Pollen cone anomalies in *Pinus sylvestris* and *Tsuga canadensis* (Pinaceae): can they give new insights in the evolution of microsporangiophores in conifers?

Abstract

In several pollen cones of *Pinus sylvestris* and *Tsuga canadensis*, in addition to the typical hyposporangiate microsporangiophores forming usually two microsporangia, several anomalous multisporangiate and perisporangiate microsporangiophores were also found. The results of the morpho-anatomical investigations clearly show that the adaxial scutellum is formed by strongly reduced adaxial microsporangia, which became sterile and scale-like. Thus, the adaxial scutellum does not represent the tip of a possible microsporophyll. It could be also shown that the microsporangia are not formed by the scutellum, but are formed by the central stalk of the microsporangiophore. Especially in distal microsporangiophores the scutellum is nearly always strongly reduced or completely absent. In anomalous terminal microsporangiophores found in *Tsuga canadensis* the stalk produced 1-3 distal microsporangia, but a scutellum was not developed. No evidence was found supporting the idea that the hyposporangiate type of microsporangiophores in Pinaceae is derived from a perisporangiate ancestral condition. The results deliver several supporting arguments that the microsporangia bearing structure corresponds to a microsporophyll. However, it still leaves open which part of the coniferous microsporangiophore is homologous to which part of a microsporophyll.

Key words: cone, evolution, conifers, *Pinus*, *Tsuga*, sporangiophore.

1 Introduction

In Pinaceae the male reproductive structures are arranged in compact strobili, the so called “pollen cones”. All pinaceous pollen cones are unbranched structures consisting of a central cone axis and several spirally inserted sporangiophores (e.g. Lotsy 1911; Krüssmann 1955, 1983; Coulter & Chamberlain 1917; Sporne 1965; Dallimore & Jackson 1966; Mirov 1967; Liu 1971; Farjon 1984, 1990, 2005, 2010; Eckenwalder 2008). Within pinaceous pollen cones, bracts and terminal microsporangiophores are always lacking (e.g. Pilger 1926; Farjon 1990; Mundry 2000). Branched pollen cones as developed in some Taxaceae and Cupressaceae (e.g. Lemoine-Sebastian 1967; Wilde 1975; Mundry & Mundry 2001; Farjon 2005; Eckenwalder 2009; Dörken *et al.* 2011; Schulz *et al.* 2014) are always absent in Pinaceae. Among extant Pinaceae the pollen cones differ significantly in size and shape from each other. Pollen cones in e.g. *Cathaya argyrophylla* are column-like, up to 70 mm long and consisting of 97-152 hyposporangiate microsporangiophores (Dörken & Nimsch 2015a). Pollen cones in e.g. *Tsuga canadensis*, however, are about 5-7 mm long and consist of 10-14 hyposporangiate microsporangiophores.

Within pollen cones of extant Conifers two different types of microsporangiophores are developed:

(1) perisporangiate, radial sporangiophores, with sporangia all around a central stalk;

(2) hyposporangiate, dorsiventral sporangiophores, with sporangia only on the abaxial side of a central stalk and an adaxial scutellum. The majority of extant Conifers are hyposporangiate. In Pinaceae only hyposporangiate sporangiophores are developed with always two abaxial microsporangia. Perisporangiate microsporangiophores as developed within Taxaceae (e.g. Wilde 1972; Mundry & Mundry 2001; Dörken *et al.* 2011; Schulz *et al.* 2014; Dörken & Nimsch 2015b) are always absent in Pinaceae. Even today the “true nature” of coniferous microsporangiophores is still discussed controversially; two conflicting major hypotheses exist:

(**1**) all microsporangiophores are homologous structures; the hyposporangiate (dorsiventral) type is derived from a perisporangiate one (e.g. Wordsell 1901; Dupler 1919; Dluhosch 1937);

(**2**) perisporangiate microsporangiophores represent a radial synangium consisting of several dorsiventral reduced microsporangiophores (e.g. Thomson 1940; Wilde 1975; Mundry & Mundry 2001;

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Dörken *et al.* 2011; Dörken & Nimsch 2015b). For Taxaceae it could be shown that the perisporangiate microsporangiophores represent radial synangia, consisting of several fused hyposporangiate microsporangiophores (Dörken & Nimsch 2015b). When regarding only typical shaped pollen cones and microsporangiophores, it is nearly impossible to make suggestions about the evolutionary pathway of pinaceous pollen cones and their sporangiophores. By chance several anomalous pollen cones in *Pinus sylvestris* and *Tsuga canadensis* were found, for example cones terminating with a microsporangiophore or cones forming multisporangiate microsporangiophores with a varying number of microsporangia instead of the typical two. Some of the anomalies seem to be quite helpful for suggesting evolutionary scenarios. The morpho-anatomical investigations will focus mainly on the question do the microsporangiophores correspond to microsporophylls and if so which parts in microsporangiophores and microsporophylls are homolog to each other. Furthermore the investigations should solve if the central stalk or the adaxial scutellum is responsible for producing the microsporangia. Investigations will be done with SEM and paraffin technique.

2 Material & Methods

2.1 Material

To exclude the possibility that the detected anomalies were random artifacts depending on genetic mutations or disturbances in the physiology of a single individual, pollen cones were collected from 10 different trees per species and also from different branches within the crown. Typical and anomalous pollen cones of *Pinus sylvestris* L. were collected from trees growing on the campus of the University of Konstanz (Germany). *Tsuga canadensis* (L.) Carrière was collected from trees cultivated in the Botanic Garden of the Ruhr-University Bochum (Germany), the Heinrich-Heine University Düsseldorf (Germany) and also on the Isle of Mainau (Konstanz, Germany).

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 (Gerstberger & Leins 1978) and critical point dried. Sputter coating was done with a sputter coater SCD 50 BAL-TEC (BALZERS). The pollen cones were examined with an AURIGA ZEISS TM. Microphotography was accomplished using 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 coniferous microsporangiophores as microsporophylls. Here I avoid using the term “sporophyll” or “microsporophyll” for the sporangia bearing structure as otherwise a homology that is applied to it would be introduced *a priori*. In general it is also still unclear which parts within the microsporangiophore can be regarded as homologous to which part of a leaf. Thus, the green adaxial scale-like structure in hyposporangiate microsporangiophores will be termed as scutellum and not as “phylloid rest” as is frequently done.

3 Results

Different types of pollen cone anomalies were found which can be classified into two morphological groups.

3.1 Anomalous multisporangiate microsporangiophores

3.1.1 *Pinus sylvestris*

Pollen cones of *Pinus sylvestris* consist of several spirally arranged hyposporangiate microsporangiophores. Each microsporangiophore consists usually of two abaxial sporangia and a distinct adaxial scutellum (fig. 1). Within 8.5% of the 300 investigated pollen cones, microsporangiophores bearing more than the usual two sporangia were found (figs 2E & F). They are developed in all regions of the pollen cone, but most frequently in distal parts (figs 2B-F). The majority of the multisporangiate microsporangiophores had three sporangia (figs 2A-D). The sporangia and the adaxial scutellum are similar in size and shape to these formed in typical bisporangiate microsporangiophores. Anomalous multisporangiate microsporangiophores bearing four sporangia were only found in most distal parts of

the pollen cones close to the tip of the cone axis (figs 1E & F). The scutellum of these microsporangiophores is deeply notched into two halves. Each of the halves is fused with two microsporangia (fig. 1F). The sporangia have the same dimensions as those of typical microsporangiophores.

3.1.2 *Tsuga canadensis*

Typical pollen cones of *Tsuga canadensis* consist of a varying number of spirally arranged, hyposporangiate microsporangiophores, each of them bearing two sporangia and a small adaxial scutellum (fig. 3). The scutellum is generally quite small (figs 3A-E). In 7% of the 300 investigated pollen cones, also some lateral microsporangiophores showing 3 fertile sporangia (fig. 4) were found. The adaxial scutellum is nearly completely reduced. These anomalous microsporangiophores are supplied with a single collateral vascular bundle strand.

3.2 Formation of an anomalous terminal microsporangiophore

3.2.1 *Tsuga canadensis*

In typical pollen cones, the microsporangiophores are developed exclusively lateral at the cone axis (fig. 5), so that the apex of the cone axis can still be observed as a small tip (fig. 5C). In 8% of the 300 investigated pollen cones a terminal microsporangiophore was developed at the end of the cone axis (fig. 6). Most of the terminal microsporangiophores are perisporangiate. All sporangia are fertile. Apart to the perisporangiate type some terminal microsporangiophores were found consisting only of a stalk and 1 (figs 6E & F) or 2 (fig. 6D) sporangia. A scutellum is not developed.

4 Discussion

Within Conifers the morphological identity of the microsporangium-bearing structure is still controversial. Even defining a clear border between the different parts within the microsporangiophores e.g. between the central stalk and the scutellum is very difficult. Thus, it is still quite problematic to determine if the microsporangiophores are displaying homologous structures among all extant Conifers (Schulz *et al.* 2014). Several authors regard the microsporangiophores in Conifers as reduced fertile leaves. Thus they are often called “microsporophylls” (e.g. Lotsy 1911; Krüssmann 1955, 1983; Coulter & Chamberlain 1917; Sporne 1965; Dallimore & Jackson 1966; Mirov 1967; Liu 1971; Farjon 1984, 2005, 2010: 1990; Eckenwalder 2009). However, the terminal position of microsporangiophores at the cone axis as found in some anomalous shaped pollen cones of *Tsuga canadensis* would exclude a leaf character for this structure, because leaves are always developed as lateral organs at the apex and can therefore never be developed terminal. However, they can be shifted by secondary growing processes out of the primary lateral position into a terminal one. Within mature pollen cones it cannot be recognized if the terminal position is the original one or if the sporangiophore has secondarily been shifted out of its original lateral position into the terminal one. This can only be solved in ontogenetic studies, but these anomalies are too rare, so that ontogenetic studies are nearly impossible. Such anomalous terminal microsporangiophores were described also for other coniferous groups, e.g. *Podocarpus* (Dörken & Nimsch 2015c). Terminal microsporangiophores are usually absent in Pinaceae, but typical for pollen cones of *Cephalotaxus* and *Torreya* (Taxaceae). In both *Cephalotaxus* and *Torreya* the terminal perisporangiate microsporangiophore represents a radial synangium consisting of 2-4 fused hyposporangiate microsporangiophores that get in physical contact and finally fuse to a single radial structure (Dörken & Nimsch 2015b). Depending on the number of hyposporangiate microsporangiophores that are involved in forming the perisporangiate terminal microsporangiophore the number of vascular bundle strands supplying the microsporangiophore varies strongly. Thus, the perisporangiate microsporangiophores in Taxaceae do not represent a peltate-like microsporophyll (Dörken & Nimsch 2015b). The anomalous perisporangiate terminal microsporangiophores found in *Tsuga canadensis* are quite different from that. In *Tsuga canadensis* the perisporangiate microsporangiophore are supplied by only a single collateral vascular bundle strand as is typical for hyposporangiate microsporangiophores. There is no evidence to regard the anomalous perisporangiate microsporangiophores of *Tsuga canadensis* as a Taxaceae-like radial synangium. Thus, two completely different development pathways are distinguishable between perisporangiate microsporangiophores of Taxaceae on one side and the anomalous perisporangiate ones in *Tsuga canadensis* on the other side. The results clearly show that in *Tsuga canadensis* the third adaxial microsporangium does not belong to a possible second, fused microsporangiophore. Here the central stalk developed a further fertile microsporangium instead of an adaxial scutellum. This explains why only a single collateral vascular

bundle is developed within the anomalous perisporangiate sporangiophores of *Tsuga canadensis*. The collateral structure of the vascular bundle indicates that the sporangia bearing structure has to be a leaf and excludes a shoot- or synangium-character. It remains, however, open, which part of the microsporangiophore belongs to which part of the leaf.

Within extant Conifers forming hyposporangiate microsporangiophores the number of microsporangia developed per sporangiophore varies strongly between the different systematic groups. Among e.g. Pinaceae, Sciadopityaceae and Podocarpaceae the microsporangiophores are always bisporangiate as is also the case for some taxa within Cupressaceae (e.g. *Athrotaxis* and some *Cupressus* species). However, in most Cupressaceae and Taxaceae the number of sporangia developed per hyposporangiate microsporangiophore varies strongly not only between the different taxa, also even within a single pollen cone (Farjon 1984, 1990, 2010). In several of the investigated pollen cones of *Pinus sylvestris* (fig. 2) and *Tsuga canadensis* (fig. 4) also several multisporangiate sporangiophores bearing 3 or 4 microsporangia, were inserted lateral at the cone axis. Within *Tsuga canadensis* the number of anomalous multisporangiate microsporangiophores was especially high. Such multisporangiate microsporangiophores are also described for other Pinaceae e.g. *Picea asperata* (Mundry 2000) and *Cathaya argyrophylla* (Dörken & Nimsch 2015a). Within several of the lateral anomalous *Tsuga canadensis* microsporangiophores showing 3 instead of the usually 2 sporangia, the additional third fertile one is developed exactly in the position where usually the scutellum would be formed. It seems that in Pinaceae the scutellum is formed by a reduced microsporangia that becomes sterile and scale-like. This is conforming to the results of earlier studies on microsporangiophores in *Pseudotaxus* (Taxaceae). Dörken & Nimsch (2015b) found apart from the typical perisporangiate microsporangiophores, several anomalous hyposporangiate ones with a distinct adaxial scutellum that is varying strongly in size and shape. It could be shown that in these anomalous hyposporangiate microsporangiophores the adaxial scutellum is formed by strongly reduced adaxial microsporangia, which became sterile. This explains why the abaxial microsporangia are not attached to the adaxial scutellum but exclusively to the central stalk. In this case the adaxial scutellum does not represent the tip of a possible microsporophyll. Some of the detected multisporangiate microsporangiophores of *Pinus sylvestris* are representing a fusion product of 2 hyposporangiate microsporangiophores that were fused laterally to each other so that the adaxial scutellum is still developed (figs 2E & F). Thomson (1940) observed such fusions at or near the apex of the pollen cones in all coniferous families. Such fusions are representing a simple contact parastichy of directly neighboured microsporangiophores and not of ontogenetic subsequent microsporangiophores. Their fusion takes place in earliest ontogenetic stages favoured by the broad bases of the central stalk of the microsporangiophores and due to a lack of space in distal parts of the cone axis. First, within a parastichy the primordia of directly neighboured microsporangiophores get in physical contact. Secondary, they fuse completely with each other so that the fusion product has only a single very broad stalk and also a very broad but conspicuously notched scutellum (figs 2E &F). Each half of the scutellum belongs to one of the involved microsporangiophores. Such contact parastichys are also random accidental artifacts and therefore without evolutionary relevance.

Apart from the strongly varying number of sporangia per microsporangiophore, the size and shape of the scutellum varies significantly between extant Conifers. Within the investigated anomalous multisporangiate microsporangiophores of *Tsuga canadensis* and *Pinus sylvestris* the central sporangia are not fused with the scutellum as is a typical feature for microsporangiophores forming usually more than 2 sporangia (e.g. several Cupressaceae). The central sporangia are exclusively attached to the stalk, as is also the case in the investigated *Pinus sylvestris* (figs 2A-D) and *Tsuga canadensis* (figs 2A-E). Especially at microsporangiophores developed in distal parts of the pollen cone the scutellum is mostly completely reduced so that only stalked microsporangia are formed (figs 6D-F). This clearly indicates that microsporangia are not formed by the scutellum but by the central stalk of the microsporangiophore. This idea is supported by anomalous terminal microsporangiophores of *Tsuga canadensis*, consisting of only a stalk and distal sporangia. Especially the anomalous microsporangiophores consisting of a central stalk terminating with a single microsporangium demonstrate quite well that the scutellum is not needed for forming the microsporangia (figs 6E & F).

5 Acknowledgements

I am grateful to Dr. Michael Laumann and Mrs. Lauretta Nejedli (SEM-Center, Department of Biology, University of Konstanz, Germany) for technical support (SEM and paraffin technique). Furthermore I thank all visited Botanic Gardens for generous providing of research material.

6 Bibliography

Coulter, J. M & C.J. Chamberlain (1917). *Morphology of Gymnosperms*. 2nd edition. University of Chicago Press, Chicago (reprint 1982).

Dallimore, W. & A.B. Jackson (1966). *A handbook of Coniferae and Ginkgoaceae*. London: Arnold.

Dluhosch, H. (1937). Entwicklungsgeschichtliche Untersuchungen über die Mikrosporophylle der Koniferen. *Bibliot. Bot.* 114(3): 1-24.

Dörken V. M., Z.X. Zhang, I.B. Mundry & Th. Stützel (2011). Morphology and anatomy of male reproductive structures in *Pseudotaxus chienii* (W.C. Cheng) W.C. Cheng (Taxaceae). *Flora* 206: 444-450.

Dörken, V.M. & H. Nimsch (2015a). Morpho-anatomical investigations of cones and pollen in *Cathaya argyrophylla* Chung & Kuang (Pinaceae, Coniferales) under systematical and evolutional aspects. *Feddes Repert.* 125: 25-38.

Dörken, V.M. & H. Nimsch (2015b). About the origin of perisporangiate sporangiophores in taxaceous pollen cones. *Bull. CCP* 5: 3-21.

Dörken, V.M. & H. Nimsch (2015c). Morphology and anatomy of pollen cones and pollen in *Podocarpus gnidiodes* Carrière (Podocarpaceae, Coniferales) and its evolutionary relevance. *Bull. CCP* 4: 36-48.

Dupler, A.W. (1919). Staminate strobilus of *Taxus canadensis*. *Bot. Gaz.* 68: 345-366.

Eckenwalder, J.E. (2009). *Conifers of the World*. Timber Press, Portland.

Farjon, A. (1984). *Pines, drawings and descriptions of the genus* Pinus. Brill, Leiden.

Farjon, A. (1990). *Pinaceae. Drawings and descriptions of the genera* Abies, Cedrus, Pseudolarix, Keteleeria, Nothotsuga, Tsuga, Cathaya, Pseudotsuga, Larix *and* Picea. Koeltz Scientific Books, Königstein.

Farjon, A. (2005). *A monograph of Cupressaceae and* Sciadopitys. Royal Botanic Gardens, Kew.

Farjon, A. (2010). *A handbook of the world's conifers*. Vol. I & II. Brill, Leiden.

Gerlach, D. (1984). *Botanische Mikrotomtechnik, eine Einführung*. 2nd ed. Thieme, Stuttgart.

Gerstberger, P. & P. Leins (1978). Rasterelektronenmikroskopische Untersuchungen an Blütenknospen von *Physalis philadelphia* (Solanaceae). *Ber. Deutsch. Bot. Ges.* 91: 381-387.

Krüssmann, G. (1955). *Handbuch der Nadelgehölze*. 1st ed. Parey, Berlin, Hamburg.

Krüssmann, G. (1983). *Handbuch der Nadelgehölze*. 2nd ed. Parey, Berlin, Hamburg.

Lemoine-Sébastian, C. (1967). Appareil reproducteur mâle de *Juniperus*. *Trav. Lab. Forest. Toulouse* 1(6): 1-35.

Liu, T. S. (1971). *A monograph of the genus* Abies. National Tapei University, Taipei.

Lotsy, J. P. (1911). *Vorträge über die Botanische Stammesgeschichte, ein Lehrbuch der Pflanzensystematik*. Dritter Band: Cormophyta Siphonogamia, erster Teil. Fischer, Jena.

Mirov, N. T. (1967). *The Genus* Pinus. Ronald Press, New York.

Mundry, I. (2000). Morphologische und morphogenetische Untersuchungen zur Evolution der Gymnospermen. *Bibliot. Bot.* 152.

Mundry. I. & M. Mundry (2001). Male cones in Taxaceae s.l. - an example of Wettstein's pseudanthium concept. *Plant. Biol.* 3(2001): 405-416.

Page, C.N. (1990). Gymnosperms. In Kubitzky, K. (ed.). *The Families and Genera of Vascular Plants.* Vol. 1. Berlin, Heidelberg. u.a.: Springer: 279-391.

Pilger, R. (1926). Coniferae. In: Engler, A. *Die natürlichen Pflanzenfamilien nebst ihren Gattungen und wichtigeren Arten insbesondere der Nutzpflanzen*. Leipzig: Engelmann.

Schulz, C., K.V. Klaus, P. Knopf, M. Mundry, V.M. Dörken & Th. Stützel (2014). Male cone evolution in conifers: not all that simple. *Amer. J. Pl. Sci.* 5: 2842-2857.

Sporne, K.R. (1965). *The morphology of gymnosperms: The structure and evolution of primitive seed-plants*. Hutchinson & Co., London.

Thomson, R.B. (1940). The structure of the cone in the Coniferae. *Bot. Rev.* 6: 73-84.

Wilde, M.H. (1975). A new interpretation of microsporangiate cones in Cephalotaxaceae and Taxaceae. *Phytomorphology* 25: 434-450.

Wordsell, W.C. (1901). The morphology of the "flower" of *Cephalotaxus*. *Ann. Bot.* 15: 637-652.

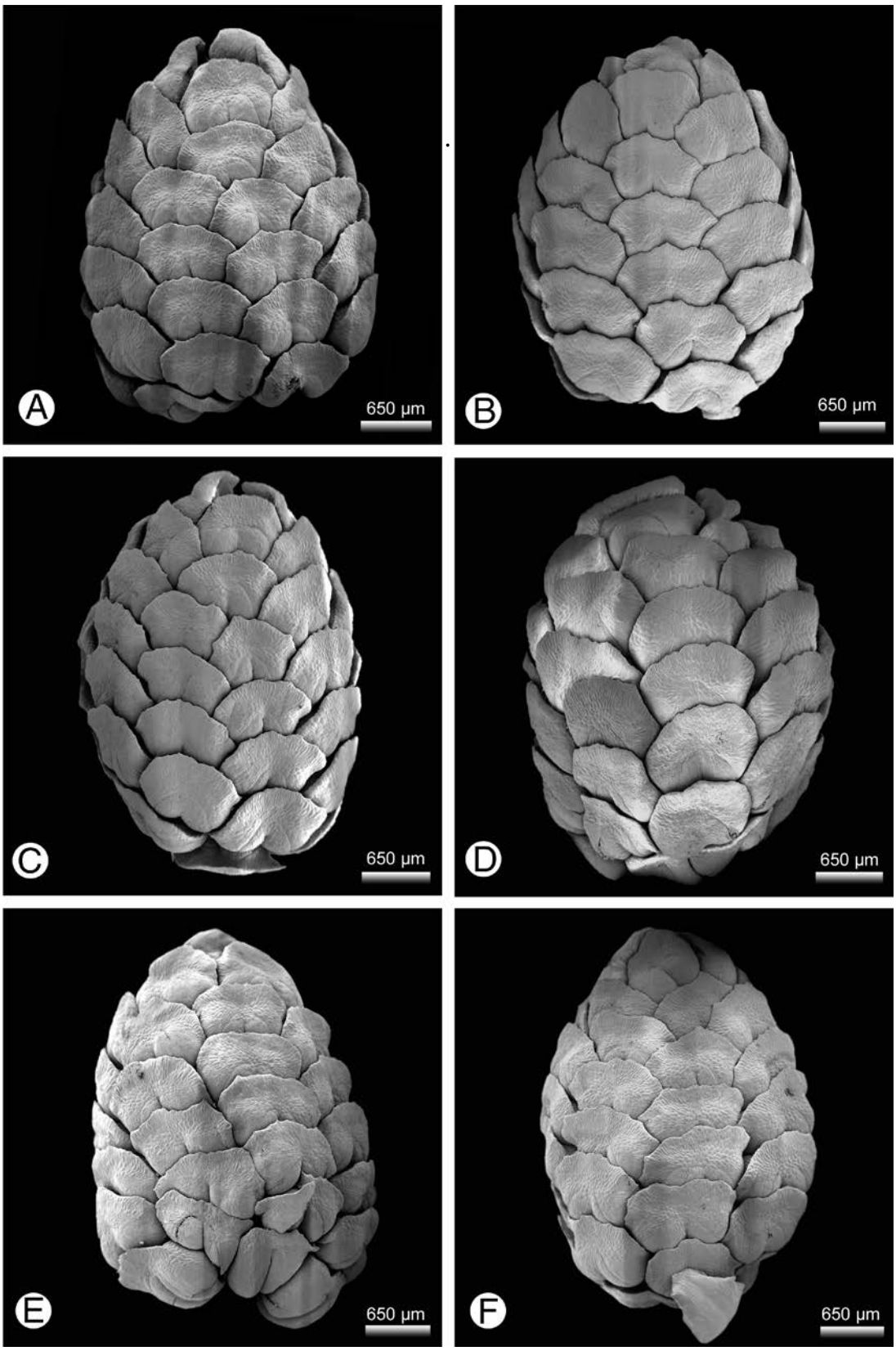

Fig. 1: *Pinus sylvestris*.
Lateral view of typical pollen cones carrying several spirally arranged hyposporangiate microsporangiophores; each sporangiophore with two sporangia and a distinct adaxial scutellum.

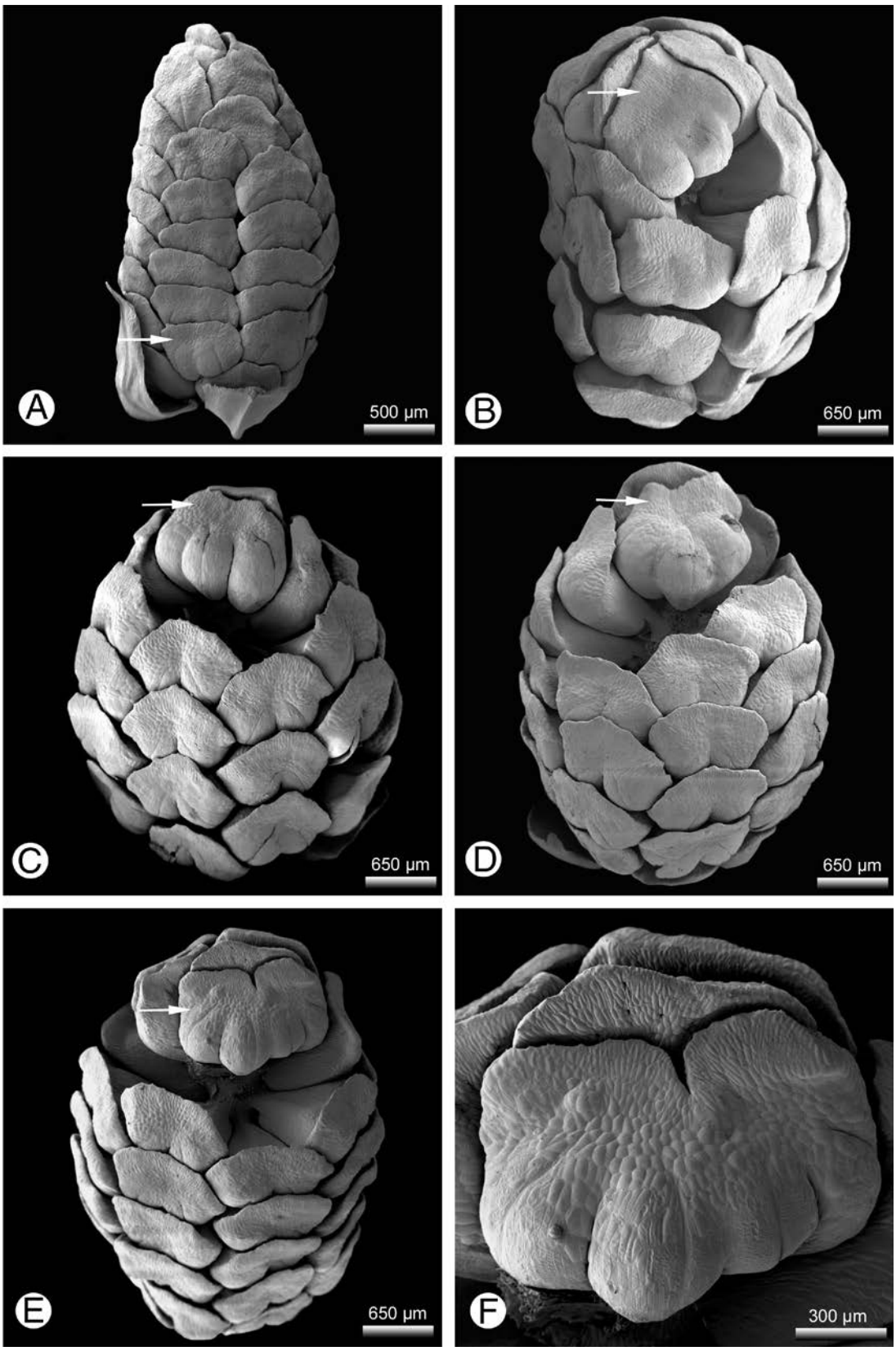

Fig. 2: *Pinus sylvestris*.

Pollen cones with anomalous multisporangiate microsporangiophores showing three (**A**-**D**) or four (**E** & **F**) sporangia and a distinct scutellum; scutellum at microsporangiophores with four sporangia deeply notched (**E** & **F**); for a better overview some microsporangiophores removed.

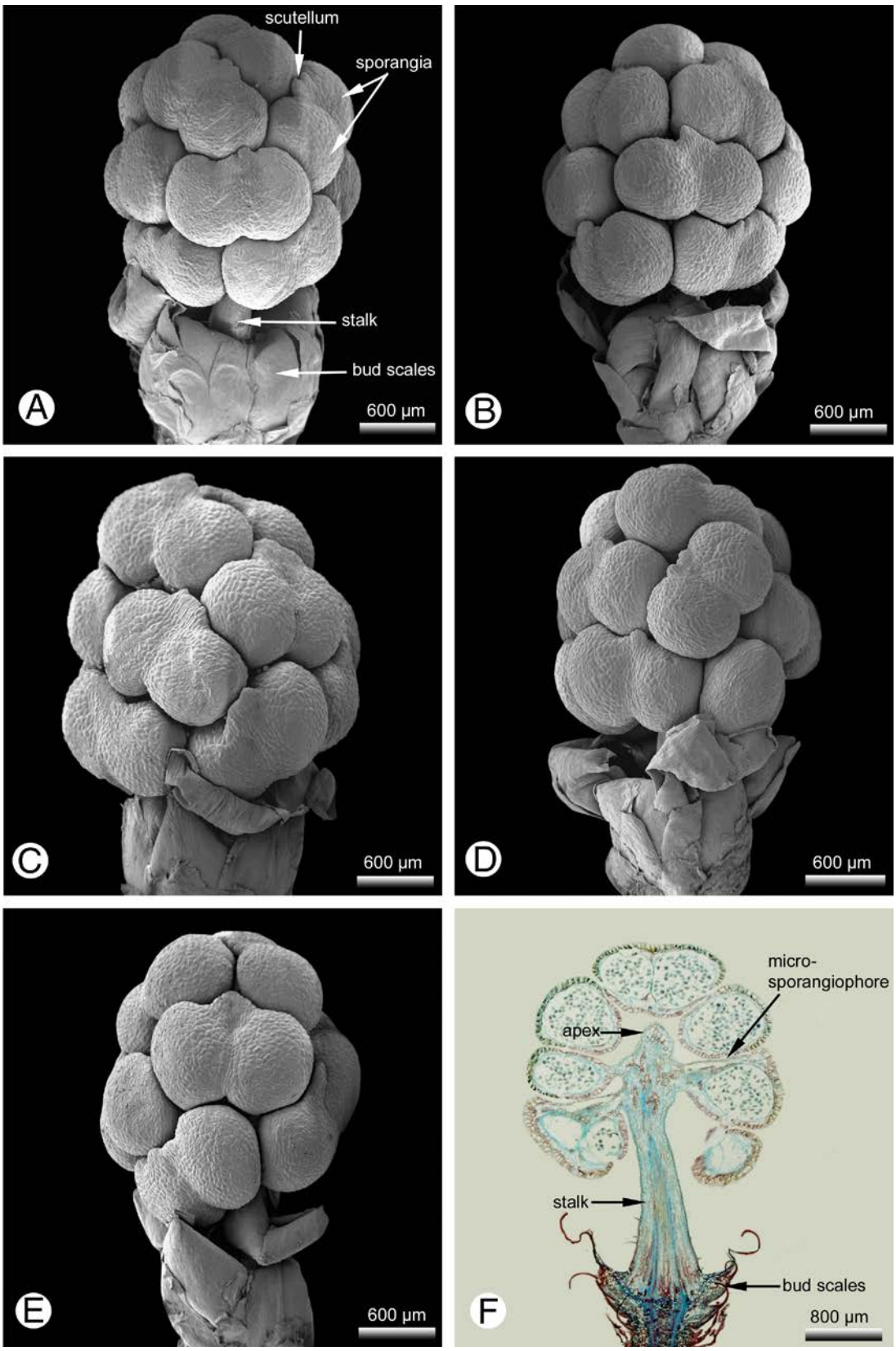

Fig. 3: *Tsuga canadensis*.

Typical pollen cones in lateral view; the cones consist of several hyposporangiate microsporangiophores; each with two microsporangia and a small adaxial scutellum **(A-E)**; longitudinal section of a pollen cone (**F**).

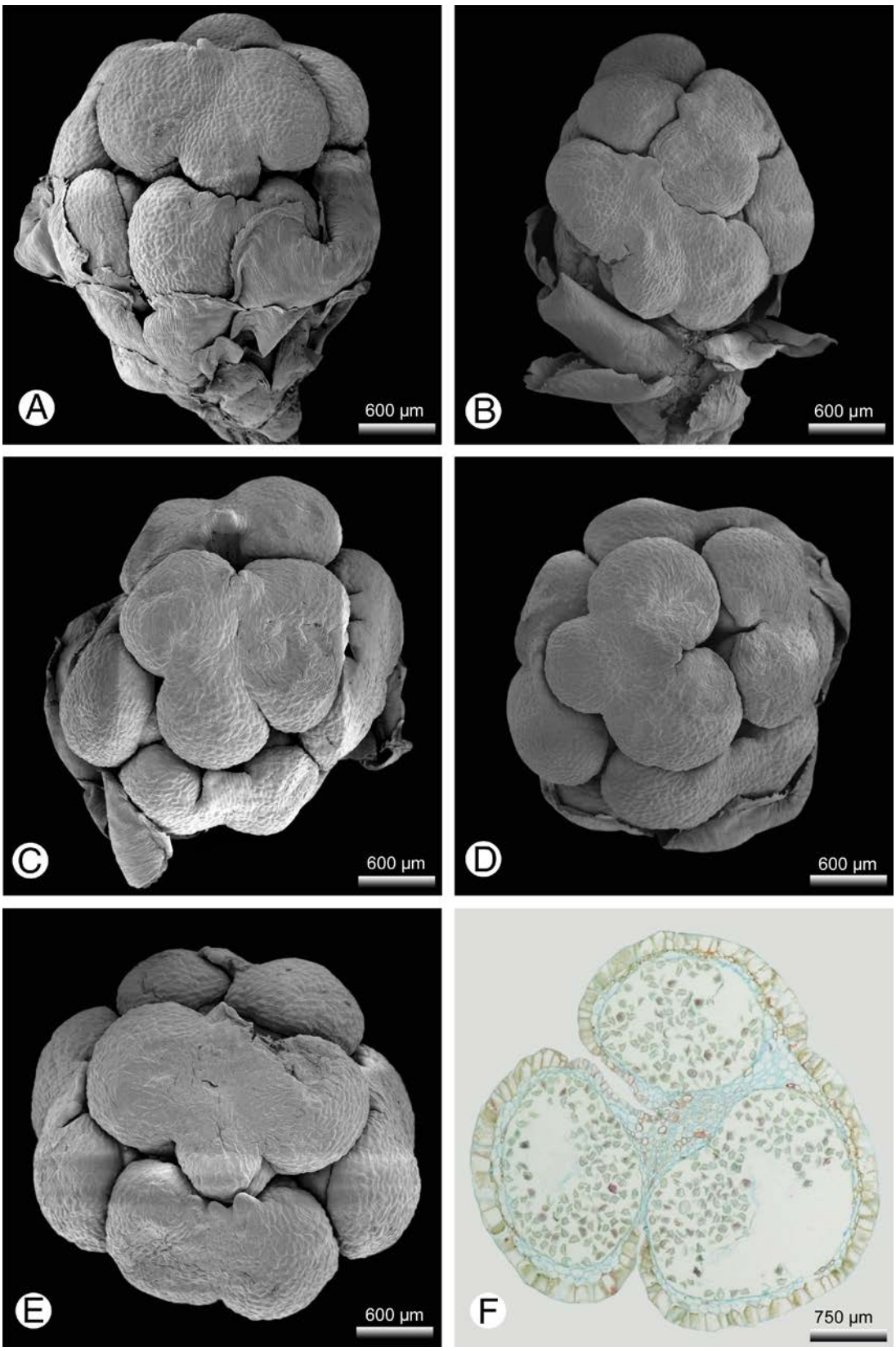

Fig. 4: *Tsuga canadensis*.

Pollen cones with anomalous microsporangiophores showing three instead of the usual two sporangia (**A**-**E**); all 3 sporangia are fertile (**F**).

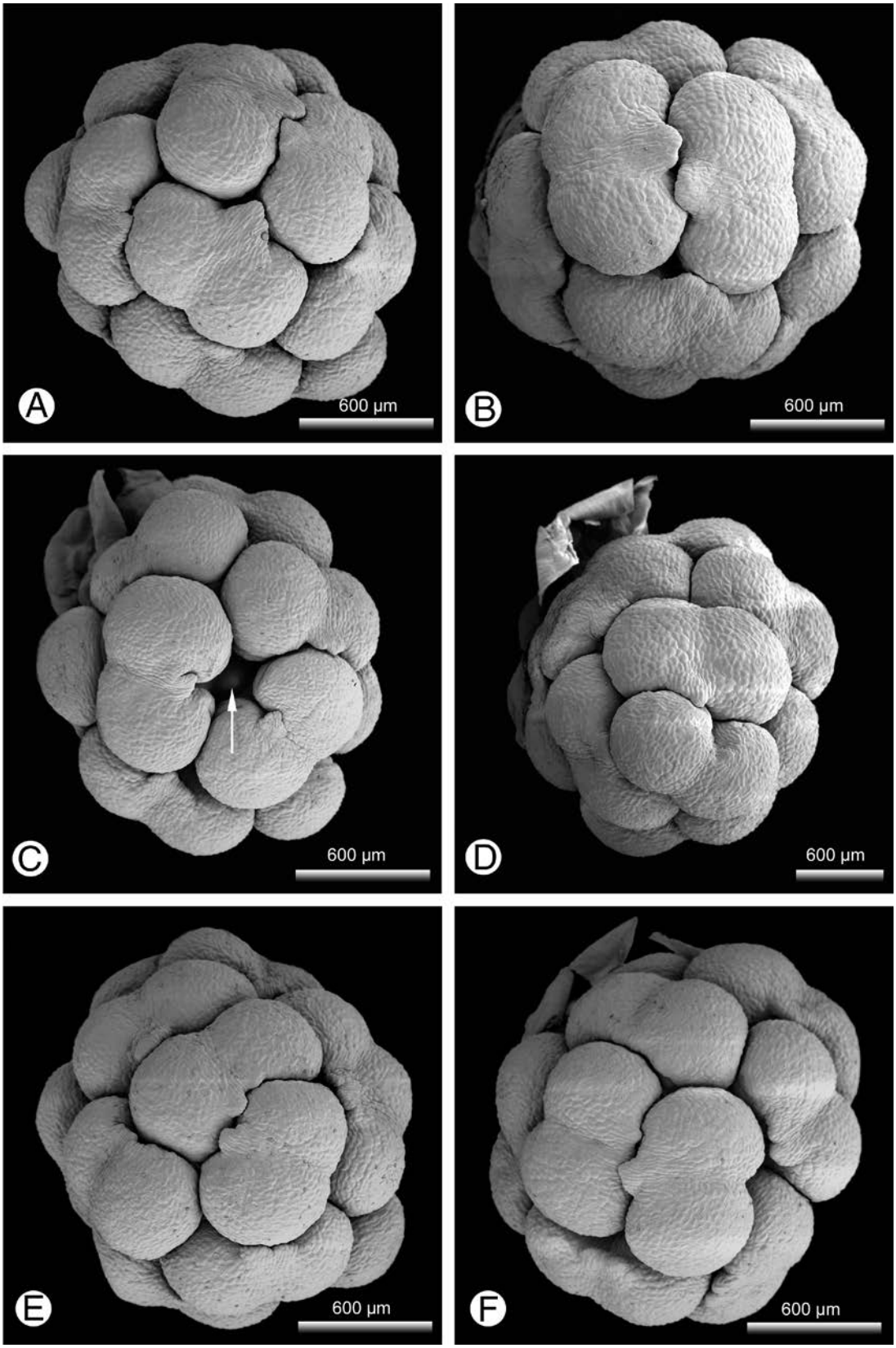

Fig. 5: *Tsuga canadensis.*

Top view of typical shaped pollen cones; a terminal microsporangiophore is always absent, sometimes the tip of the cone axis can be observed (**C**, arrow).

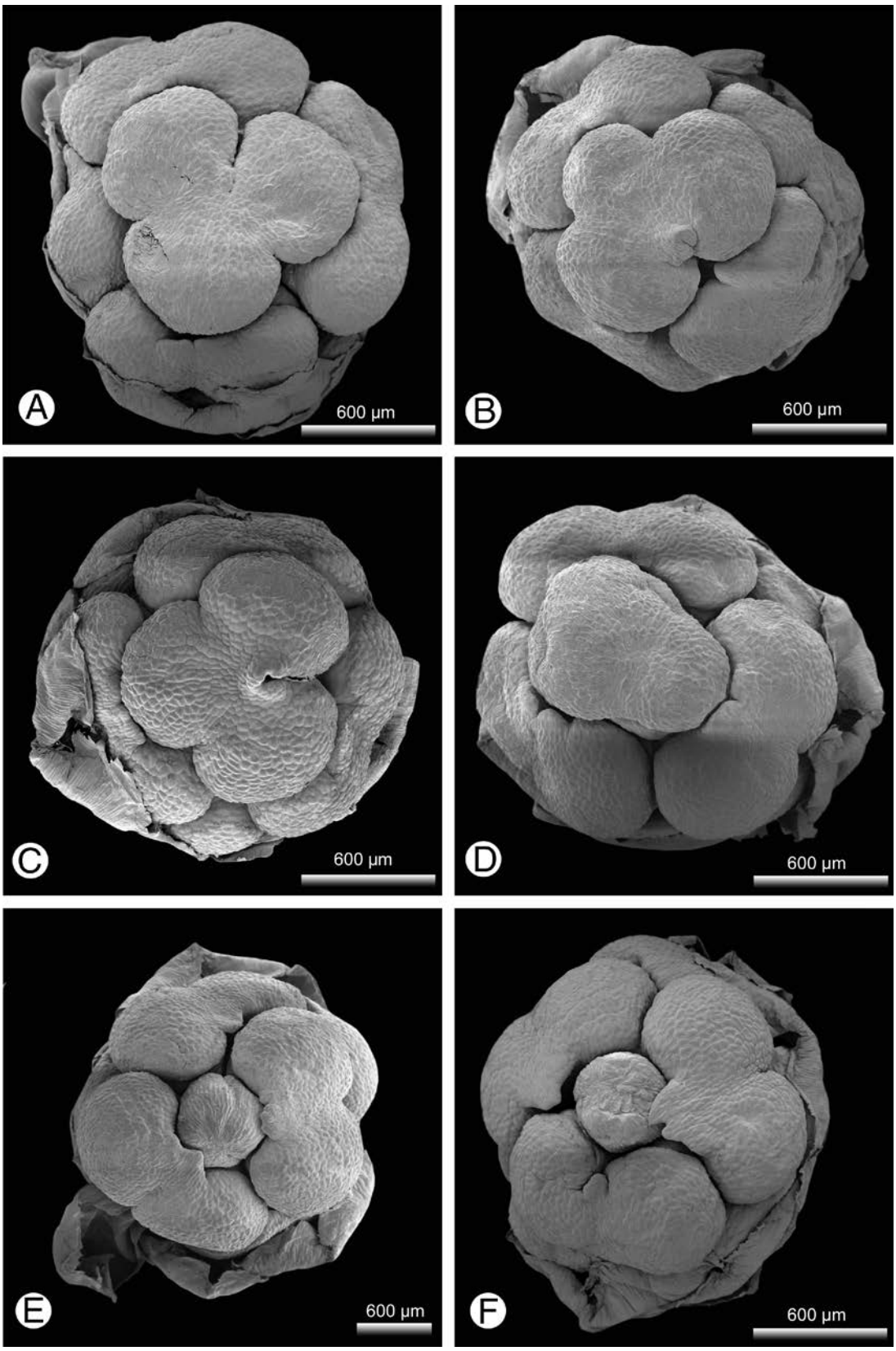

Fig. 6: *Tsuga canadensis*.

Anomalous shaped pollen cones terminating with a sporangiophore; within most of the microsporangiophores three sporangia are developed; the scutellum is strongly reduced (**A-C**); within microsporangiophores showing two (**D**) or one (**E-F**) sporangia, a scutellum is absent.