universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Pollen Morphology and Ultrastructure of Selected Zingiberaceae and Costaceae Species"

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2017 / Vienna 2017

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 832
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Botanik
Betreut von / Supervisor:	ao. Univ.-Prof. Mag. Dr. Martina Weber

Abstract

About structure and sculpture of Zingiberaceae and Costaceae pollen few information is available. In the present study six species of Zingiberaceae and three species of Costaceae were investigated. Various light microscopic (LM), scanning- (SEM) and transmission electron microscopic (TEM) techniques were applied to elucidate pollen wall stratification.

Zingiberaceae pollen is inaperturate and exine-less. The wall structure consists of a three-layered intine and a polysaccharide surface layer. Ornamentation elements are echini, and not resistant to acetolysis, which indicates polysaccharides.

Compared to Zingiberaceae, Costaceae pollen is aperturate and psilate. The wall comprises a sporopollenin exine (tectum, infratectum) and a mono-layered intine.

The pollen wall structure of other Zingiberales, like Strelitziaceae, Heliconiaceae or Cannaceae, are well comparable to Zingiberaceae. The common feature is a thick, channeled ektintine, whereas the presence of an exine is the major difference.

Kurzfassung

Zingiberaceae und Costaceae gehören zur Ordnung der Zingiberales (Ingwerartige) und sind unter anderem als Zier-, Arznei- oder Gewürzpflanzen bekannt. Aus palynologischer Sicht sind diese Familien wenig erforscht. Die Pollenwand ist im Vergleich zu der, vieler anderer Angiospermen sehr ungewöhnlich strukturiert. Es konnte gezeigt werden, dass die Pollenwand der inaperturaten Zingiberaceae aus einem 3-schichtigen Intine-Komplex besteht. Nach außen hin schließt ein dünner „surface layer“ an. Die Ornamentierung ist psilat bis echinat. Im Gegensatz zu herkömmlichen Pollenwänden bestehen die Skulptur-Elemente aus Polysacchariden, und nicht aus widerstandsfähigem Sporopollenin. Dieses Merkmal grenzt die Zingiberaceae klar von den anderen Familien der Zingiberales ab.

Um diese komplexe Struktur der Pollenwand aufzuklären, wurden mit unterschiedlichsten licht- und elektronenmikroskopischen Methoden, sechs Zingiberaceae- sowie drei Costaceae-Arten untersucht.

Trotz naher Verwandtschaft unterscheiden sich die Pollenwände der Costaceae deutlich von denen der Zingiberaceae. Costaceae besitzen ein acetolyseresistentes Sporoderm, bestehend aus einer massiven sporopollenin-haltigen Exine und einer einschichtigen Intine. Im Gegensatz zu den inaperturaten Zingiberaceae haben Costaceae Aperturen.

Acknowledgements

First, I would like to express sincere gratitude to my supervisor ao. Univ.-Prof. Mag. Dr. Martina Weber, who gave me the opportunity of investigating such an interesting palynological topic. She always took time to advise me, no matter if my problems result from technical or logical issues and simultaneously gave me a free hand to realize my own ideas of this thesis.

I would also like to thank the Core Facility Botanical Garden HBV at the University of Vienna, the Austrian Federal Gardens (Schönbrunn), o. Univ.-Prof. i.R. Dr. Anton Weber and my supervisor for supplying me with plant material. The Department of Botany and Biodiversity Research of the University of Vienna and ao. Univ.-Prof. Mag. Dr. Ingeborg Lang from the Core Facility of Cell Imaging and Ultrastructure Research (CIUS) at the University of Vienna for providing working time on their microscopy equipment.

Furthermore, thanks go out to Andrea Frosch-Radivo, Dr. Heidemarie Halbritter and Mag. Dr. Silvia Ulrich, for sharing their technical know-how and their patience, while imparting this knowledge to me.

Mag. Susanne Sontag and Ursula Schachner, who encouraged me in times of desperation and always had a friendly ear for any kind of problems.

Dipl.-Ing. Erich Egert and Ursula Schachner, thank you for proof reading and constructive criticism.

Finally, special thanks go out to my family, particularly my mother Martina and my brother Florian for their support during the years of study and process of writing this thesis, as well as to my friends for all inspiring conversations. Without them, this accomplishment would not have been possible.

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1 Introduction

Zingiberaceae and Costaceae are both members of the monocot order Zingiberales (Stevens, 2001 onwards). Zingiberales are distributed pantropical and separated into 8 families with 92 genera and approximately 2,000 species (Kress et al., 2001).

With 53 genera and more than 1,200 species, Zingiberaceae, commonly known as gingers, represent the largest family of this order (Kress, 1990; Kress et al., 2002). Whereas the highest diversity is found in the old world tropics, with focus on Southeast Asia (Kress, 1990), the family is also represented in the Neotropics and Africa (Kress et al., 2002). Based on molecular data, Kress et al. (2002) divided the family into four subfamilies: While Siphonochiloideae and Tamijioideae are represented by only one genus each, Alpinioideae and Zingiberoideae are responsible for the main diversity. Samples investigated in this study belong to the latter two subfamilies.

According to Wu & Larsen (2000b) Zingiberaceae are perennial herbs, living either terrestrial or rarely epiphytic. Furthermore, they are characterized by fleshy rhizomes and pseudostems formed by leaf sheaths. Phyllotaxis is distichous and leaf blades are gradually reduced towards the plants base.

Flower morphology is showing three sepals and petals, each in a fused whorl as well as a trimerous inferior gynoecium (Wu & Larsen, 2000b). Contrary to these characteristics, fitting well to the basal monocot flower model $P_{3+3}\ A_{3+3}\ G_3$ (Strasburger et al., 2008), the androecium is more derived. Out of basally 6 stamen, one is aborted, 4 are reduced to staminodes and only one remains fertile (Kress, 1990; Wu & Larsen, 2000b). While two lateral staminodes of the inner anther whorl are fused to a prominent labellum (Kress, 1990), the other two turn petaloid as well and are located as small teeth at the labellum base (Wu & Larsen, 2000b). The presence of a single tetrasporangiate anther and a slender style that is located between the thecae, are features shared with Costaceae (Kress, 1990).

This strongly derived flowers, often shift from insect pollination to other syndromes, especially bird pollination (Specht et al., 2012). From the sample set used in this study *Alpinia vittata*, *Etlingera elatior* and *Hedychium gardnerianum* are pollinated by Nectariniidae (sunbirds), whereas *Alpinia foxworthii*, *Globba winitii* and *Globba schomburgkii* are bee-pollinated (Specht et al., 2012).

Due to special flower morphology and the content of secondary plant metabolites, many Zingiberaceae species were used as ornamental plants/cut flowers and for medicinal purposes or cooking, e.g. *Curcuma longa*, *Zingiber officinale* or *Alpinia galanga* (Larsen & Wong, 1999; Kress et al., 2005; Chan et al., 2007).

Contrary to Zingiberaceae, the Costaceae, are a small group in the order Zingiberales. It comprises 7 genera and approximately 100 species (Specht et al., 2001; Specht, 2006; Specht & Stevenson, 2006). For a long time they were treated as members of Zingiberaceae. Nakai (1941), separated Costaceae from Zingiberaceae, supported by anatomical investigations of Tomlinson (1962). Costaceae are distributed pantropical with its center of diversity in the Neotropics. Some taxa are also found in Africa, Asia and northern Australia (Kress, 1990).

Costaceae are perennial herbs with fleshy rhizomes Wu & Larsen (2000a). Contrasting to Zingiberaceae, the simple leaves, including a closed leaf sheath are arranged as monistichous spiral (Kress, 1990; Wu & Larsen, 2000a). Concerning the flowers, similarities to gingers can be found. They show fused whorls of sepals and petals, an inferior gynoecium and only a single fertile stamen (Wu & Larsen, 2000a). While the fertile stamen is equipped with petaloid connective and filament, the five remaining stamens are reduced to staminodes and fused to a showy labellum (Kress, 1990; Wu & Larsen, 2000a).

Also in case of pollination, Costaceae display similarities to Zingiberaceae. *Cheilocostus speciosus* is pollinated by bees, *Costus pictus* by Trochilidae (hummingbirds) and *Tapeinochilos ananassae*, by Nectariniidae (sunbirds) (Specht et al., 2012).

Taxa of Costaceae are also known as ornamental plants e.g., *Tapeinochilos ananassae* (Gavillán-Suárez et al., 2015) and medicinal plants e.g., *Cheilocostus speciosus* (Duraipandiyan et al., 2012).

Palynological studies on Zingiberaceae and Costaceae are meagre (Liang, 1988; Mangaly & Nayar, 1990). More palynological data are available for other families of Zingiberales: Strelitziaceae (Hesse & Waha, 1983; Kronestedt-Robards & Rowley, 1989), Heliconiaceae (Kress et al., 1978; Simao et al., 2007; Kress & Stone, 2009) and Cannaceae (Skvarla & Rowley, 1970; Kress & Stone, 2009).

Comprehensive studies by Liang (1988) and Mangaly & Nayar (1990) include LM and SEM methods, but only a few species were investigated using TEM, e.g. *Curcuma* sp., *Boesenbergia* sp. (Chen & Xia, 2011) and *Tapeinochilos ananassae* (Stone et al., 1981).

Pollen of Zingiberaceae, including Costaceae, are described as spherical, sub-spherical, ovoid or prolate in shape, inaperturate or aperturate and a size from 36 µm to 225 µm (Liang, 1988). According to Liang (1988) the pollen wall is composed of a thin exine and a thick intine, which is not resistant to acetolysis. Furthermore Mangaly & Nayar (1990) postulated that all Zingiberaceae have an exine, except *Kaempferia* sp. According to the aperture condition the authors divided the family in an inaperturate and an aperturate group. Theilade et al. (1993) and Chen & Xia (2011) refuted the presence of apertures for three of the previously aperturate species *Curcuma* sp., *Boesenbergia* sp. and *Zingiber* sp. All the studies using TEM are based on standard contrast methods.

In the present study, a variety of LM, SEM and TEM techniques were applied, to clarify pollen wall structure and sculpture. Similarities or differences with standard angiosperm pollen walls will be discussed. The results are compared to other Zingiberales families as well as to Araceae, demonstrating that such a derived pollen wall is no unique feature in angiosperms.

2 Material and Methods

Plant Material

Since neither Zingiberaceae nor Costaceae are native to central Europe, sampling of fresh material, that is essential for ultrastructural investigations, was delimited to the Botanical Garden of Vienna and The Austrian Federal Garden Schönbrunn. To complete the sample set, ethanol fixations of o. Univ-Prof. i.R. Dr. Anton Weber were used for light microscopic (LM) and scanning electron microscopic (SEM) investigations, as well as resin embedded glutaraldehyde fixations of ao. Univ.-Prof. Mag. Dr. Martina Weber for transmission electron microscopic (TEM) purposes.

Table 1: Sample set; **WA** Anton Weber, **WM** Martina Weber, **DL** Lukas Dirr, **AFG** The Austrian Federal Gardens, **HBV** Botanical Garden Vienna; **WAB** Waimea Arboretum and Botanical Garden (Hawaii, Oahu)

Species	Plant ID	Condition	Collector	Location
Alpinia foxwothii	1532	Fresh	WM	WAB
Alpinia vittata	1314	Fresh	WM	HBV
Alpinia vittata	1724	Fresh	DL	HBV
Cheilocostus speciosus	1713	Fresh	DL	HBV
Costus pictus	1726	Fresh	DL	AFG
Etlingera elatior	1715	Fresh	DL	HBV
Globba schomburgkii	1239	Fresh	WM	HBV
Globba winitii	1711	Fresh	DL	HBV
Hedychium gardnerianum	1236	Fresh	WM	HBV
Hedychium gardnerianum	1718	Prefixed	WA	HBV
Tapeinochilos ananassae	1712	Fresh	DL	HBV
Tapeinochilos ananassae	1723	Fresh	DL	HBV

Chemicals

- Acetic anhydride (Loba)
- Acetone (Merck)
- Deionized water (H_2O)
- 2,2-Dimethoxypropane (DMP; Sigma-Aldrich)
- 3 % Glutaraldehyde (GA; Merck) in 0.1 M Phosphate buffer (pH 7.4)
- 0.2 N Hydrochloric acid (HCl; Loba)
- 5 % Hydrogen peroxide (H_2O_2; Loba)
- 3 % Lead citrate (PbC; Ultrostain 2, Leica)
- 1 % Osmium tetroxide (OsO_4, Agar Scientific)
- 1 % Periodic acid (PA; Fluka)
- Phosphate buffered saline tablets (Sigma-Aldrich)
- 1.5 % Pioloform (agarscientific) in Chloroform (Loba)
- 0.8 % Potassium hexacyanoferrate (III) ($K_4[Fe(CN)_6]$; Fluka)
- 1 % Potassium permanganate ($KMnO_4$; Merck)
- 1 % Silver proteinate (SP; Fluka)
- Sodium hydroxide (NaOH) pellets (Carl Roth)
- Spurr Low-Viscosity Embedding Kit (Sigma-Aldrich)
    - D.E.R. 736
    - Dimethylaminoethanol
    - ERL 4221
    - Nonenylsuccinic anhydride
- 96 % Sulfuric acid (H_2SO_4; Carl Roth)
- 0.2 % Thiocarbohydrazide (TCH; Fluka)
- 0.5 % Uranyl acetate (UAc; Ultrostain 1, Leica)
- Xylene (Loba)

Light Microscopy

Before any more elaborate analysis of the samples was done, pollen of dried or ethanol fixed anthers was rehydrated or washed with H_2O, prior to observation under a “Nikon Eclipse Ni” or an “Olympus BX50-F” light microscope (LM) for checking quality of the material.

Acetolysis

For observing sporopollenin containing parts of the pollen wall samples were treated with an acetolysis mixture that was produced, according to Erdtman (1960), of 9 parts acetic anhydride and 1 part H_2SO_4. Due to the small amount of available anthers this procedure was done directly on a glass slide, following the single-grain technique by Zetter (1989) and Ferguson et al. (2007). Heating the samples with an open flame for short times was intermitted by checking the progress of acetolysis through a “Motic SMZ-168“-binocular. After these steps that all should be conducted under a fume hood. The acetolyzed pollen grains were either transferred into a drop of H_2O on a new glass slide or stayed in the acetolysis mixture for observation under the “Olympus BX50-F” LM, depending on the pollen wall stability. For documentation pictures were taken with the mounted “Color View IIIu” camera (Soft Imaging System), controlled by “analySIS docu” software (Soft Imaging System).

Autofluorescence

Air dried and ethanol fixed samples were transferred into a drop of H_2O on a glass slide, for observation with a “Nikon Eclipse Ni” LM. Beside bright field, an epifluorescence analysis was done. Fluorescence analysis was used to detect the sporopollenin containing exine (van Gijzel, 1971) or exine-like structures by using UV-light to excite autofluorescence (Yeloff & Hunt, 2005).

The used “DAPI-filter square” excites fluorescence in a wavelength area from 340 nm to 380 nm and allows blue light with a wavelength of 435 nm to 485 nm to pass the barrier filter. Extended focus, a “DS-Ri2” high definition color camera (Nikon) and “NIS-Elements” software (Nikon) were used for documentation.

Scanning Electron Microscopy

Following Halbritter (1998), anthers were packed into small baskets made of filter paper and immediately transferred into acidified DMP (1 drop of 0.2N HCl added to 30 ml DMP) for dehydration. While air dried anthers should be rehydrated carefully, with H_2O before dehydration, fresh or ethanol fixed samples can be used without any preliminary treatment. After 30 minutes the filter baskets were washed in 100 % acetone for 10 minutes before critical point drying in a "tousimis Autosamdri-815" is done by CO_2, with 100 % acetone as intermediate fluid. After drying, the samples were mounted onto aluminum stubs by using a "Scotch double stick tape" and sputter coated with gold in a "BAL-TEC SCD050" sputter coater for 5 minutes. For observation, a "JEOL JSM-IT300" scanning electron microscope at 10 kV was used.

Transmission Electron Microscopy

To elucidate ultrastructural pollen wall properties by transmission electron microscopy (TEM), fresh or rehydrated air dried anthers were used.

As a first step the samples were fixed in 3 % GA (in 0.1 M, pH 7.4 phosphate buffer) for 6 hours at 20 °C in a rotating device, followed by washing in phosphate buffer (2 times, 5 minutes each) and H_2O (2 times, 5 minutes each). Postfixation was done in a 2:1 mixture of 1 % OsO_4 and 0.8 % $K_4(Fe[CN]_6)$ at 4 °C.

After 12 hours, the fixative got discarded and the samples were washed in H_2O for 3 times, 5 minutes each followed by dehydration in acidified DMP (3 times, 10 minutes each). As a next step a 1:1 mixture of DMP and pure acetone acts as pre-stage (for 5 minutes) before the samples were transferred into pure acetone.

For starting infiltration with Spurr Low-Viscosity resin (Sigma-Aldrich, 2010) the samples were transferred into a mixture of 0.5 ml fresh pure acetone and 5 drops of resin. The closed sample tubes were kept under the fume hood all the time and were only opened shortly every 12 hours, for adding 3 drops of resin to each of them. After 48 hours (adding 3 drops of resin, 4 times) the sample tubes were not closed anymore, allowing the remaining acetone to evaporate. After 6 hours, the anthers were finally embedded in freshly prepared resin and put into an oven (Memmert) at 70 °C for 2 days, to ensure complete resin polymerization.

After trimming the polymerized blocks, approximately 90 nm ultra-thin sections were cut in a "Leica EM UC6" ultra-microtome with a "DiAtome ultra 45°" diamond

knife. Floating sections were stretched by Xylene vapor and then transferred onto 50 mesh copper or gold grids (Agar Scientific) that were coated by a Formvar support film.

To gain as much information as possible from these sections, different chemicals and protocols for contrast enhancement were applied. Consumption of chemicals gets minimalized by placing only small drops of them on the hydrophobic surface of Parafilm M (Sigma-Aldrich), mounted into a petri dish. For reducing sample damage due to sunlight exposure, the petri dishes were shaded all the time. Between 2 contrasting steps the grids were washed for 15 minutes in large drops of H_2O or for 15 minutes in 7 % acetic acid and 2 times 10 minutes H_2O after applying TCH.

Conventional staining for contrast enhancement was performed by applying 0.5 % UAc for 30 minutes followed by 5 minutes 3 % PbC (in CO_2 free atmosphere, guaranteed by placing NaOH pellets beneath the drops of staining solution) on copper grids (Hayat, 1989) as well as a modified Thiéry-test on gold grids. For the latter one samples were treated with 1 % PA for 10 minutes, 0.2 % TCH for 15 minutes and 1 % SP for 10 minutes (Weber & Frosch, 1995).

Furthermore, a conventional Thiéry-test was performed on gold grids to label neutral polysaccharides. To obtain OsO_4 free sections, which were needed for this contrasting method, the grids were treated with 5 % H_2O_2 for 10 minutes (Böck, 1984).

Following Thiéry (1967), sections were stained with 1 % PA for 30 minutes, 0.2 % TCH for 5 hours and 1 % SP for 30 minutes. By performing this test without previous H_2O_2 and PA treatment, unsaturated lipids can be detected (Rowley & Dahl, 1977). For detecting endexine or endexine-like structures copper grids were stained with 1 % $KMnO_4$ for 5 minutes (Lawn, 1960; Weber & Ulrich, 2010; Ulrich et al., 2016)

All observations of the ultrathin sections were done with a “Zeiss EM 109” or a “ZEISS 900” TEM at 50 kV and documented by a “Mega View III” camera (Soft Imaging System), controlled with “iTEM” (Soft Imaging System) software.

For description of pollen features terminology follows Hesse et al. (2009).

3 Results

Zingiberaceae

Pollen of Zingiberaceae is inaperturate. The psilate, echinate or micro-echinate pollen grains are distributed as monads. Pollen size varies between approximately 45 µm (*Globba winitii*) and 70 µm (*Etlingera elatior*) and are therefore classified as medium or large pollen grains. Pollen walls are not resistant to acetolysis and consist of a multilayered intine that is partly transversed by channels. Pollenkitt is present.

Alpinioideae

Alpinia vittata

A complete data set, is available, due to enough fresh material. Ornamentation is echinate (Fig. 1 A-B, D). Pollen walls are not resisting acetolysis. After 1 minute of heating in acetolysis mixture only the protoplast is left (Fig. 1 E). Prolonging this procedure for another 30 seconds, destroyed the whole pollen grain, indicating absence of sporopollenin containing wall layers. Amyloplasts in the vegetative cytoplasm, indicate starch as reserve substance (Fig. 1 C, F).

Staining behavior (Fig. 2) elucidates structure of the pollen wall. Its separated into inhomogeneous endintine (In1), followed by a thin intine layer 2 (In2) and a thick, channeled ektintine (In3). Following this multi-layered intine complex, echini and a surface layer (Sl) were found. In2 and In3 react to Thiéry-test only (Fig. 2 C) whereas In1 appears stained independent of the applied method (Fig. 2 A-D). The surface layer is shown most prominent after Thiéry-test (Fig. 2 C) indicating polysaccharide nature, which is also suggested for echini (Ec), which are destroyed after acetolysis (Fig. 1 E).

Alpinia foxworthii

Availability of resin embedded material allows TEM investigations only (Fig. 3). Ornamentation is echinate (Fig. 3 A-D). Sporoderm is separated in endintine (In1), intine layer 2 (In2), channeled ektintine (In3), echini (Ec) and surface layer (Sl). In1 and In3 are stained after Thiéry-test (Fig. 3 C), whereas In2 stains additionally to this treatment electron dense after $KMnO_4$ (Fig. 3 B) as well.

Etlingera elatior

Fresh material for a complete data set was available. It represents the psilate ornamentation type (Fig. 4 A-D, F). Faint autofluorescence (Fig. 4 D), and missing resistance to acetolysis indicate absence of sporopollenin. After 60 seconds in acetolysis mixture only the protoplast remains (Fig. 4 E), which is also destroyed after prolonging the procedure for another minute. Amyloplasts in the vegetative cytoplasm store starch as reserve substance (Fig. 4 C, F).

Sporoderm is stratified into endintine (In1), a thin intine 2 (In2) and a channeled ektintine (In3). Followed by a thin surface layer (Sl). The whole intine complex reacts positively to Thiéry-test (Fig. 5 C-D). Contrary the surface layer (Sl) can be detected best after lipid-test (Fig. 5 A) and $KMnO_4$ (Fig. 5 B). Missing resistance to acetolysis (Fig. 4 E) emphasizes polysaccharidic nature of this zone.

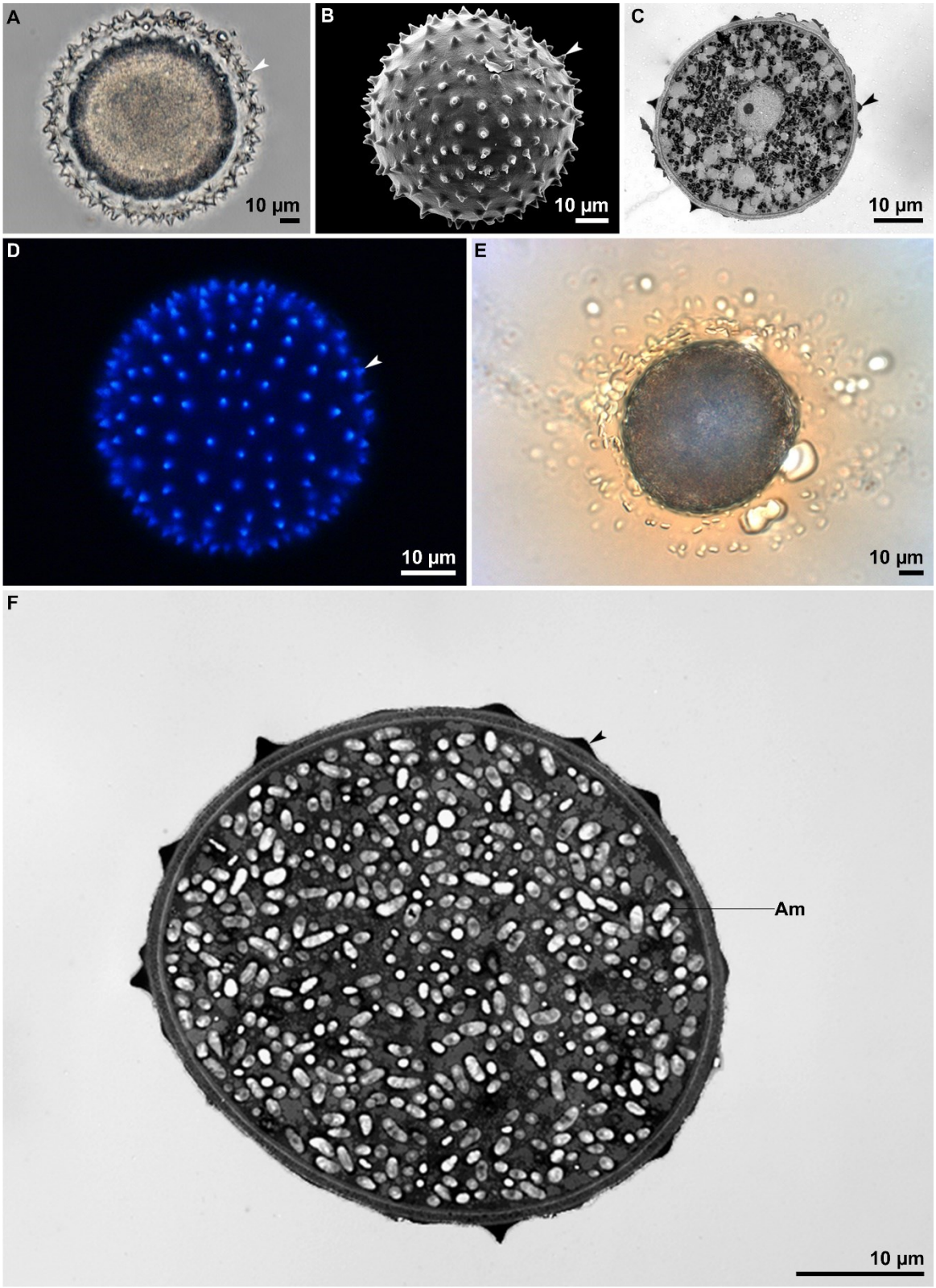

Figure 1: *Alpinia vittata*. **A** hydrated pollen grain, bright field LM, **arrowhead** echinus; **B** hydrated pollen grain, SEM, **arrowhead** echinus; **C** overview of pollen grain, modified Thiéry-test, TEM, **arrowhead** echinus; **D** autofluorescence of polysaccharidic echini, epifluorescence LM with DAPI filter cube, **arrowhead** echinus; **E** after 60 seconds of acetolysis protoplast left, bright field LM; **F** overview of pollen grain, UAc-PbC, TEM, **Am** amyloplast, **arrowhead** echinus

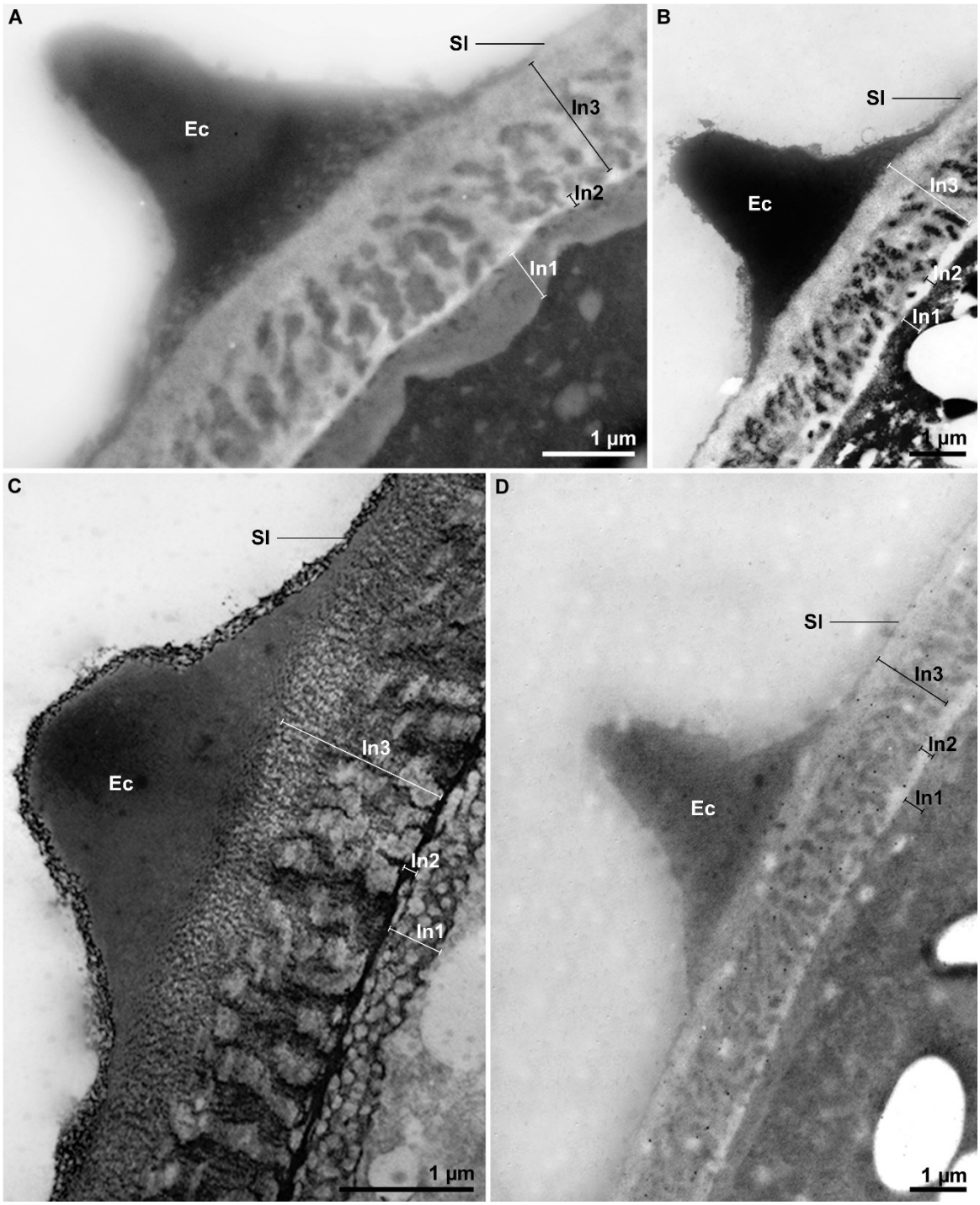

Figure 2: *Alpinia vittata*. Pollen wall stained with different methods. **A** unsaturated lipids; **B** $KMnO_4$; **C** Thiéry-test; **D** Thiéry-test control; **Ec** echini, **In1** endintine, **In2** intine layer 2, **In3** ektintine, **Sl** surface layer

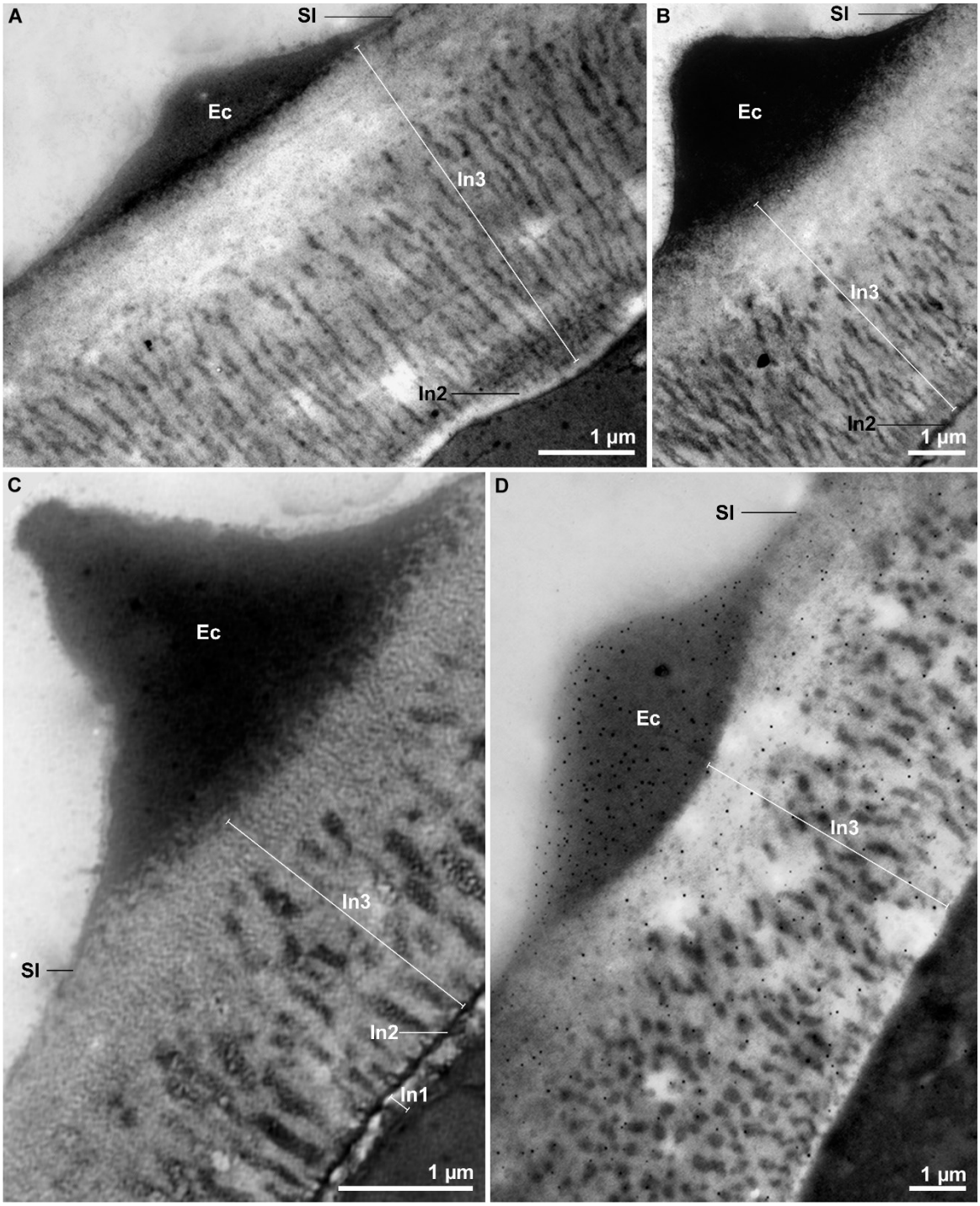

Figure 3: *Alpinia foxworthii*. Pollen wall stained with different methods. **A** unsaturated lipids; **B** $KMnO_4$; **C** Thiéry-test; **D** Thiéry-test control; **Ec** echini, **In1** endintine, **In2** intine layer 2, **In3** ektintine, **Sl** surface layer

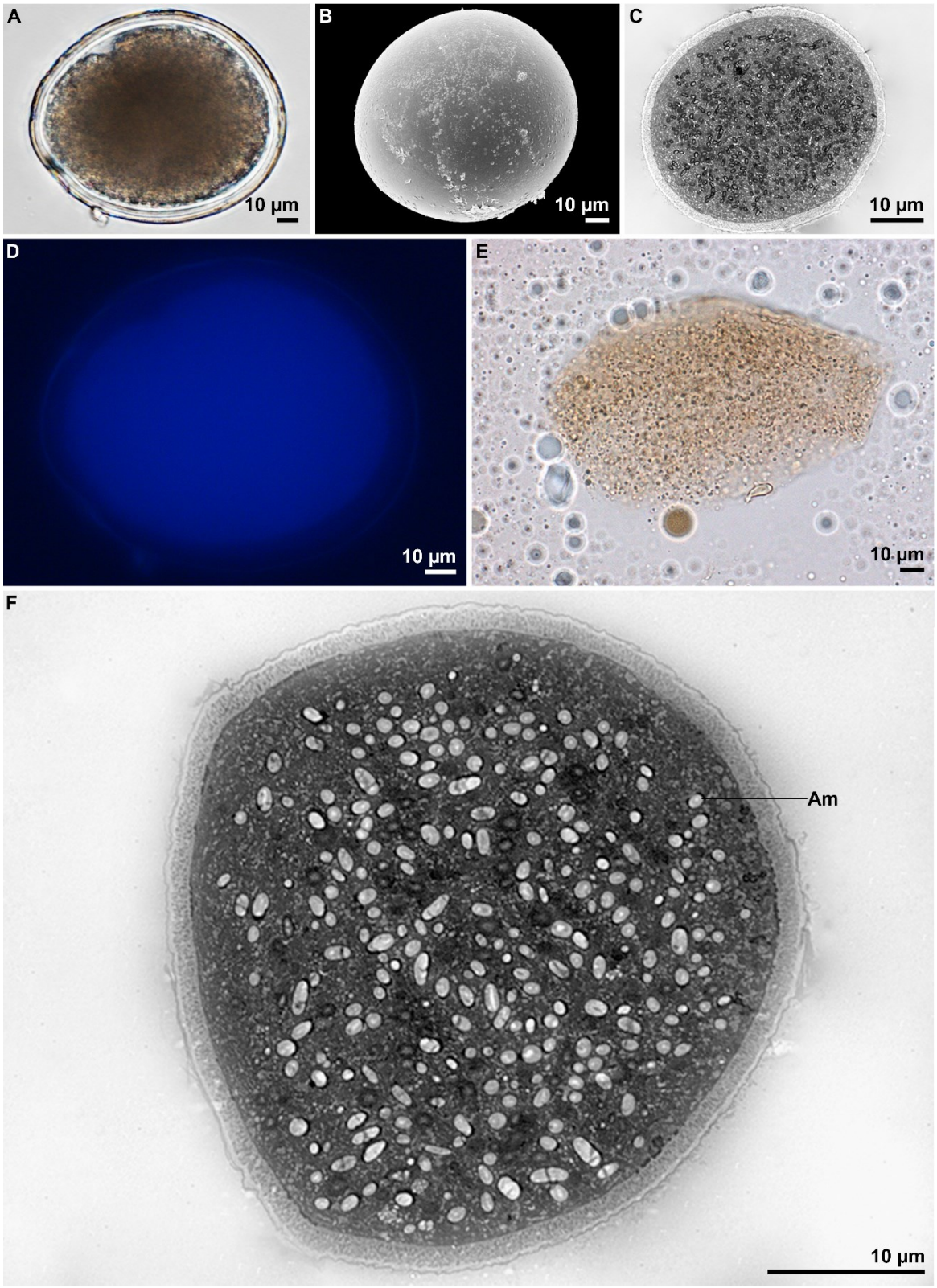

Figure 4: *Etlingera elatior*. **A** hydrated pollen grain, bright field LM; **B** hydrated pollen grain, SEM; **C** Overview of pollen, modified Thiéry-test, TEM; **D** Autofluorescence of psilate pollen grain surface, epifluorescence LM with DAPI filter cube; **E** after 60 seconds of acetolysis pollen wall already strongly damaged, bright field LM; **F** overview of pollen grain, UAc-PbC, TEM, **Am** amyloplast

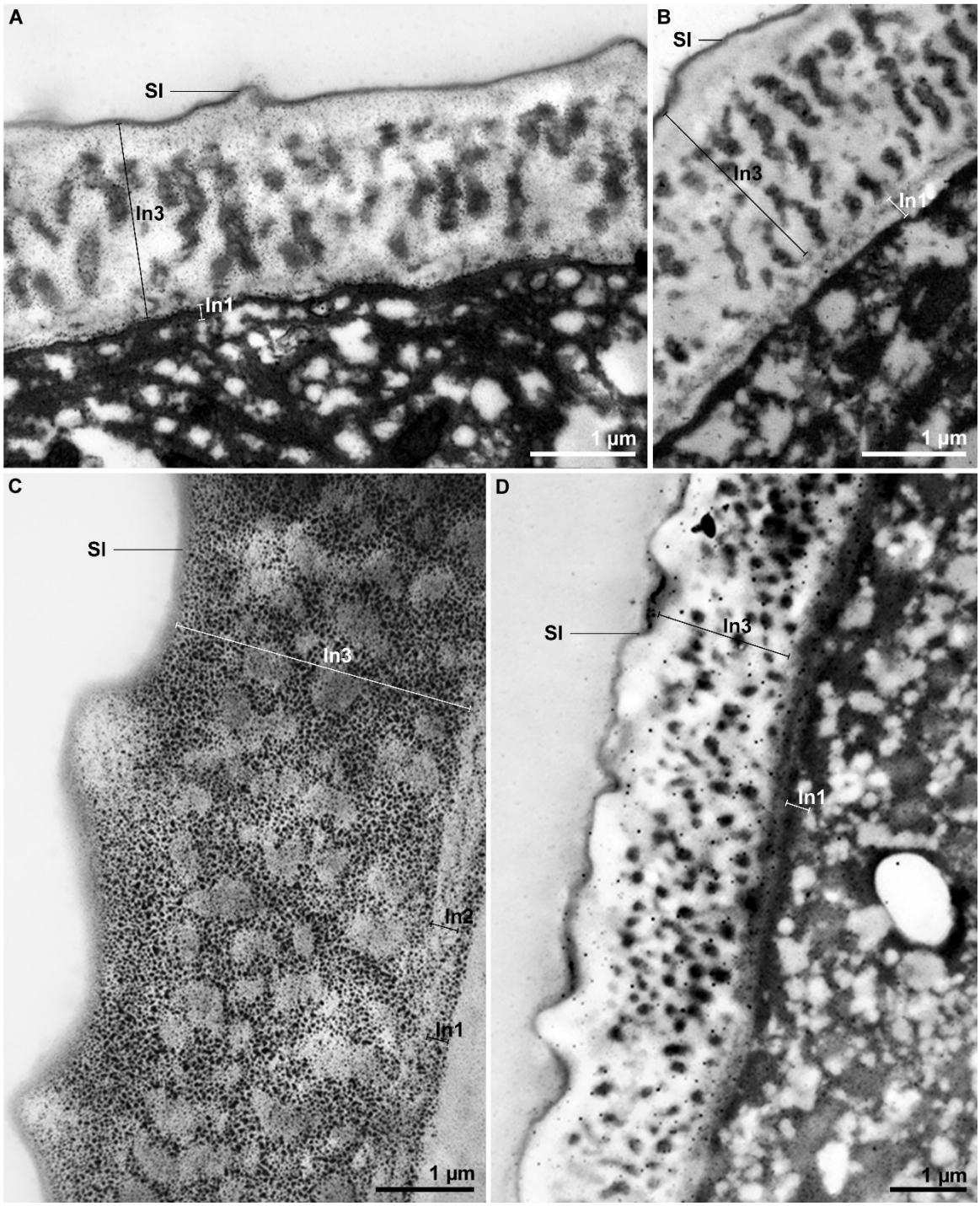

Figure 5: *Etlingera elatior*. Pollen wall stained with different methods. **A** unsaturated lipids; **B** $KMnO_4$; **C** Thiéry-test; **D** Thiéry-test control; **In1** endintine, **In2** intine layer 2, **In3** ektintine, **Sl** surface layer

Zingiberoideae

Globba winitii

This species, located in the second large subfamily of Zingiberaceae, represents the third pollen type found in this taxonomic order. Availability of enough fresh material, allows a complete data set. Ornamentation is micro-echinate (Fig. 6 A-D, F). Pollen wall is not resistant to acetolysis. After approximately 30 seconds of heat exposure in the acetolysis mixture, only the protoplast remained (Fig. 6 E), which was destroyed after another 30 seconds. This indicates, absence of sporopollenin in the pollen wall layers. Cytoplasm contains numerous amyloplasts (Fig. 6 F) as nutrient reserve, but in lower quantity as in the species examined before.

In *Globba* the pollen wall is formed by the intine, which is separated into three zones: endintine (In1), intine layer 2 (In2) and ektintine (In3). The endintine (In1) appears inhomogeneous and electron dense throughout all applied contrasting techniques (Fig. 7 A-D). Intine layer 2 (In2) is extremely thin (Fig. 7 D), whereas the ektintine (In3) forms a massive layer and stains strongly electron dense after the Thiéry-test (Fig. 7 C). Echini (Ec) are small and destroyed during acetolysis, indicating their polysaccharidic nature (Fig. 6 E). The outermost part of the pollen wall is a thin surface layer (Sl; Fig. 7 A-D).

Globba schomburgkii

Only resin embedded anthers are available and used for TEM-investigation. Good fixation quality allows to differentiate all wall layers perfectly (Fig. 8). The inhomogeneous endintine (In1) is followed by an extremely thin intine layer 2 (In2) and a massive, channeled ektintine (In3). Next to this complex intine, small echini (Ec) and a thin surface layer (Sl) can be recognized.

All intine layers as well as echini are positive for polysaccharides (Fig. 8 C-D). Additionally, In1 stains electron dense after lipid-test and $KMnO_4$ (Fig. 8 A-B).

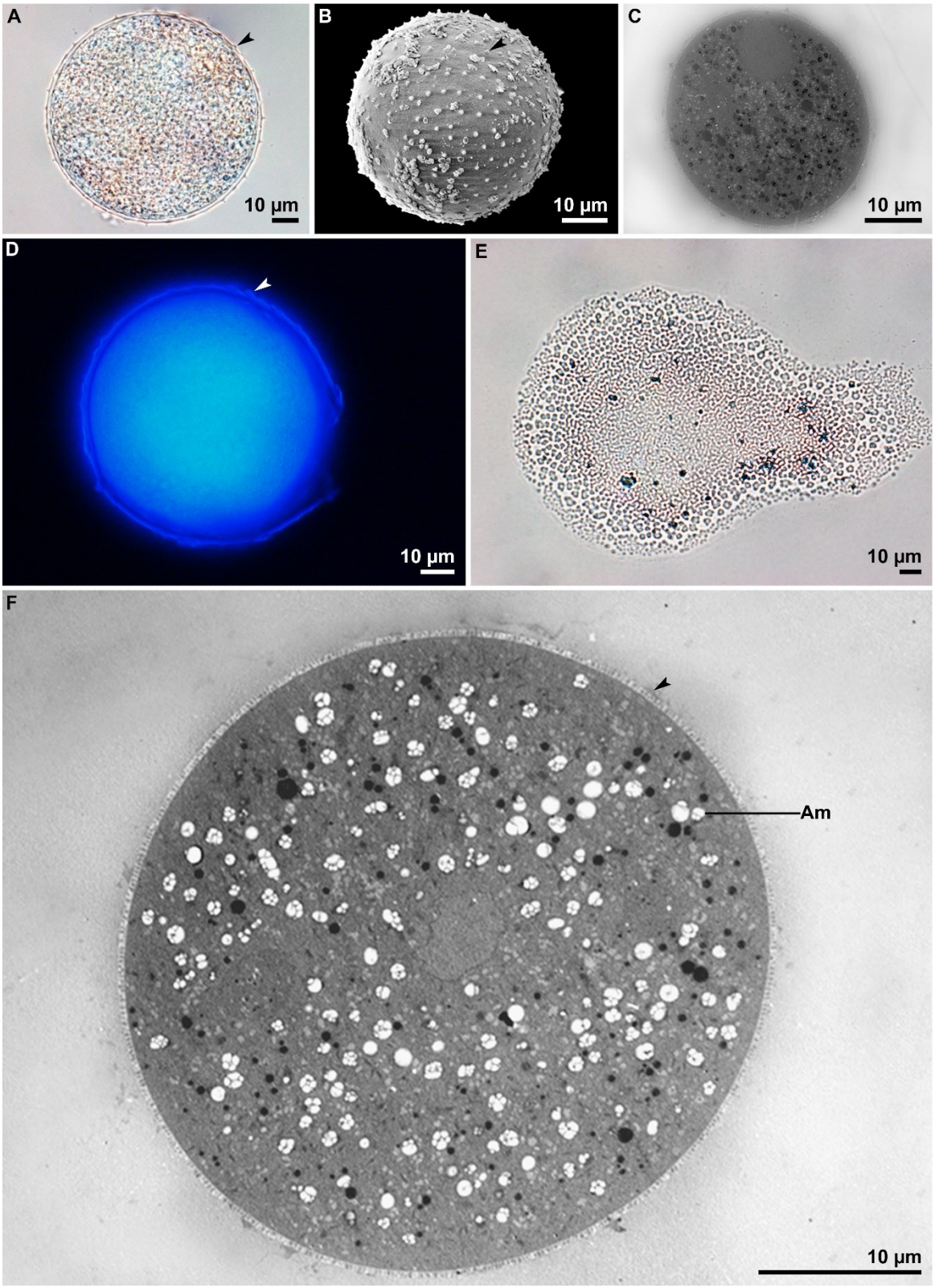

Figure 6: *Globba winitii*. **A** hydrated pollen grain, bright field LM, **arrowhead** micro-echinus; **B** hydrated, micro-echinate, pollen grain, SEM, **arrowhead** micro-echinus; **C** overview, modified Thiéry-test, TEM; **D** auto-fluorescence of the pollen wall with hardly visible micro-echini, epifluorescence LM with DAPI filter cube, **arrowhead** micro-echinus; **E** pollen wall strongly disintegrated after 30 seconds of acetolysis, bright field LM; **F** overview, UAc-PbC, TEM, **arrowhead** micro-echinus, **Am** amyloplast

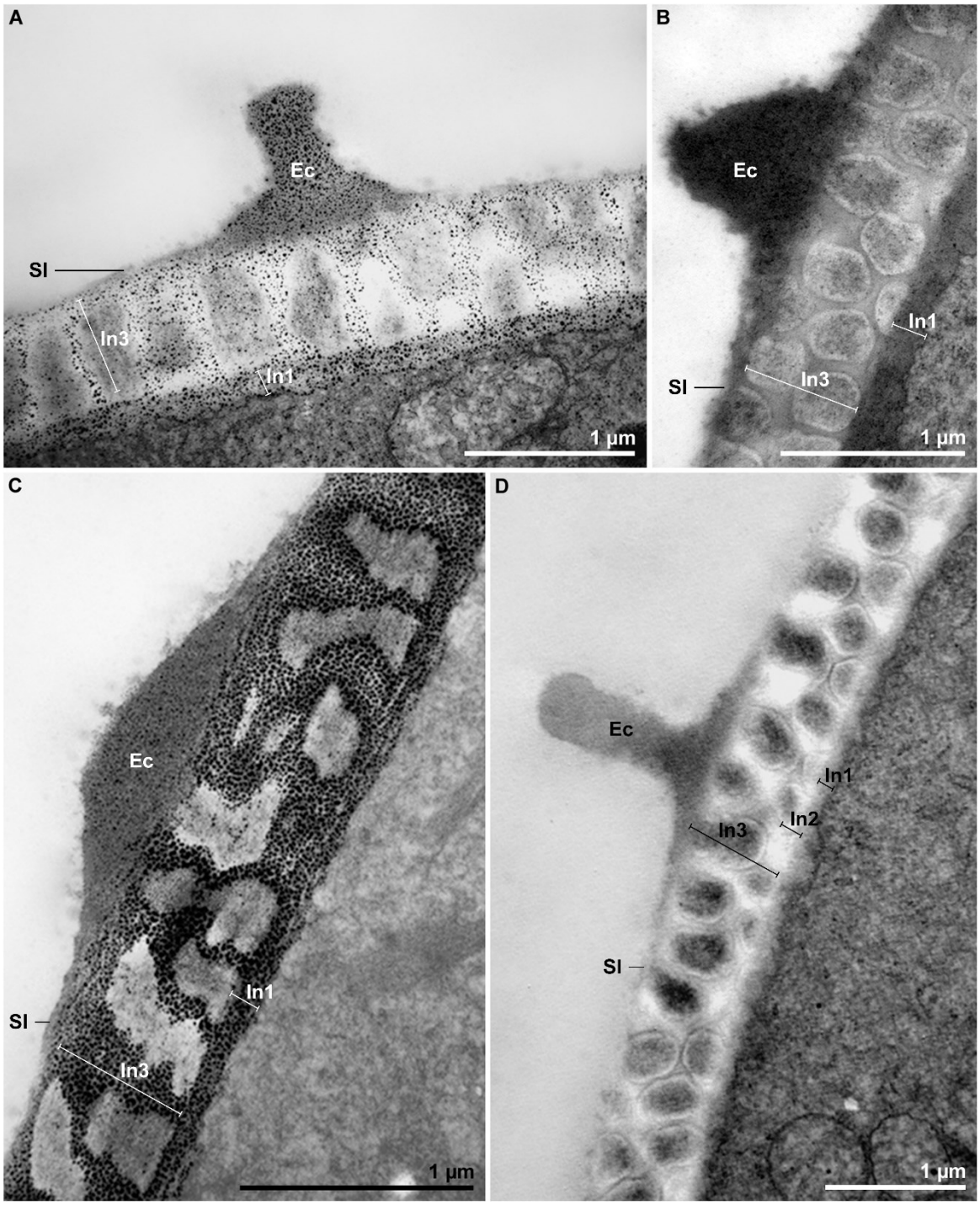

Figure 7: *Globba winitii*. Pollen wall stained with different methods. **A** unsaturated lipids; **B** $KMnO_4$; **C** Thiéry-test; **D** uranyl acetate/lead citrate; **Ec** micro-echini, **In1** endintine, **In2** intine layer 2, **In3** ektintine, **Sl** surface layer

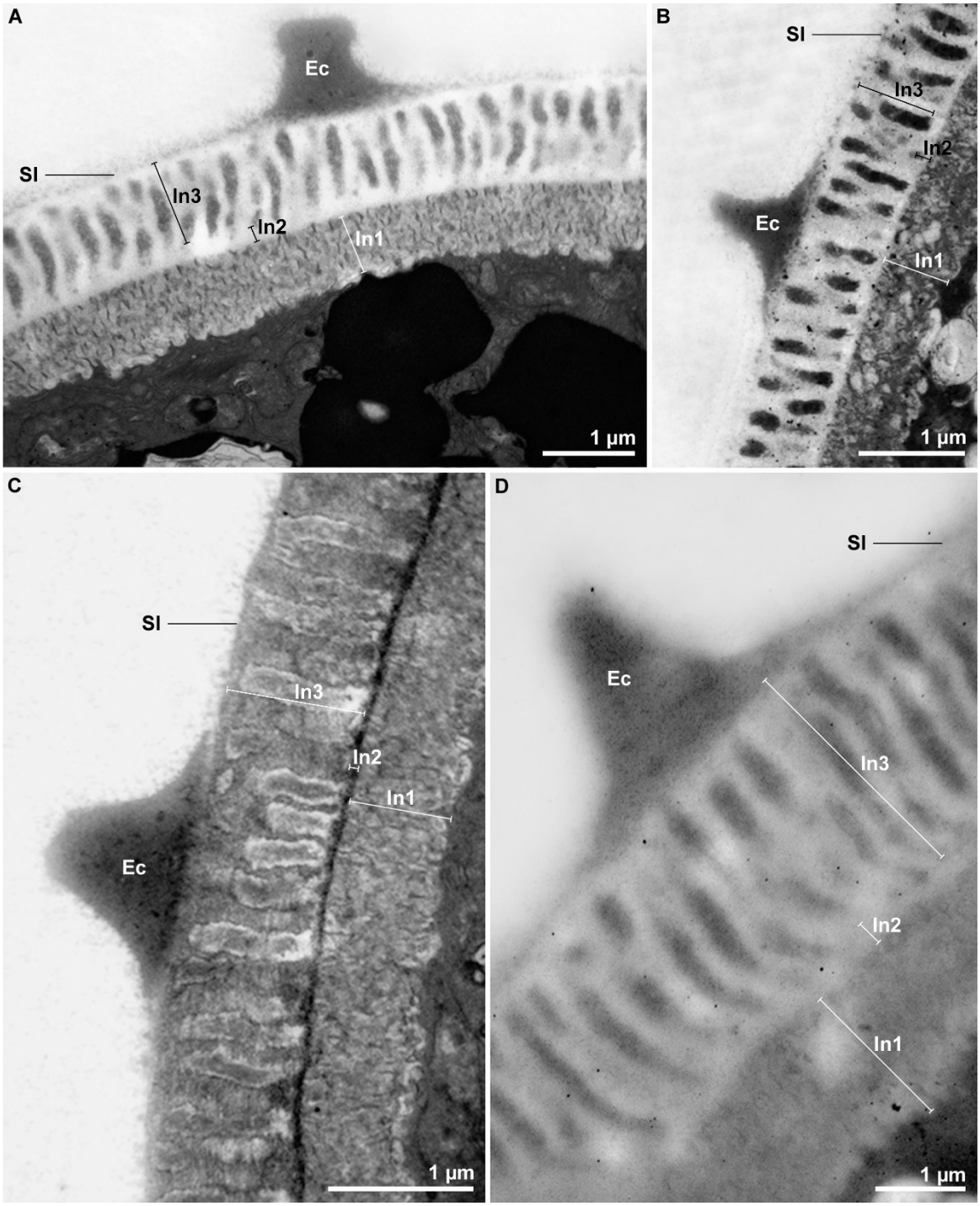

Figure 8: *Globba schomburgkii*. Pollen wall stained with different methods. **A** unsaturated lipids; **B** $KMnO_4$; **C** Thiéry-test; **D** Thiéry-test control; **Ec** micro-echini, **In1** endintine, **In2** intine layer 2, **In3** ektintine, **Sl** surface layer

Hedychium gardnerianum

Pollen is inaperturate (Fig. 9 A-B) and has starch and lipids as reserves in the vegetative cytoplasm (Fig. 9 F). Adding water to a dry pollen grain initiates detachment of the outer wall layer(s), which are finally shed, while the protoplast remains small (Fig. 9 A, D).

Pollen wall does not resist acetolysis. After 30 seconds in the acetolysis mixture the detached wall layers are dissolved (Fig. 9 E). After another 30 seconds, also the protoplast gets destroyed.

The pollen wall is separated into endintine (In1), thin intine layer 2 (In2), massive, channeled, ektintine (In3) and a surface layer (Sl). All layers are clearly distinguishable (Fig. 10 C). In3 shows a distinct reaction to the Thiéry-test (Fig. 10 C-D). Intine 1 as well as the surface layer (Sl) react positive for lipids (Fig. 10 A) and $KMnO_4$ (Fig. 10 B).

Table 2: TEM staining behavior of Zingiberaceae. "**?**" ambiguous data; "**-**" layer missing; **K** $KMnO_4$ (green); **L** lipid-test (red); **T** Thiéry-test (yellow); **C** control for Thiéry-test (purple); Electron dense zone, marked in treatment specific color

Species	Intine 1 (Endintine)				Intine 2 (Thin layer)				Intine 3 (Ektintine)				Surface layer				Orna-mentation			
	K	L	T	C	K	L	T	C	K	L	T	C	K	L	T	C	K	L	T	C
Alpinia foxworthii	?	?		?				?												
Alpinia vittata																				
Etlingera elatior					?	?		?									-	-	-	-
Globba schomburgkii																				
Globba winitii				?	?	?	?	?				?				?				?
Hedychium gardnerianum						?											-	-	-	-

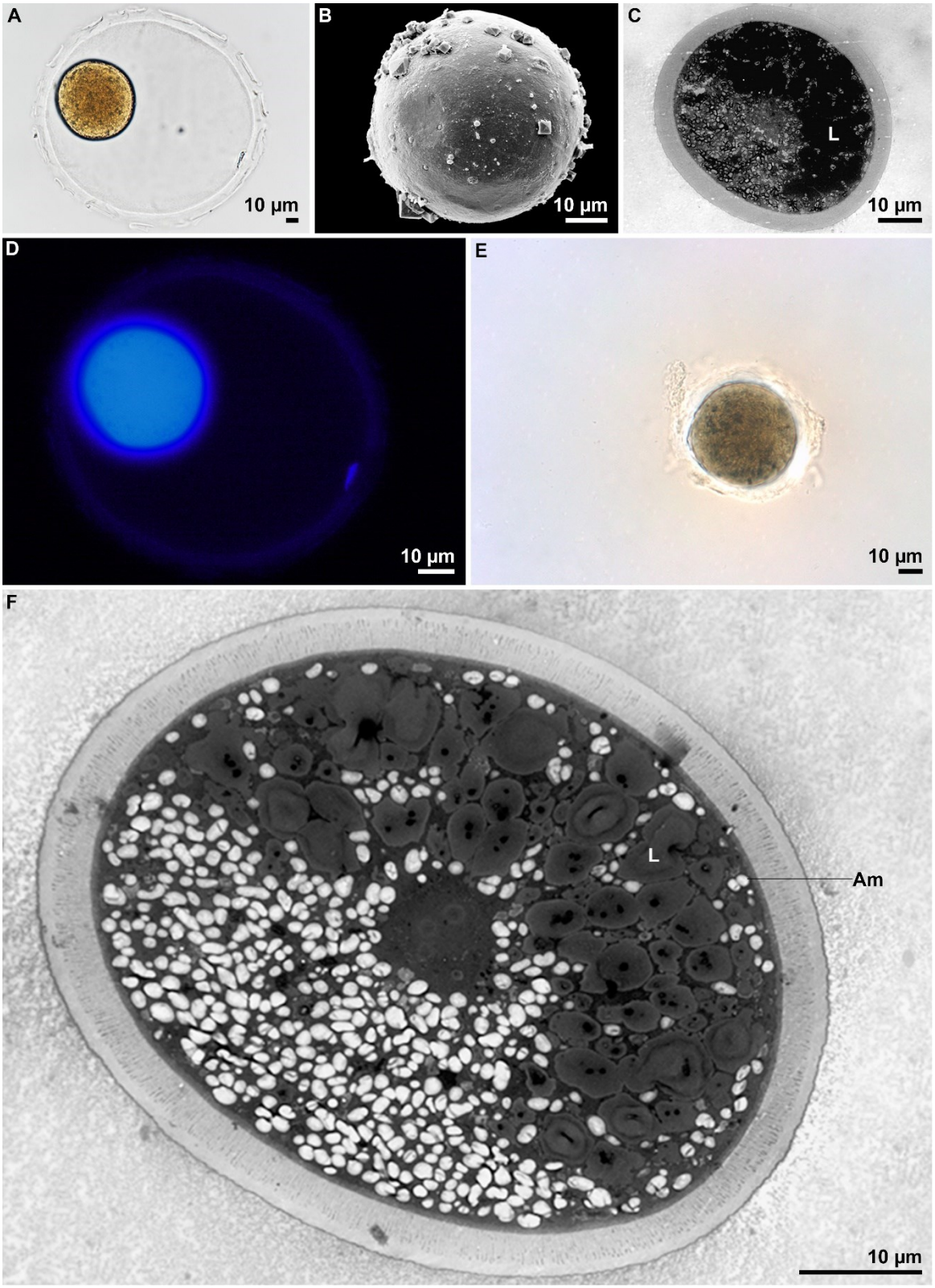

Figure 9: *Hedychium gardnerianum*. **A** hydrated pollen grain, bright field LM; **B** hydrated, psilate, pollen grain, SEM; **C** overview pollen grain, modified Thiéry-test, TEM, **L** lipid droplet; **D** autofluorescence of pollen wall, epifluorescence LM with DAPI filter cube; **E** acetolysis for 30 seconds, bright field LM; **F** overview of pollen grain, UAc-PbC, TEM, **Am** amyloplast, **L** lipid droplet

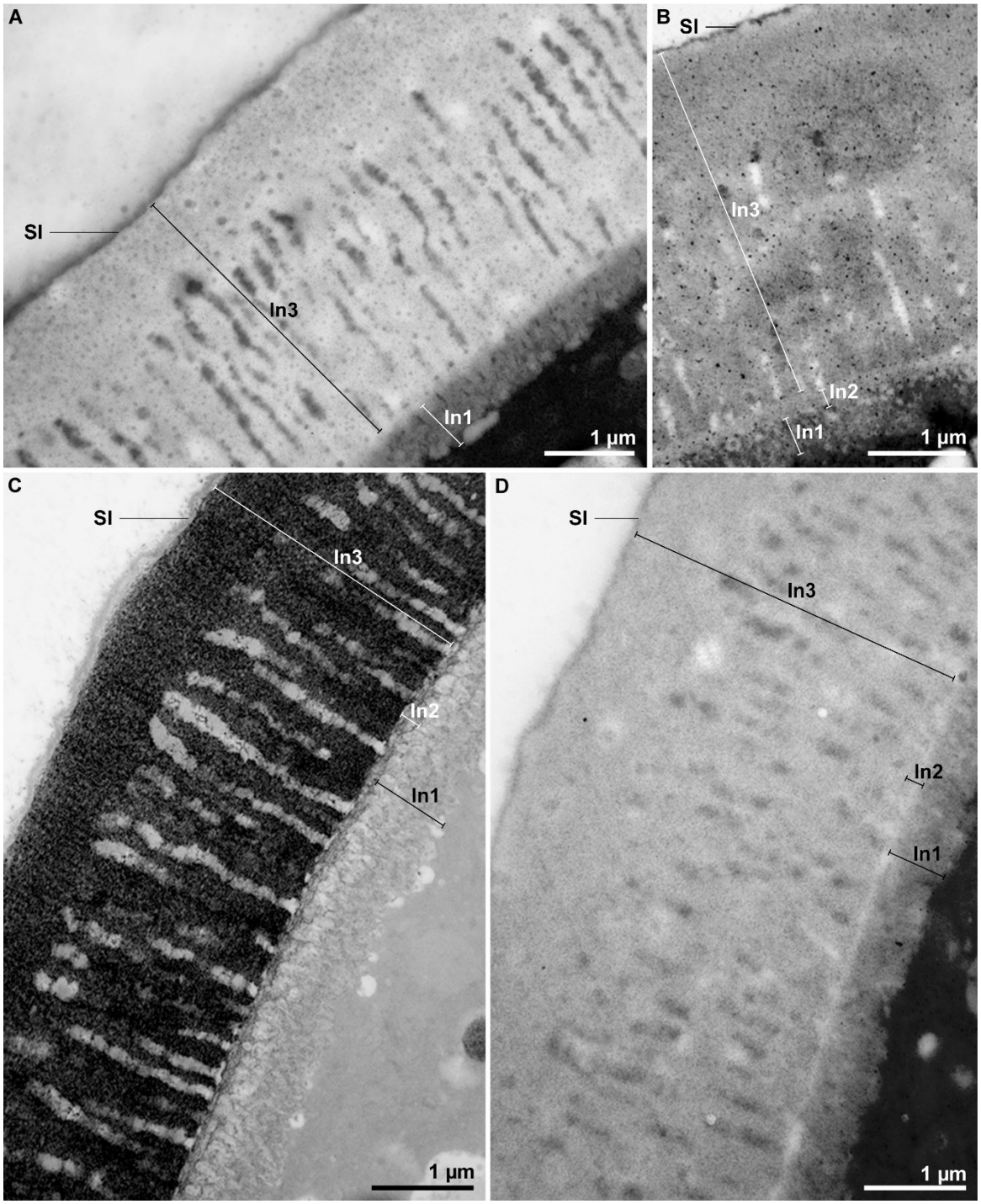

Figure 10: *Hedychium gardnerianum*. Pollen wall stained with different methods. **A** unsaturated lipids; **B** $KMnO_4$; **C** Thiéry-test; **D** Thiéry-test control; **In1** endintine, **In2** intine layer 2, **In3** ektintine, **Sl** surface layer

Costaceae

Pollen of the investigated species is aperturate, psilate and distributed as monads. Their size ranges from 60 µm (*Tapeinochilos ananassae*) to 105 µm (*Costus pictus*), which classifies them as large to very large pollen grains. Pollen wall is resistant to acetolysis, due to a sporopollenin containing pollen wall. Pollenkitt is present.

Cheilocostus speciosus

Hydrated pollen grains a spheroidal (Fig. 11 A-D). Aperture condition is pantoaperturate with 5 or more pori (Fig. 11 B-F, asterisks). Within the vegetative cytoplasm prominent lipid droplets are found (Fig. 11 C, F).

The pollen wall is resistant to acetolysis, indicating the presence of sporopollenin (Fig. 11 E). It is formed by a very compact exine, including a massive tectum (Te) and an infratectum (It), whereas a foot layer and an endexine is missing. The granular infratectum is highly compressed (Fig. 12 A-B). The intine is mono-layered and thickened (bi-layered) at the apertures (Fig. 11 B, F) and stains for polysaccharides (Fig. 12 C-D).

Costus pictus

Hydrated pollen grains a spheroidal (Fig. 13 A-F). Aperture condition is pantoporate with 5 or more pori (Fig. 13 B, E, asterisks). Starch is stored in the vegetative cytoplasm as reserves (Fig. 13 C, F).

The pollen wall is resistant to acetolysis (Fig. 13 E). The structure of the pollen wall includes a spongy tectum (Fig. 14 B, C), a granular infratectum (Fig. 14 B, C) and an intine. Foot layer and endexine are missing.

Tapeinochilos ananassae

Pollen grains in hydrated condition are spheroidal to triangular (Fig. 15 A-D, F). Ornamentation is psilate (Fig. 15 C, F). Aperture condition is unknown. Reserves in the vegetative cytoplasm are starch granules (Fig. 15 C, F).

The pollen wall is resistant to acetolysis (Fig. 15 E). The ektexine consists of a thick tectum and a thin granular infratectum. Foot layer and endexine are missing.

Table 3: TEM staining behavior of Zingiberaceae. **K** $KMnO_4$ (green); **L** lipid test (red); **T** Thiéry-test (yellow); **C** control for Thiéry-test (purple); Electron dense zone, marked in treatment specific color

	Intine				Granular Infratectum				Tectum			
Species	K	L	T	C	K	L	T	C	K	L	T	C
Cheilocostus speciosus												
Costus pictus												
Tapeinochilos ananassae												

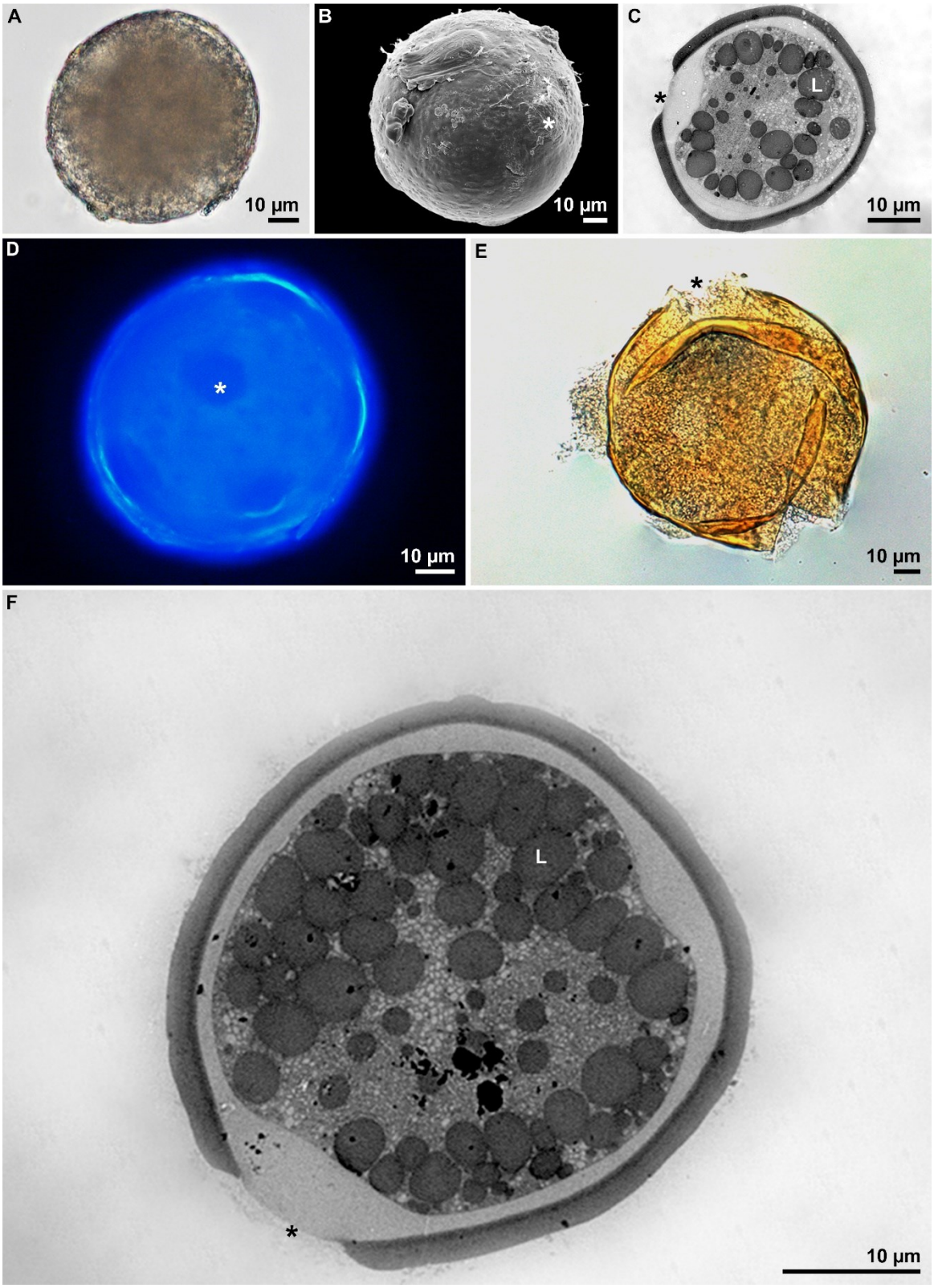

Figure 11: *Cheilocostus speciosus*. **A** hydrated pollen grain, bright field LM; **B** hydrated pollen grain, SEM, **asterisk** aperture; **C** overview pollen grain, modified Thiéry-test, TEM, **asterisk** aperture, **L** lipid droplet; **D** autofluorescence of ektexine, apertures appear dark, epifluorescence LM with DAPI filter cube, **asterisk** aperture; **E** result of acetolysis, bright field LM, **asterisk** aperture; **F** overview pollen grain, UAc-PbC, TEM, **asterisk** aperture

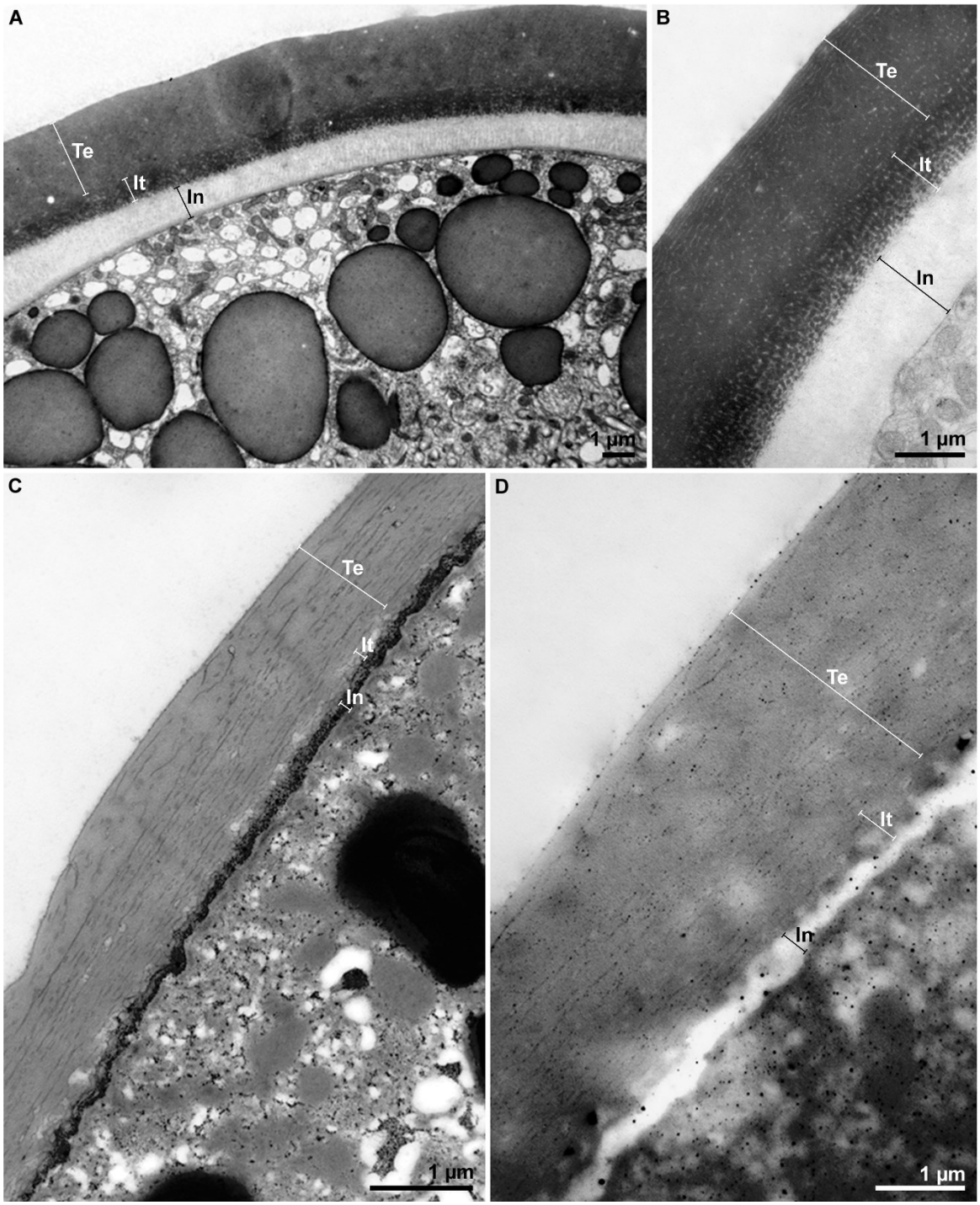

Figure 12: *Cheilocostus speciosus*. Pollen wall stained with different methods. **A** unsaturated lipids; **B** $KMnO_4$; **C** Thiéry-test; **D** Thiéry-test control; **In** intine, **It** infratectum, **Te** tectum

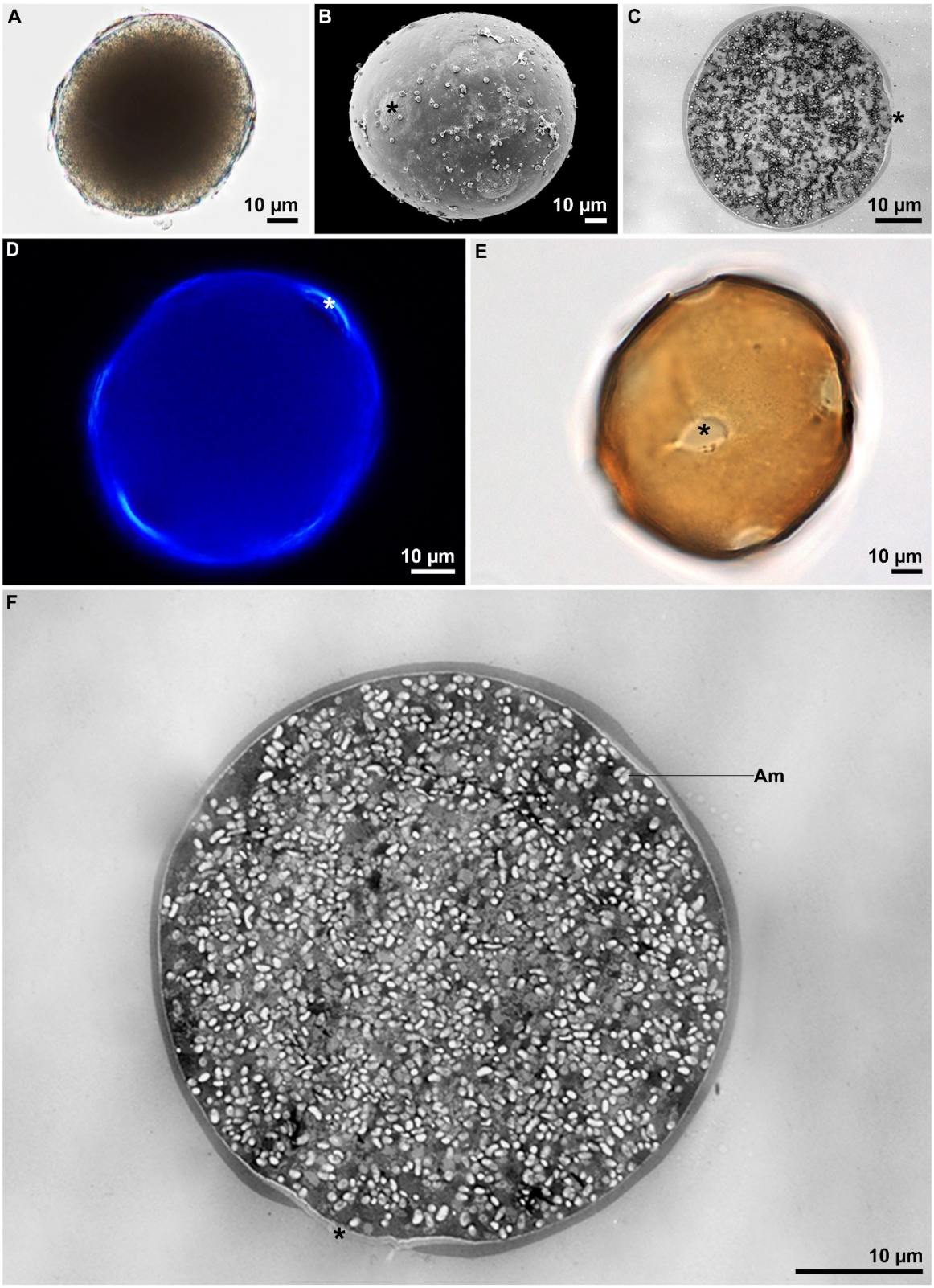

Figure 13: *Costus pictus*. **A** hydrated pollen grain, bright field LM; **B** hydrated porate pollen grain, SEM, **asterisk** aperture; **C** overview pollen grain Thiéry-test, TEM, **asterisk** aperture; **D** autofluorescence of ektexine, epifluorescence LM with DAPI filter cube, **asterisk** aperture; **E** after acetolysis psilate exine with pores remains, bright field LM, **asterisk** aperture; **F** overview pollen grain, UAc-PbC, TEM, **Am** amyloplast, **asterisk** aperture

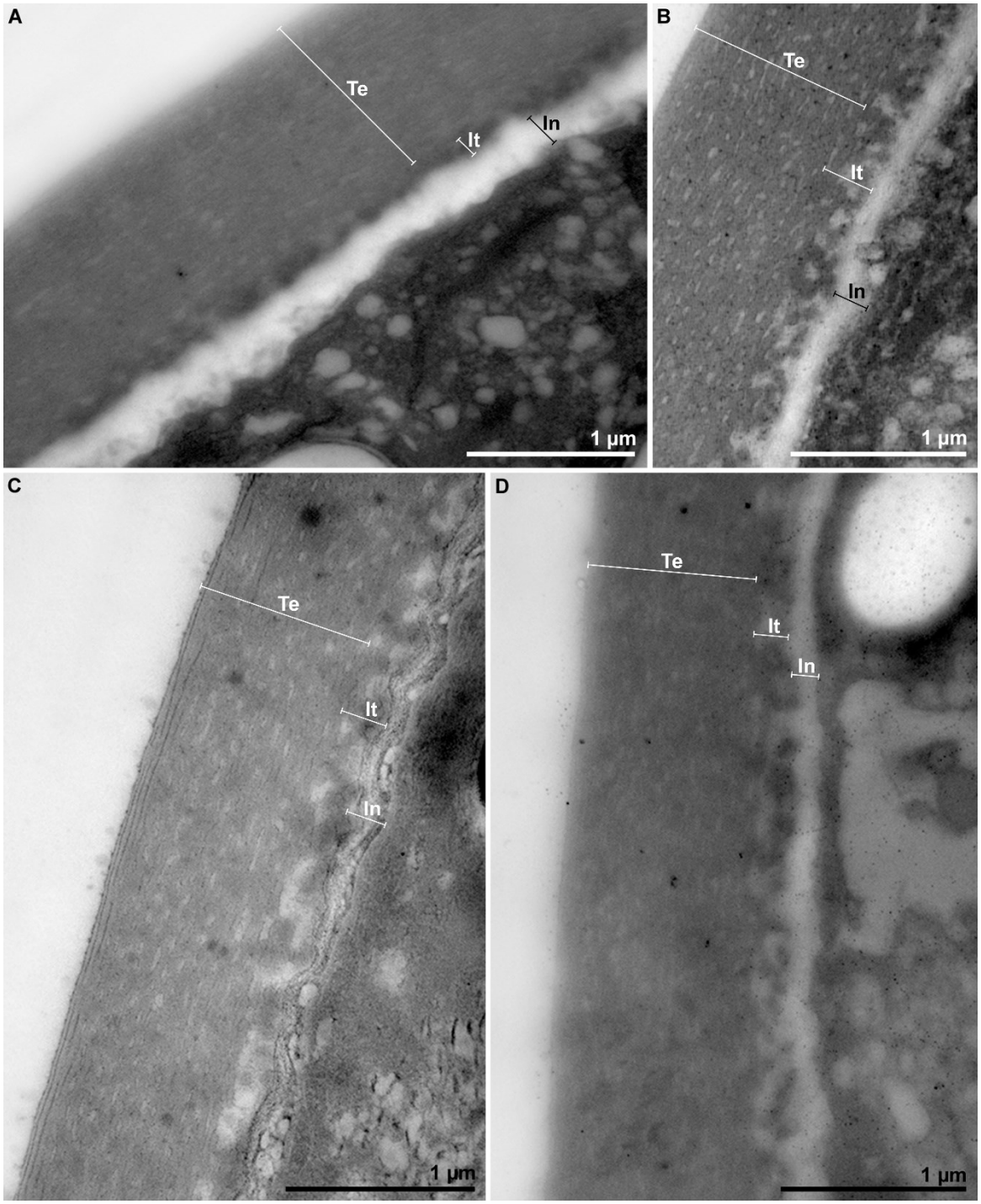

Figure 14: *Costus pictus*. Pollen wall stained with different methods. **A** unsaturated lipids; **B** $KMnO_4$; **C** Thiéry-test; **D** Thiéry-test control; **In** intine, **It** infratectum, **Te** tectum

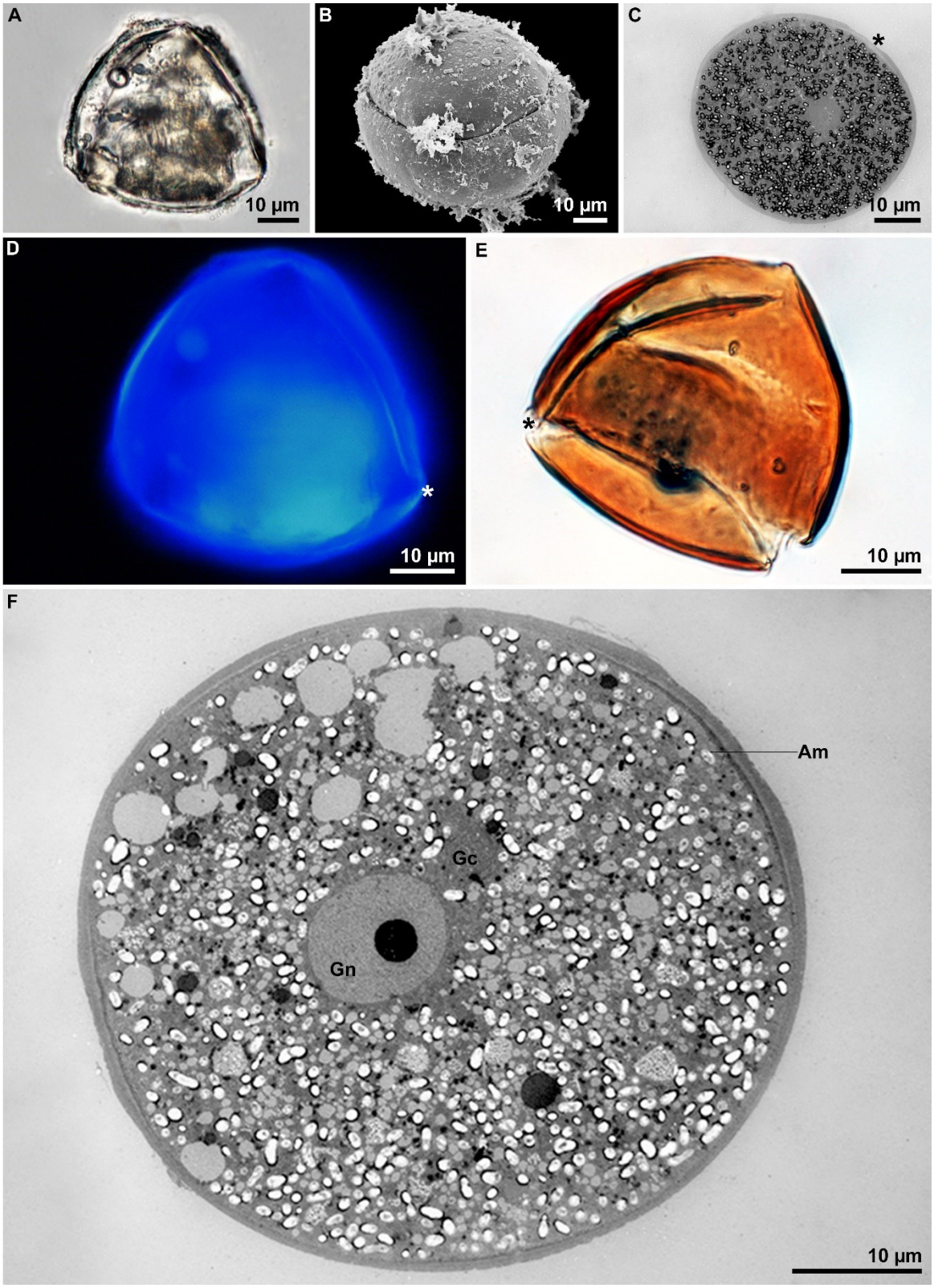

Figure 15: *Tapeinochilos ananassae*. **A** hydrated pollen grain, bright field LM, **asterisk** aperture; **B** hydrated pollen grain, SEM; **C** overview pollen grain, modified Thiéry-test, TEM, **asterisk** aperture; **D** autofluorescence of ektexine, apertures hard to see, epifluorescence LM with DAPI filter cube, **asterisk** aperture; **E** acetolysis, bright field LM, **asterisk** aperture; **F** Overview pollen grain, UAc-PbC, TEM, **Am** amyloplasts, **Gc** generative cell, **Gn** generative nucleus

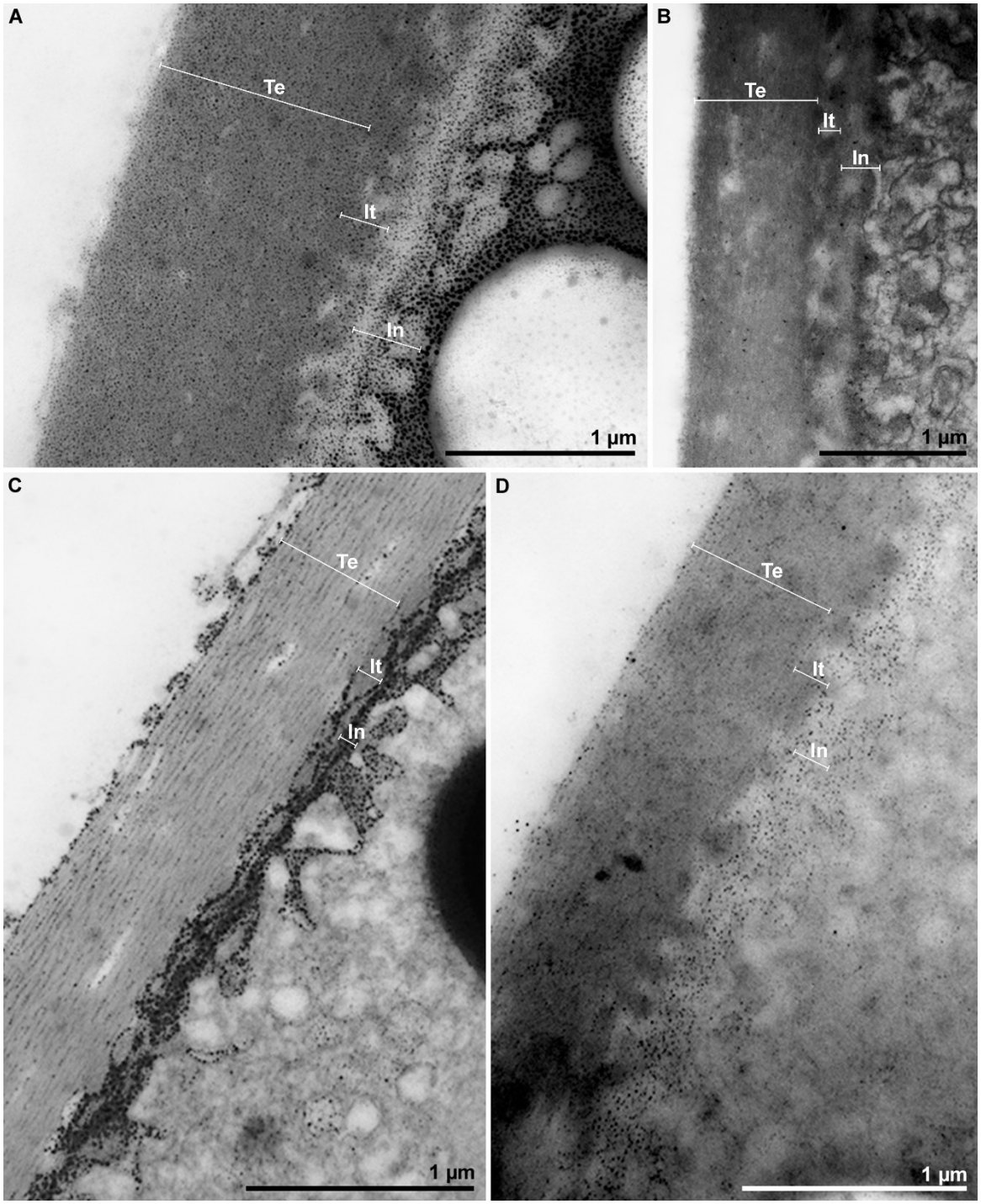

Figure 16: *Tapeinochilos ananassae*. Pollen wall stained with different methods. **A** unsaturated lipids; **B** $KMnO_4$; **C** Thiéry-test; **D** Thiéry-test control; **In** intine, **It** infratectum, **Te** tectum

4 Discussion

Pollen wall structure of the investigated species

According to Liang (1988) Zingiberaceae pollen can be separated into different types, based on their ornamentation: areolate, echinate, micro-echinate, psilate and striate (terminology follows Hesse et al., 2009). All Zingiberaceae in this study are inaperturate and belong either to the psilate, echinate or micro-echinate pollen type. Even though they are sculptured differently, the stratification of the sporoderm is similar.

The pollen wall consists of 3 intine layers and a surface layer. Next to the cell membrane, an endintine (In1) is located. Following this innermost layer a very thin intine layer 2 (In2) and a thick, channeled ektintine (In3) is found. Although their staining behavior is different, all of them are recognized as intine. A similar wall structure for Zingiberaceae was described by (Mangaly & Nayar, 1990), based on investigations with light microscopy. Mangaly & Nayar (1990) suggest a high content of cellulose in the endintine. This means the endintine should react strongly on the Thiéry-test, indicating polysaccharides. Normally the intine is electron-lucent throughout all common staining techniques, except for Thiéry-test (Weber & Ulrich, 2010) due to its main components cellulose, hemicellulose, pectins and proteins (Ariizumi & Toriyama, 2011). In the present study, the inhomogeneous structured endintine faintly stains after the Thiéry-test, while In2 and In3 show a much stronger reaction.

Stratification into a homogeneous endintine and a thick, channeled ektintine was reported by Chen & Xia (2011) for *Curcuma* sp. (Zingiberaceae) and *Boesenbergia* sp. (Zingiberaceae). But in difference to the present study, they did not separate the intine complex into three layers, even though some images would indicate the presence of a third layer.

The most prominent intine layer is In3, characterized by its thickness and radially arranged channels. In3 resembles a classical ektintine, usually found in apertural regions or over the whole pollen wall in inaperturate pollen grains (Hesse et al., 2009). The channeled ektintine stores several proteins discharged on the stigma during pollen germination (Heslop-Harrison, 1987).

A thin surface layer (Sl) is covering the 3-layered intine. Depending on the staining methods, it appears as distinct layer. The layer belongs neither to the exine nor to the intine. It seems to be polysaccharidic in nature. An exine is excluded, as the layer does not resist acetolysis, indicating absence of sporopollenin (Hesse & Waha, 1989; Jones, 2014). Based on the fact, that the layer is also covering the ornamentation elements an affiliation to the intine is excluded as well. Additionally, faint autofluorescence of this surface layer, compared to the strong autofluorescence of the sporopollenin-containing exine in e.g., *Cheilocostus speciosus* also indicates absence of sporopollenin.

Similar surface layers were described for other species of Zingiberaceae (Sakhanokho & Rajasekaran, 2010) and Araceae (Ulrich et al., 2016). Sakhanokho & Rajasekaran (2010) doubt that there is any elaborate exine present in *Hedychium* sp., which is in accordance with the present study.

While pollen wall structure within the Zingiberaceae is almost uniform, ornamentation varies. The “simplest” sporoderm type is found in *Etlingera elatior* and *Hedychium gardnerianum*. In both species, ornamentation is psilate. Within the other investigated species echini are present: *Alpinia* sp. is echinate, *Globba* sp. micro-echinate. TEM staining behavior is giving contradicting evidences about the nature of echini. While a positive reaction to the Thiéry-test indicates polysaccharides (Thiéry, 1967; Ulrich et al., 2016), $KMnO_4$, a stain for phospholipid-protein complexes and lignin in cell walls, as well as a lipid-test suggest endexine origin (Hayat, 2000; Weber & Ulrich, 2010). Missing resistance to acetolysis is evidence against the presence of endexine material, because this wall layer normally consists of sporopollenin, lipids and proteins (Heslop-Harrison, 1968a, 1968b; Heslop-Harrison et al., 1973; Weber & Ulrich, 2010).

In the investigated species ornamentation elements (echini, micro-echini) are polysaccharidic in nature. The origin of the echini in Zingiberaceae is so far unknown. Ontogenetic studies of *Arum* sp., revealed that the amoeboid tapetum is responsible for formation of the echini, in the late tetrad stage (Anger & Weber, 2006). Eventually this is also the case for the echini in Zingiberaceae, as an amoeboid tapetum type is also reported (Dahlgren et al., 1982).

Mangaly & Nayar (1990) reported a distinct, interrupted exine layer and echinate exine ornamentation for the whole genus *Alpinia*, except for the *Alpinia vittata*, described as psilate. But the present study revealed that this

species is missing an exine and has echini as ornamentation elements. For the genus *Globba*, Mangaly & Nayar (1990) described an exine with papillate ornamentation. The current investigation of *G. schomburgkii* and *G. winitii* disproved literature. An exine is missing and the ornamentation element should be re-named to micro-echinus (according to Hesse et al., 2009), due to its pointed appearance and a size less than 1 µm, this term is more adequate.

Similar observations on the structure of Zingiberaceae pollen walls, were done by Theilade et al. (1993) for *Zingiber* sp. or by Chen & Xia (2011) for *Curcuma* sp. and *Boesenbergia* sp. Although the authors separated this layer only into endintine and channeled ektintine, the TEM images suggest that there is a third layer located between these two zones, corresponding to In2 in the present study.

Concerning the surface layer of Zingiberaceae, interpretation of other authors diverges strongly from the findings in this study. While Liang (1988), Mangaly & Nayar (1990) and Theilade et al. (1993) describe the surface layer as a smooth or sculptured exine layer, the present findings strongly emphasize the absence of sporopollenin.

Literature is coincident in opinion that the aperture condition as inaperturate. Only for *Zingiber* sp. results are conspicuous, it was classified either as monosulcate (Dahlgren et al., 1985; Mangaly & Nayar, 1990) or as inaperturate (Liang, 1988; Theilade et al., 1993), which seems to be more appropriate.

According to pollen wall structure, Costaceae represented here by *Cheilocostus speciosus*, *Costus pictus* and *Tapeinochilos ananassae*, show huge differences, compared to Zingiberaceae. Costaceae are aperturate. Apertures are defined as regions of the pollen wall, that differ significantly from the rest of the wall in its morphology and anatomy (Hesse et al., 2009). *Cheilocostus speciosus* and *Costus pictus* are pantoporate, while the aperture condition of *Tapeinochilos ananassae* remains still unclear. According to Stone et al. (1981) pollen *of T. ananassae* is spiraperturate combined with a small colpus.

Pollen of Costaceae, show contrary to the walls of their sister family Zingiberaceae, a typical two layered wall structure with exine and intine, like in most angiosperms. The exine is highly modified. A clear differentiation of the ektexine into tectum, infratectum and foot layer is not possible. The tectum is less compact, than in other species, and shows a smooth transition to a granular infratectum. This part of the pollen wall is resistant to acetolysis, indicating

sporopollenin, already mentioned by Erdtman (1986). Furthermore, strong autofluorescence, except for the apertural region, indicates an exine as well. Foot layer and endexine are missing.

Stone et al. (1981) studied the ontogeny of *Tapeinochilos ananassae*. According to them, the outer pollen wall is a stratified primexine, a layer, reaching maturity during the tetrad stage of microspore development. They interpret this neotenic character as well as the type of aperture as secondary derived traits.

Costaceae show a typical intine, mono-layered in the interapertural areas and bi-layered at the apertures (ektintine and endintine). Both layers react to the Thiéry-test, indicating polysaccharides (Thiéry, 1967; Weber & Ulrich, 2010).

To summarize, the pollen wall stratification of investigated species within the two Zingiberales families strongly varies from the classical pollen wall scheme (Erdtman, 1986; Hesse et al., 2009; Ariizumi & Toriyama, 2011).

Investigated species vs. other extraordinary pollen walls

Zingiberaceae are the largest family (53 genera, approx. 1,200 species) of the order Zingiberales (92 genera, approx. 2,000 species; (Kress, 1990; Kress et al., 2001), but still not covering the complete variety in pollen morphology. Similarities between the families can be found.

In the following, present results are compared with *Strelitzia* sp. (Strelitziaceae, Strelitziineae), *Heliconia* sp. (Heliconiaceae, Heliconiineae) and *Canna* sp. (Cannaceae, Zingiberineae).

Strelitzia reginae, commonly known as bird of paradise, is another species with psilate ornamentation. Hydrated sporoderm is translucent, fragile and homogeneous in LM and appears slightly wrinkled at higher magnifications using SEM or TEM (Hesse & Waha, 1983). While the fragile outermost layer of *Strelitzia* sp. is resistant to acetolysis (Hesse & Waha, 1983) and therefore differs from here investigated Zingiberaceae, the complex intine is similar.

Contrary to Hesse & Waha (1983), who differentiated the sporoderm into endintine, channeled ektintine, and skin-like exine, Kronestedt-Robards & Rowley (1989) described the pollen wall differently. They suggested a stratification into a simple intine, followed by a 3 layered channeled zone, called onciform zone (Rowley et al., 1997) and a very thin exine. Additionally Kronestedt-Robards & Rowley (1989) studied pollen development and found evidence for unusual

sporoderm formation. A thick primexine is formed during the tetrad stage of pollen development. Passive stretching of this wall results in a very thin exine layer. According to Kronestedt-Robards & Rowley (1989), insufficient sporopollenin production or a failure of the primexine sporopollenin-receptors are responsible for this process. Nevertheless, they were still not able to explain the function of such a derived pollen wall.

Neither Hesse & Waha (1983) nor Kronestedt-Robards & Rowley (1989) used specific staining methods, like a Thiéry-test or other compound specific contrasting techniques, for TEM investigations. Application of more techniques would eventually lead to a better differentiation of these wall layers.

Though the sporoderm of *Strelitzia* sp. is classified differently than investigated Zingiberaceae, structural similarities are given, indicating a commonly applied basal pattern of pollen wall zonation among different Zingiberales families.

Another member of Zingiberales, *Heliconia* sp. has a more complex sporoderm stratification. Large pollen size, fragile pollen wall and an oblate to spheroidal outline, are basal characteristics (Kress et al., 1978). A unique feature within the pollen of Zingiberales is their heteropolarity. While the proximal polar area, that faces to the tetrad center during sporogenesis, is psilate, the distal area is echinate (Stone et al., 1979; Simao et al., 2007; Kress & Stone, 2009). Besides differences in sculpture, also incongruities in the pollen wall structure are found. The endintine is continuously present as a solid layer around the whole pollen grain. Whereas the ektintine is compact at the proximal polar area and channeled at the distal area (Kress & Stone, 2009). As *Strelitzia* sp., also, *Heliconia* sp. pollen has a thin exine layer (Simao et al., 2007). It is thicker at the proximal area and turns thinner distally (Kress & Stone, 2009). Resistance to acetolysis proves the presence of sporopollenin in the exine. Due to the differences in thickness, the proximal part is more resistant to this treatment (Stone et al., 1979). Pollen of *Heliconia* sp. was functionally classified as monoaperturate or inaperturate, like most other Zingiberales (Kress et al., 1978; Stone et al., 1979; Simao et al., 2007).

The structure of the distal polar area of *Heliconia* sp. is very similar to the pollen wall of *Alpinia* sp. and *Globba* sp. due to the thick, channeled ektintine. Contrary to the investigated Zingiberaceae, the intine layer 2 is missing. A thick, channeled ektintine is also characteristic for an aperture. This is in agreement with PalDat (Halbritter, 2016) where *Heliconia* sp. is described as ulcerate, with an ornamented

(micro-echini) aperture membrane. Contrary to Zingiberaceae, the thin surface layer of *Heliconia* sp. pollen is an ektexine, confirmed by the partially resistance to acetolysis, as well as positive reactions to fuchsine and auramine O (Kress & Stone, 2009).

With *Canna* sp., another genus with echinate pollen is found within Zingiberales. As most other members within this order, pollen grains are spheroidal and inaperturate (Skvarla & Rowley, 1970; Kress & Stone, 2009). The intine is separated into a more or less homogeneous endintine and a channeled ektintine (Kress & Stone, 2009), very similar to Zingiberaceae. The major difference in pollen wall ornamentation are echini, composed of sporopollenin. Acetolysis destroys the whole pollen grain after 10 minutes, while echini remain intact (Skvarla & Rowley, 1970). Missing resistance to 2-amino-ethanol, a substance that degrades sporopollenin (Skvarla & Rowley, 1970), and the positive reaction to fuchsine and auramine O, confirms the presence of sporopollenin as well (Kress & Stone, 2009).

Concerning the sporoderm stratification *Canna* sp. is almost the same as in other discussed Zingiberales. The main difference to the Zingiberaceae is the presence of sporopollenin in the pollen wall.

All investigated species of Zingiberaceae, as well as discussed examples from Strelitziaceae, Heliconiaceae and Cannaceae represent a very similar intine structure. Concerning the surface layer and the nature of the ornamentation elements, remarkable differences are found. Within these families, a gradient in exine reduction can be observed. *Heliconia* sp. has a discontinuous exine that turns thicker at the proximal polar area (Kress & Stone, 2009), *Strelitzia* sp. only has a delicate skin-like sporopollenin exine (Hesse & Waha, 1983; Kronestedt-Robards & Rowley, 1989), in *Canna* sp. a continuous exine is missing, but sporopollenin echini are found (Skvarla & Rowley, 1970; Kress & Stone, 2009). In the investigated Zingiberaceae species, no sporopollenin was detected at all.

This exine reduction and the neotenic primexine formation in Costaceae is eventually an evolutionary adaptation to moist environments (Knox, 1984; Simao et al., 2007).

Pollen walls show a huge variety in sculpture and structure (Hesse et al., 2009). Submerged water pollinated angiosperms, for example, show sometimes extreme structural wall reduction (Pettitt & Jermy, 1974; McConchie et al., 1982; Weber et

al., 1998). But also terrestrial living plants are sometimes missing wall layers, e.g. the foot layer in Haemodoraceae (Simpson, 2009) or the endexine in *Geranium* sp. (Geraniaceae; Weber, 1996). Even stronger exine reduction can be found at *Arisaema* sp. (Araceae). In this case the pollen wall consists mainly of endexine and only a thin, two-layered ektexine membrane (Ohashi et al., 1983). Weber et al. (1999) distinguish two basic pollen wall types in Araceae, each with two subtypes: Type 1 is defined by presence of an ektexine and a more or less classical structured pollen wall, whereas in type 2 the ektexine is missing and the endexine is forming the outmost wall layer. A surface layer as well as ornamentation elements composed of polysaccharides found in Zingiberaceae are also described for some Araceae species (Weber et al., 1998, 1999; Ulrich et al., 2016).

For a long time palynologists treated the absence of an ektexine (Hesse, 2006a; 2006b) and the presence of polysaccharidic ornamentation elements as unique features for some Aroideae (Weber et al., 1999). However, results gathered during the present investigation of Zingiberaceae disproved this uniqueness.

It can be assumed that modified pollen walls, as found in Zingiberaceae, are less durable and prone to various environmental influences, like desiccation. Concluding, Zingiberaceae and some species of Araceae found a way to protect their male gametes without incorporating huge amounts of sporopollenin, either by thickening of the endexine (Aroideae) or by evolving a highly complex intine (Zingiberaceae).

5 References

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