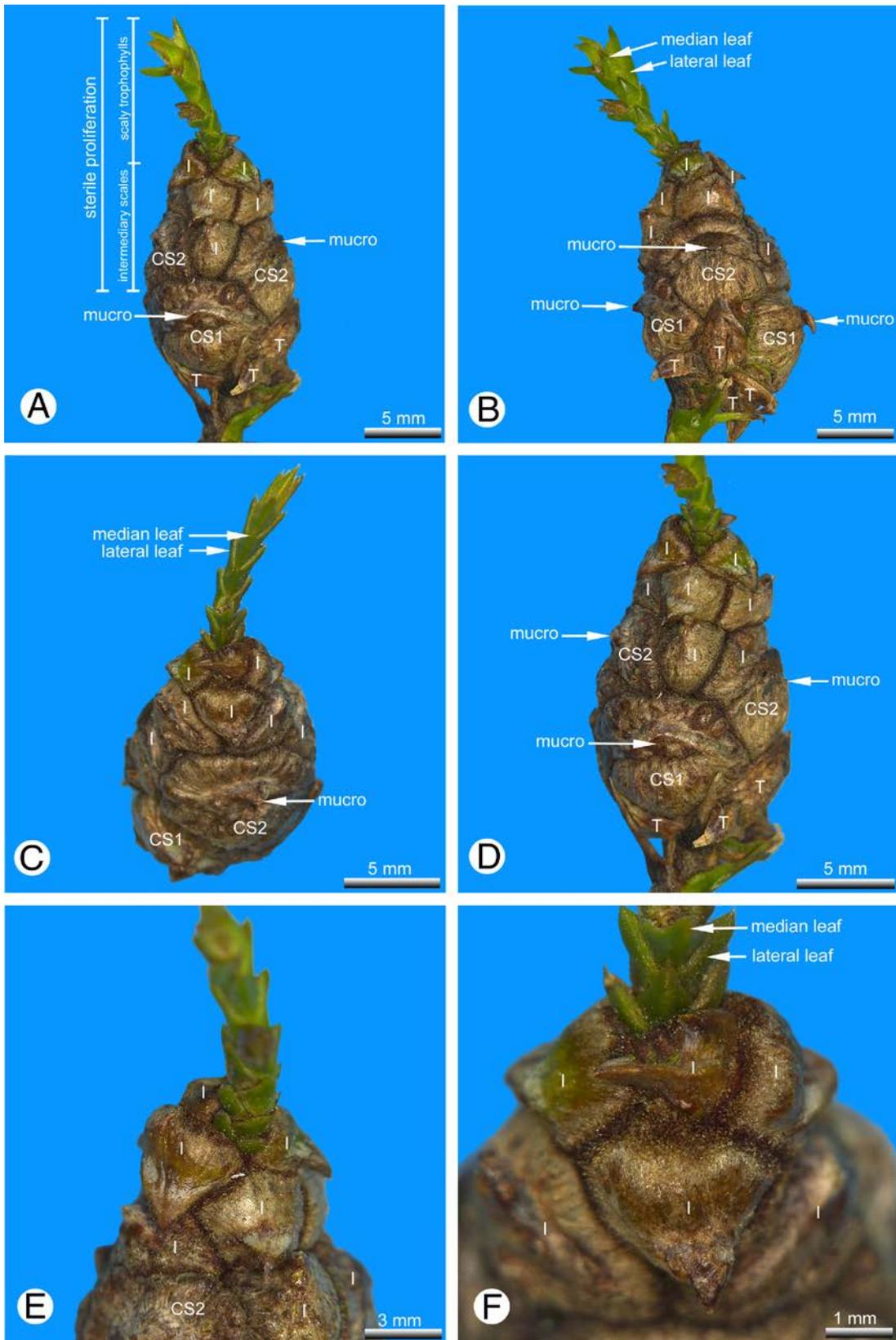
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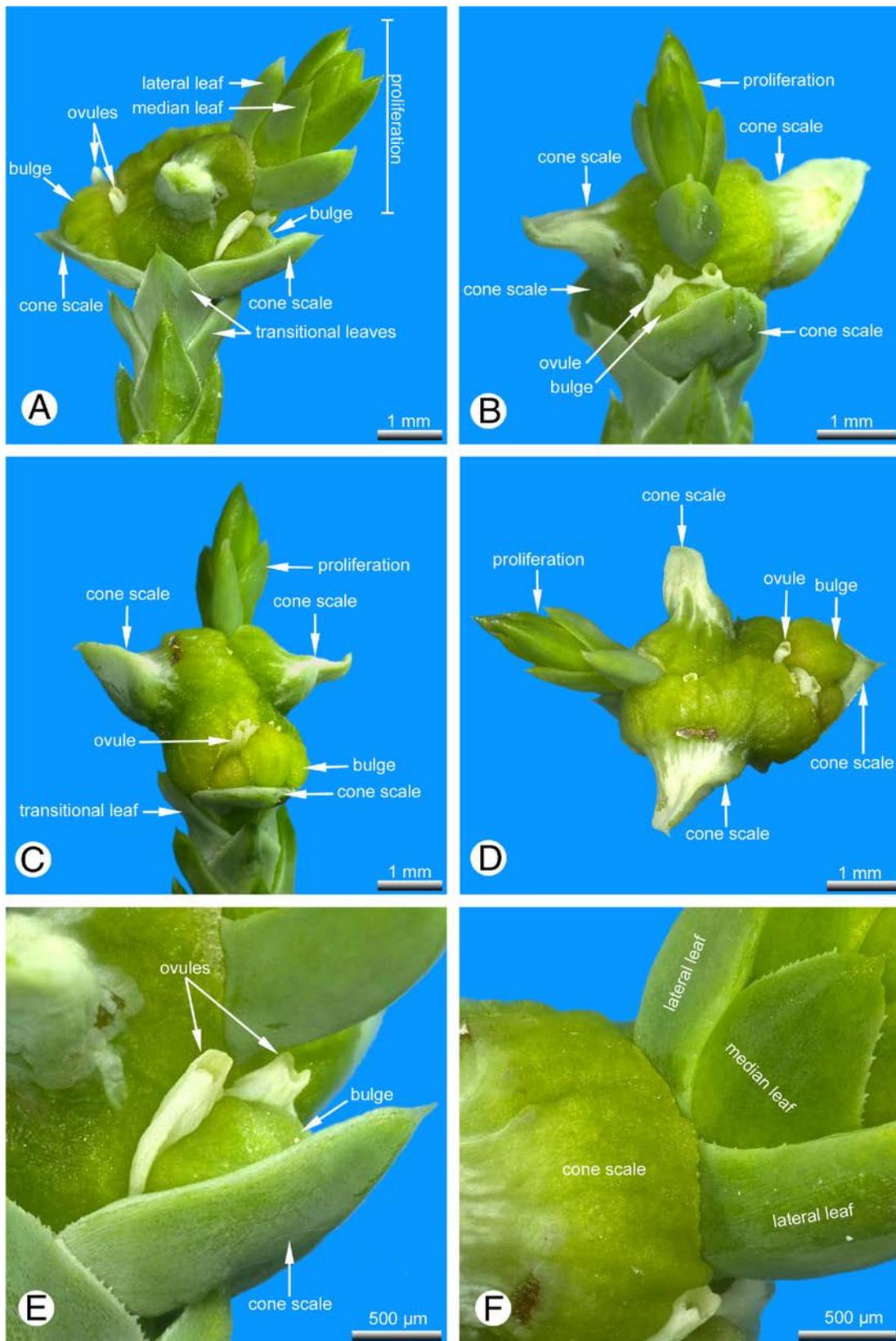
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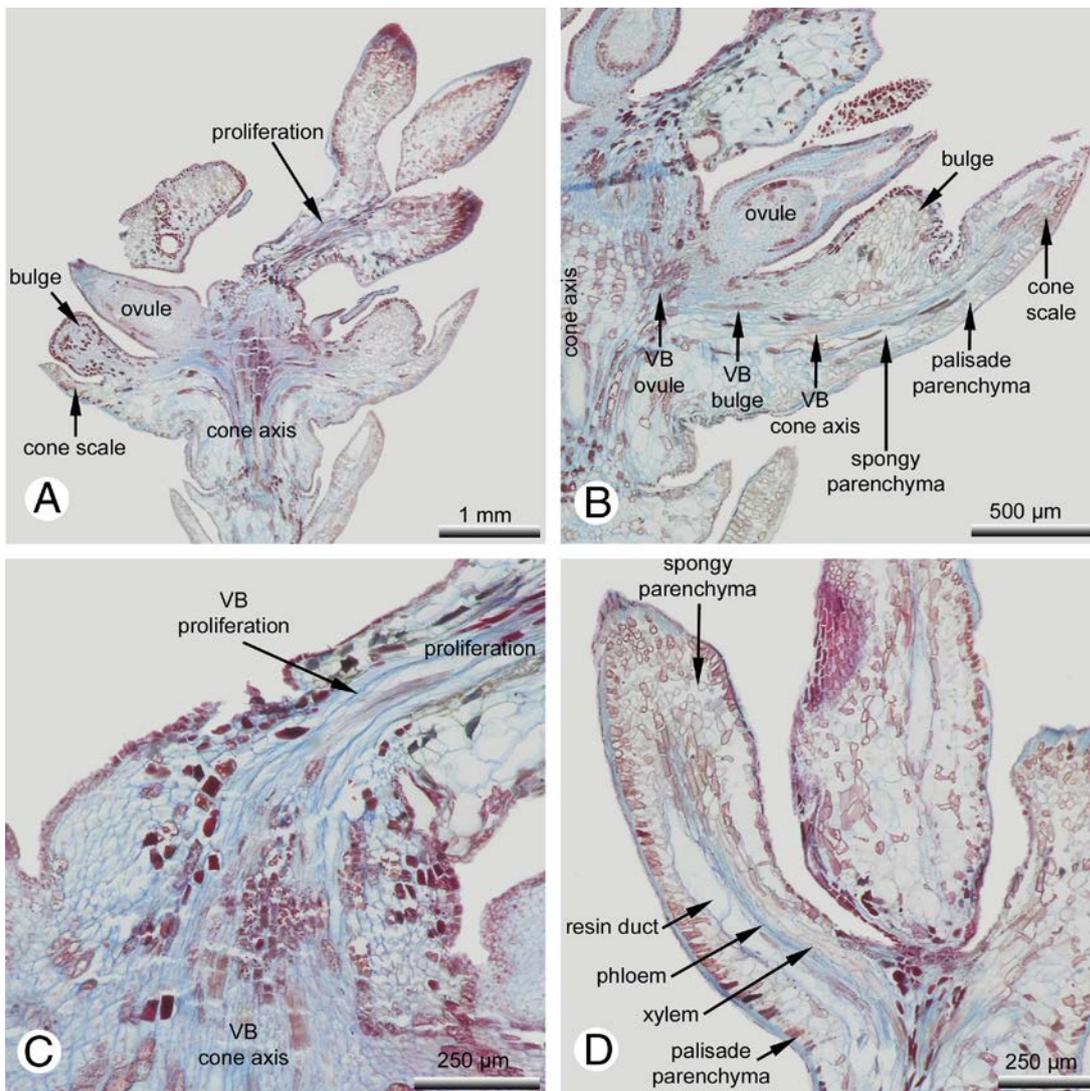
**Fig. 1:** *Cupressus vietnamensis*, apical proliferation.

**A-D:** Seed cone in different views; **E-F:** Detail of the distal part, showing several intermediary leaves leading towards the scaly trophophylls at the proliferation (CS1 = first pair of fertile cone scales; CS2 = second pair of fertile cone scales; I = intermediary scales; T = transitional leaf).



**Fig. 2:** *Cupressus vietnamensis*, axillary proliferation in one of the distal cone scales.

**A-D:** Proliferated seed cone in different views; **E:** Detail of a cone scale of the lower pair with two ovules and a distinct ventral bulge; **F:** Basal part of the proliferation.



**Fig. 3:** *Cupressus vietnamensis*, anatomy (longitudinal sections) of the seed cone illustrated in Fig. 2.

**A:** Median section of the cone axis showing the basal fertile pair of cone scales and the axillary proliferation in the subsequent pair of cone scales; **B:** Detail of a fertile cone scale of the lower pair, each the ovule, the ventral bulge and the cone scale are supplied by a separate vascular bundle (VB); **C:** Detail of A; vascular detail of the transitional zone between the cone axis and the proliferation; **D:** Detail of a scaly trophophyll inserted at the proliferation.



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### ***Cupressus vietnamensis* at the Bedgebury Pinetum, UK.**

Specimen in the shade with a majority of juvenile needles and a strong leader and no cone.

**Page 100:** Specimen in full sunlight showing mostly intermediate leaves and a great number of pollen cones.

Photos taken on the 22.04.2017.

At the Bedgebury Pinetum, *Cupressus vietnamensis* resisted to a temperature of  $-15^{\circ}\text{C}$  for a short time in December 2010.

Photos: *Cupressus* Conservation Project.

