Science is wonderfully equipped to answer the question "How?" but it gets terribly confused when you ask the question "Why?"

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Chapter 2

THE ONTOGENY AND MORPHOLOGY OF MANGO (*Mangifera indica* L.) FRUIT WAX

2.1 ABSTRACT

The epicuticular surfaces of mango fruit (cultivars 'Keitt', 'Kent' and 'Tommy Atkins') were studied. A complex layer of wax functioning in reflectance and maintenance of physiological processes, bring about the dull bloom observed on developing fruit. The development of the wax is marked by sequential formation of distinct morphologies of crystalloid structures. Initially, there is a non-homogenous layer over the embryonic cuticle and epidermis, while more intricate structures develop soon after initialisation of fruit-set. The growth rate and increase in volume leads to the destruction of the second morphological type, which is pushed to the uppermost boundary of the final wax layer. The final morphology is characterised by a dense cover of delicate sepaline crystalloids, which fuse and eventually collapse as the fruit passes physiological ripeness. Cutin plates formed during lateral expansion of the epidermal layer contribute to the pyramidal, block-like morphology of the wax layer on mango fruit. This morphology is pronounced during fruit set, but less so on mature fruit. Originating from integrated wax and cutin on the ovary, the cuticular layer rapidly increases in quantity and eventually forms an intercellular filling that is saturated with cuticular wax. No apparent relationship between plate boundaries and the subcellular arrangement of epidermal cells could be established. No cracks were observed between the cuticular plates of any of the cultivars used in this study. As far as could be ascertained, this is the first comparative study of the ontogeny and morphology of the fruit wax and cuticle of different mango cultivars from the point of anthesis to physiological ripeness. Inter boundary distances during development of plates during fruit set present a possible answer for periodic poor adherence of preharvest pesticides.

2.2 INTRODUCTION

Fruit are transient aerial plant structures, with their singular importance being agents for propagation, and species survival giving their evolutionary impetus. The fruit surface is a specialised and intrinsic part of this evolution and its characteristics are regarded as species specific and persistent (Hietala *et al*., 1997; Griffiths *et al*., 2000). This implies that the architecture, composition and abundance of surface characteristics are genetically programmed (Lemieux, 1996; Jetter *et al*, 2000; Long *et al*., 2003). Various elements contribute to the complex entity of the fruit surface, but it consists mostly of cutin, cuticular wax and epicuticular wax crystalloids (Holloway, 1982; Jeffree, 1996; Jetter *et al*, 2000). Structural surface features may also include oil glands, a variety of trichomes and stomata (Esau, 1977; Stern, 1994).

As the interface between the atmosphere and the internal components, the importance of the fruit surface cannot be overestimated. The epicuticular wax is the first barrier presented by this interface, protecting the fruit and its physiological processes against injury by high levels of ultraviolet (UV) radiation, microbial attack, predation and mechanical injury (Stephanou & Manetas, 1997). Several wax crystalloid types (Barthlott *et al*., 1998) and chemical compositions (Hietala *et al*., 1997; Griffiths *et al*., 1999; Griffiths *et al*., 2000, Goodwin *et al*., 2003) have been described, attesting to its varied and species-specific nature. The importance of epicuticular wax has lead to its use in several studies, such as monitoring environmental pollution (Bakker *et al*., 1998; Evans *et al*., 2001; Pal *et al*., 2002), development of biological control measures (Rutledge *et al*., 2003; Eigenbrode, 2004) and quantification of physical attributes involved in crop improvement (Beattie & Marcell, 2002; Grant *et al*., 2003).

These studies underline the effects of human intervention, whether through physical control or genetic development of monoculture crops (Hietala *et al*., 1997; Beattie & Marcell, 2002). Such interventions are often part of both pre- and postharvest management of fruit crops. The success and financial feasibility of any intervention forming part of crop management depends on effective and successful implementation thereof, whether genetic, chemical or physical. However, successful implementation not only depends on precise management, but also on innate qualities and characteristics of the plant surface (Bally *et al*., 1996; Barthlott & Neinhuis, 1997; Mouloungui & Gauvrit, 1998; Bauer *et al*., 2004). Due to its hydrophobic properties, the presence of superficial wax layers affects all pre- and postharvest programs that rely on application of chemical or biocontrol measures. In this respect, epicuticular and cuticular wax has a particular role to play in the regulation of substances moving in or out through superficial plant structures (Chamel & Gambonne, 1997). Various features of the cuticular membrane are all involved in determining the extent to which the fluidity of the carrier matrix and plant wax achieves successful interaction, absorbance, penetration and translocation (Mouloungui & Gauvrit, 1998; Liu, 2004). The physical characteristics of surface wax are as important as the chemical nature thereof, as illustrated by Liu (2004), who found that improvement of the amount of droplet contact area and herbicide absorbance requires matching of surfactant and plant wax properties.

Various interactions with epicuticular and cuticular waxes not only affect disease and pest control, but also plant physiology through its determination of moisture loss (Oliveira *et al*., 2003) and reflectance of UV-B radiation (Rozema *et al*., 1997; Frohnmeyer & Staiger, 2003). Long-term effects of elevated UV-B levels are not yet clear, although Evans *et al*. (2001) proved that UV-B radiation penetrates the mesophyll, damaging cuticular and subcuticular tissue. Rozema *et al*. (1997) reported that UV-B damaged genetic material were excised as part of cellular maintenance, and consider the possibility that physiological damage due to UV-B may surpass negative effects related to predation or competition. Holmes and Keiller (2002) compared the reflectance of a range of plants that were hairless, hairy, woolly or waxy, and showed that epicuticular waxes effectively reflect UV radiation. They concluded that the substantial differences in the ability of waxes to reflect various wavelengths are based on chemical and morphological characteristics of the waxes. This corresponds with findings by Grant *et al*. (2003) that leaf reflectance is directly related to the leaf wax cover, and particularly important in studying the effects of increasing UV-B levels on the biochemistry and physiological processes of plants.

Fruit, as the fertilised ovary, is regarded as a modified leaf (Cutter, 1980), and is biochemically and physiologically complex through its dedicated functionality. To protect these biochemical and physiological processes, allowing embryo maturation and eventual dissemination thereof (Esau, 1977; Stern, 1994), control of external effects such as light and heat is required (Núňez-Elisea & Davenport, 1995). As a subtropical crop, mangoes are subjected to intense solarisation and heat levels (Barkstrom, 2004). A dense waxy bloom covers mango fruit as it develops, giving some protection against these environmental threats. In a study of the surface and lenticels of cultivars ‘Keitt’, ‘Kent’ and ‘Tommy Atkins’, particularly dense and complex epicuticular wax structures were observed. Normal scanning electron microscope (SEM) observations of mature fruit concurred with those made by Bally (1999). He gave an overview of wax ontogeny of the cultivar ‘Kensington’, mentioning cracks in the cuticle proper (*sensu* Holloway, 1982) as the source of rind discoloration. It is felt that the chronological morphology of various wax fractions needs further investigation, but no other work on wax ontogeny was encountered. This study aims to contribute to better understanding of the ontogeny and morphology of fruit wax from major South African commercial mango cultivars.

2.3 MATERIALS AND METHODS

2.3.1 Plant material

Mango fruit samples of different developmental stages of three polyembryonic cultivars (‘Tommy Atkins’, ‘Kent’ and ‘Keitt’) were collected fortnightly from the orchards of Bavaria Fruit Estates (Hoedspruit, Limpopo Province, South Africa) throughout the 2002/2003 and 2003/2004 seasons. Samples were collected by macroscopic verification alone, necessitating several collections of matching fruit sizes to prevent misinterpretation of wax development due to mismatched data. Each fortnight, five to seven fruit sizes, with five fruit each, were collected per cultivar. Three sections per fruit was sampled, prepared and viewed for each round of collection. Dissections were done immediately upon and at the point of collection. Fruit sizes sampled at each collection were noted (straight line from stem end to stamen end), ensuring an overlap of sizes with the previous round. This assisted in creating a continuous timeline for the developmental events observed.

2.3.2 Methods

Samples for both normal scanning electron microscopy (SEM) (JSM 840, JEOL, Tokyo, Japan) and high-resolution field emission scanning electron microscopy (FE-SEM) (JSM 6000FE, JEOL, Tokyo, Japan) were dissected from fruit at anthesis to mature mango fruit. For normal SEM, sections were viewed at 5 kV and a working distance of 12 mm, while for FE-SEM 5 -10 kV was used.

Samples were prepared using two parallel methods in order to exclude the interpretation of artefacts from preparation. According to the first method, sections (5 x 5 mm) were cut and fixed in a 1:1 mixture of 2.5 % glutaraldehyde and 2.5 % formaldehyde in 0.1 M $NaPO_4$ buffer ($pH = 7.3 \pm 0.05$), postfixed with 1 % aqueous OsO4, and dehydrated in an ethanol dilution series (30, 50, 70, 90 and 3 x 100 %). The material was critically point dried (Biorad E3000, Polaron, West Sussex, UK), mounted on stubs and made conductive in the vapour of a 0.5 % RuO_4 solution (Van der Merwe & Peacock, 1999).

For the second method, small sections (5 x 5 mm) of fresh material were plunge frozen in liquid propane at -180 °C, vacuum dried (Custom built, Tshwane University of Technology, Pretoria, South Africa) at -80 °C and 10-7 mBar for 72 hours, mounted on stubs and exposed to 0.5 % Ruthenium vapour to render material conductive.

To expose the architecture of the cuticle beneath the epicuticular wax, sections of mature 'Keitt' rind (5 x 5 mm) were soaked in 78 % H_2SO_4 for 48 hours, followed by three rinses in distilled water. Thereafter the sections were soaked in chloroform ($CHCl_3$) for 72 hours, air-dried, mounted on stubs and made conductive in the vapour of a 0.5 % RuO_4 solution.

2.4 RESULTS AND DISCUSSION

The description of chronological sequence of events during the formation of fruit epicuticular wax is based on micrographic evidence. Each micrograph, however, represents the observation of tissue that was metabolically active until the moment of fixation. The fixed material therefore gives a visual representation of the momentary structural contribution of the biochemical and metabolic processes.

Determining chronological fruit and, consequently, epicuticular wax development was particularly problematic from post-anthesis to fruit size 50 mm. Structural differences in the epicuticular wax, ontogenetically and morphologically, between the different cultivars, were indiscernible (Fig. 1A - C). All cultivars, regardless of external appearance, had a similar bi-layered epicuticular wax appearance at physiological ripeness (Fig.1D). This confirmed that some intrinsic characteristics of the species are persistent across its cultivars (Knight, 1997), and is not altered by sensorially relevant features such as colour, shape, aroma and sugar content. For this reason, it was decided to report all the results as that of mango fruit as such, and not to relate it to a specific cultivar.

The two methods run in parallel gave complementary results of similar material. This was useful in several respects: analogies pointed out the consistencies and provided necessary repetition of the sampled material, whereas differences in results contributed to understanding and explaining the observations. These differences in the two methods were most pronounced for young material and were not observed in more mature fruit. Plunge frozen sections of very young material showed some shrinkage - up to 15 %, and aldehyde fixed material up to 40 % (Boyde & Maconnachie, 1979). Specific anomalies encountered in the two methods will be indicated in the discussion of the applicable results.

All the micrographs in figure 2 were of post-anthesis fruitlets, at similar developmental stages, which were harvested and fixed at the same time. Figure 2A and B illustrates the amount of shrinking due to plunge freezing and aldehyde fixation followed by critical point drying (CPD) respectively. Because of the shrinkage, artefacts were created, making the interpretation of results more difficult. Artefact formation is less severe in plunge frozen material. Fractioning of fragile surface structures (Fig. 2C & D) was more pronounced after CPD. The composition of the plant material stayed intact with plunge freezing (Fig. 2E). Soluble components in the material, however, were lost during fixation in preparation for CPD, causing further disturbance of the integrity of the sample material (Fig. 2F). The integrity of plunge frozen material was correlated to the light microscopical findings of Bezuidenhout *et al*. (2005), but in the young fruitlets aldehyde fixation created false structural entities due to uneven shrinkage of fragile or unsupported cellular structures (Fig. 2G & H).

Nevertheless, as illustrated in Figure 3 (A - D), CPD rendered information that was valuable in understanding the chronology of the wax ontogeny. Characteristic development of the epicuticular wax was found to be dependant on the direction of deposition of wax fractions. The sequence and direction of wax deposition illustrated was only visible through the removal of soluble fractions by the aqueous buffer and ethanol dehydration during preparation. Other such instances were the fruit surfaces in Figure 4 (A - D), depicting sections of cutin from 4 - 7 mm fruit. The embryonic wax was removed during sample preparation, making the initials of the more permanent wax visible (A). Globular crystalloids (Barthlott, *et al*., 1998) extruded into nematoid wax crystalloids (B), which seemed to clump together from localised regions (C & D). These nematoid crystalloids coalesce into narrow ribbons (E); in turn, these coalesce to form sepaline lamellae (F & G). Cross-sectional fractures of plunge frozen material often revealed lamellar wax crystalloids that formed after the physical impact (H). This latter type of wax crystalloid structure was only found on fruit exceeding 10 - 15 mm and is structurally fit for light manipulation by the fruit.

An amorphous (Jeffree, 1986) or smooth (Barthlott, *et al*., 1998) wax layer covered fruit from post-anthesis (2 - 3 mm) up to a size of about 10 mm. Initially, it is undulating, adhering to the apical ends of the columnar epidermal cells. This embryonic layer seemed to be a mixture of two immiscible components (Fig. 5A), one of which was indicated as soluble during aqueous aldehyde fixation. Plunge frozen fruitlets of the same sizes show no such loss of compositional fractions. At post-anthesis, the cuticle thickness averaged < 0.5 µm, in comparison to the primary cell wall thickness of < 1 µm. The embryonic wax was observed as a liquefied moiety that oozed from the cutin (Fig. 5B) and formed a thin film on the surface. Determining a time line of events was impossible, but within a day after fertilisation the fruitlet began to expand, while the wax film seemed to become segmented (Fig. 5C) and the cutin thickened (Fig. 5D). The segment artefact arose from the shrinking of the material, the borders of each segment representing a tangential wall of an epidermal cell. Segmentation became more prominent in fruit > 4 - 5 mm (Fig. 5E). In a cross-sectional fracture, the thickness of the wax layer exceeded that of the cutin and had a longitudinal striated appearance (Fig. 5F).

The stomata of fruit > 3 mm begin to open (Fig. 6A), surrounded by a pliable, and segmented wax film (Fig. 6B). Within a further 2 mm diameter increase, the formation of a new wax fraction was exposed in aldehyde fixed material (Fig. 6C). Due to the removal of the soluble fraction, the embryonic wax layer tends to lift from the surface as a crust. Once segmented, it easily became detached from the cutin during processing. In (D) of Figure 6, granular to globular crystalloids, were observed on raised prominences. Shrinkage of the epidermal cells caused the tangential cell walls to appear in this fashion, giving evidence to the apoplastic transport of the wax precursors to the surface (Kunst & Samuels, 2003). These crystalloids rapidly aggregated, forming an apparently second layer of wax underneath the embryonic wax (Fig. 6E). The presence of amorphous, inter cell wall and cuticular wax (Fig. 6F) was not reflected in the supra-cuticular wax, which appeared as irregular threads after removal of the soluble fraction (Fig. 6F). Once again, this was an artefact, demonstrated by the flaking effect of the wax detaching from the surface in (E), but more so in embryonic wax film segments (Fig. 6G). These became separated due to surface expansion of the growing fruit, forming radial dendrites originating from each segment, similar to cogs of a gear. The resultant space was filled by (at this stage) disorientated platelets that also encroached on the dendritic structures. The dendritic appearance of the laterally separated segments resulted from longitudinal depositions in the now proper wax layer (Fig. 6H).

Aldehyde fixed material of > 10 mm became very prone to disruption of surface structures (Fig. 7A). The irregular margins of the detached segments became the template for the formation of the dendritic structures (Fig. 7B). The disrupted wax film was useful in the determination that the small film segments each became the focus for the development of the radial dendrites, resembling overturned cookie cups. The central area where the embryonic wax segment was attached seemed free of platelets, with the zones where dendrites have developed containing scattered deposits. The perimeter of each segment enlarged significantly through growth of the dendrites (Fig. 7C), and the total structure increased in height. (Fig. 7D). The wax layer and cutin layer thicknesses were now equivalent, exceeding the cell wall thickness. These structures were also described by Bally (1999). During expansion of the fruit surface, most ‘cookie cups’ became fractured, indicating the inelasticity of the embryonic film remnants (Fig. 7E). Longitudinal wax deposition still contributed to the increase in thickness, and continued to be structurally simple (Fig. 7F). In contrast, platelets became organised in parallel rows. These were organised into stacked concentric rings around each ‘cookie cup’, and furrows demarcating the opposing directions of expansion. The result resembled fissured layers (Fig. 7G). The development of a bi-layered epicuticular wax is not true syntopism (Barthlott *et al*., 1998), but rather the sequential appearance of structures from which the multilayered epicuticular wax of mango fruit is finally composed. Similar multilayered epicuticular wax structures were described by Jetter *et al*. (2000) in *Prunus laurocerasus* L. At fruit size > 12 mm the cutin layer (3 - 5 µm), exceeds both the cell wall and wax layer thickness. Up to this point, the epidermal cells were arranged in a very regular layer of columnar cells, (4 - 5 µm periclinal width and 10 - 12 µm anticlinal height) (Fig. 7H).

Between 10 - 25 mm the fruit surface seemed to be covered in ‘fissured’ layers, but this appearance represented another transitional stage in the development of the epicuticular wax (Fig. 8A). The fissured appearance was only superficial; not affecting the cutin underneath (Fig. 8B); it is better described as furrows. The appearance of this transitional layer was caused by opposing bidirectional wax growth (deposition of amorphous longitudinal wax, lateral deposition of platelets) with sequential rows of platelets produced from, but never filling the furrowed zone. No cracks in the cutin could be observed in any of the cross-sections studied for the three cultivars concerned, although Bally (1999) did describe such for ‘Kensington’. It is felt that these were probably similar to the ‘fissures’ described above. This has implications for the ideas put forward regarding mango skin browning, and may require further investigation.

Although the apparent fissures did not affect the cutin, rapid surface expansion due to volumetric increase of the pulp began to push the epidermal cells out of its previously organised arrangement on top of the parenchyma/mesophyll. An uneven developing cutin layer was the first indication of the gross changes and displacement that the epicuticular layers would undergo (Fig. 8B, D & F). Externally, pyramidal wax plates began to manifest, with pronounced parallel alignment of platelets emphasising the angularity of each pyramidal structure (Fig. 8C & E). In all probability, the suture-like links between these plates as observed by O'Hare *et al*. (1999) were a pattern due to depositions of wax crystalloids that formed a barrier to protect the rapidly expanding cutin (Fig. 8A, C, & E).

Remnants of the embryonic wax film persisted on the layers developing underneath, and continued to break apart during surface expansion (Fig. 9A). The rate of growth and expansion was not 100 % equally in all directions at all times, giving the effect of lopsidedness in such cases. In (A) the newly exposed facets of the pyramidal structures were still incompletely covered in platelets, again creating an impression of surface cracking. The cross-section in (B) was cut instead of fractured, which resulted in smearing of the abundant cuticular wax. Nevertheless, penetration of the cutin to fill the space created by the displacement of the epidermal cells, were evident. During lateral expansion, the pyramidal sections split open at almost right angles to the previous division, with newly opened sections consistently filled by deposition of platelets (Fig. 9C) that will fuse into sepaline crystalloids. The epidermal cells were continuously deformed and pushed out of alignment (Fig. 9D & F), with the liberated intercellular regions simultaneously blocked by cutin deposits. From fruit size > 60 - 70 mm no further developmental changes were observed other than continued expansion and filling of existing split sites (Fig. 9E). In an abaxial view of an isolated cutin membrane, the cell boundaries were demarcated by convoluted cutin (Fig. 9G), corresponding to the protrusions of cutin observed in Figure 9 H.

The compelling relationship between cutin and wax were demonstrated by fruit with superficial damage caused by thrips (Grové & Pringle, 2000). These microscopically small insects rasp away at the surface of young fruitlets, damaging or destroying the cutin and epidermal cells, penetrating as deep as the mesophyll. The plant counters this attack by signalling living cells to deposit cutin. Thrip damage often extended several cell layers into the exodermis (*sensu* Esau, 1977), as was demonstrated in material depicted in A & B (Fig. 10). The dynamic nature of epicuticular wax became clear in (C), where even remnants of cell walls (underneath which cutin has been deposited) were covered in epicuticular wax. Several of the abnormal cutin surfaces enclosing single mesophyll cells several layers below the defunct epidermal layer were also covered in epicuticular wax (Fig. 10D). The interface of two opposing layers of cutin formed in this way was observed filled with wax crystalloids (Fig. 10E). These have most probably originated from within the enclosed mesophyll cells, since the apoplastic transport route was severely damaged. The signal and mechanism for transport of precursor wax is still unclear (Kunst &

Samuels, 2003), but these findings give support to the idea that it is a genetically programmed event actively regulated by plant cells.

These dynamics were further illustrated by the spontaneous self-assembly of lamellar crystalloids on the exposed surfaces of material cross-sectioned by snapping it while immersed in liquid nitrogen (Fig. 11A). Although structurally different from the amorphous bottom layer and the sepaline crystalloids in the top layer, these lamellae originated in a similar fashion as the other two (ref. Fig. 4). Infrequently, small (10 µm) 'cookie cup'-shaped crusts were found on top of the sepaline wax crystalloids of mature fruit (Fig. 11B). These were probably created when accumulated air bubbles formed during respiration escaped from intercellular air passages filled with liquefied cuticular wax fractions, pushing the wax out. A cross-section of a 'fissure' in the wax layer (Fig. 11C) showed it not extending into the cutin. The cutin was heavily impregnated with cuticular wax, with the globular nature of the cutin demonstrated in figure 11D.

Newly deposited cutin that had not been impregnated with cuticular wax, were found in several samples (Fig. 12). These depositions indicated where active intercellular filling during growth took place (A). The localities indicated by number 1 - 7 correspond to Figures B - H. The first locality was at the transitional zone between the old and newly deposited cutin (B, 1). To the left of the micrograph the old wax is almost completely covered by a dense, viscous layer. A single cutin globule on the right is partially covered, with small subunits of cutin beneath it. A five times higher magnification showed the wax flow in the process of covering the cutin, leaving open areas with no observed wax (C, 2). This was in contrast to the section 3, illustrated by (D, 3), where the new cutin globules and interstitial space was covered more completely. Despite even better coverage in figure 12 E (4), the presence of some holes and pits make the cutin appear more porous. The variably sized pores observed (F, 5) were most likely due to inconsistent and uneven flow when the cutin globules were enfolded with cuticular wax. Scrutiny of a section of older cutin (G, 6) revealed similar pores and openings. At very high magnification and available resolution of the new cutin, no other structural inclusions could be illustrated (H, 7).

Cutin was not only important as the reservoir for cuticular wax, but also determined the external development of morphological epicuticular detail. Bally *et al*. (1996) and Bally (1999) indicated the existence of plate-like structures from which the cutin of mango is compiled. With the information gathered by means of both the normal SEM and FE-SEM we were able to enhance this observation. What appeared as definite sets of architecturally defined ridged plates forming the base for the pyramidal wax blocks, were the footprints of laterally expanded cutin (Fig. 13A). The geometric wax arrangement therefore corresponds with the geometric cuticular development underneath. Epicuticular wax architecture is therefore dependant on the directional growth patterns of the cutin layer (Fig. 13B & C). The abaxial view of an isolated cuticular membrane (Fig. 13D) revealed that mitotic division is responsible for the formation of the adaxial architecture. The cutin layer itself thus forms a die onto which the wax plate shape is cast. No definite association between the organisation of epidermal and mesophyll cells, and the maxima and minima of the cutin plates could be established (Fig. 13E). Although some wax plates are clearly demarcated by a narrow furrow (H), no break in the cutin between the ridges and valleys could be indicated (Fig. 13G).

2.5 CONCLUSION

Fruit, a rich source of carbohydrates and phytochemicals (Plaami *et al*., 2002), are particularly vulnerable to predation and microbial attack. Protecting the developing embryo until it is ready to be disseminated is therefore important. Mango fruit is a good example of how survival measures against meteorological factors, decay and predators are combined in the epicuticular layers of the fructosphere. These survival measures are paramount to the physiological development of the fruit. Development of pesticide formulations and crop management strategies should therefore not only consider aspects such as meteorology, safety and cost (De Bie, 2004). The uniqueness of the fruit and its requirements for self-protection also needs to be taken into account, accommodating new morphogenetic adaptations in monoculture crops planted outside their natural habitat of evolutionary success.

Understanding the ontogeny and morphology of mango fruit wax more accurately will contribute to the improved management of mango production in South Africa. Epicuticular wax has been established as a self-protection mechanism in plants (Eigenbrode, 2004). For wax ontogeny, events described in this study are not successive in real time, but rather representative of a continuous process. However, collection and sample preparation terminates a very specific moment in the ontogenetic sequence, creating a visual representation of that moment. Interpreting the development or disappearance of the various structures on the fruit surface depends on this reality. Mango fruit wax development is complex from a very early stage, and static images obtained with microscopy must be interpreted to explain a continuous metabolic process. Accurate judging of the chronological developmental ages of fruit increasing in size is difficult. This is due to the fact that time intervals of individual fruit seldomly match perfectly in terms of biochemistry and development. Fruit that appear to follow sequential increases in size may differ in developmental stage, and therefore the information gathered has inconsistencies that make interpretation of observed features and dynamic changes difficult. This problem was overcome by the large number of samples collected and viewed during the very early stages of fruit development.

A common complaint during preharvest pesticide spraying is the seemingly periodic low wettability of the fruit surfaces. The pronounced angularity of epicuticular wax plates during certain stages of fruit development could present the droplets with too little surface for successful adhesion.

Using different methods in parallel was invaluable in understanding and compiling the chronology of ontogenetic events. The soluble fraction that is damaged during aldehyde fixation is most likely a sugar, or a glycosidically bound terpene or phenolic compound with possible toxic properties. Support for this idea is the observation that thrips only utilise fruit smaller than 8 - 10mm as a food source (Grové *et al*., 1999). The contribution of increasing cuticular wax fractions to thrip deterrence has to be investigated by non-destructive chemical analysis to prevent leaching of cellular content and resin. Cutin can not form part of the nutritional base during feeding, since it is known as an indigestible, inert biopolymer (Jeffree, 1986) and rapidly increases in paradermal dimensions during fruit development.

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2.7 FIGURE CAPTIONS

Figure 1 Wax morphology and ontogeny of the three cultivars studied, with similar typical surface morphology for 'Tommy Atkins' (A), 'Kent' (B) and 'Keitt' (C). Cross-section of frozen material showing bi-layered epicuticular wax (D).

Figure 2 Very young material sections prepared by plunge freezing (A) and aldehyde fixation/CPD (B), showing the degree of shrinkage. Slightly disrupted embryonic epicuticular wax layer present on plunge frozen post-anthesis fruitlets (C). More extensive disruption of the aldehyde fixed material (D). Soluble material present on the surface were retained by plunge freezing (E), with flaking wax due to the compromised composition following aldehyde fixing (F). Cell shape and size of plunge frozen material reflected that of the fresh condition (G). Artefacts created by aldehyde fixation, followed by critical point drying, in young plant material with fragile or unsupported contents (H).

Figure 3 Dendritic wax projections are overlaid with lamellar platelet moieties (A), forming concentric patterns (B). Concentric rings demarcate the horizontal growth of the epicuticular wax (C), at the same time enlarging the initial focus in a 'cookie cup' shape (D).

Figure 4 Wax structures from anthesis (ovaries 3 - 5 mm) to physiologically mature fruit. Globular to granular crystalloids (A). Extruded wax initials forming nematoid crystalloids (B). Clumping of nematoid crystalloids in localities of origin (C). Nematoid and thread-like crystalloids aggregate (D). Fusion of nematoid crystalloids creates ribbon-like structures with entire margins (E). Ribbon-like structures fuse into sepaline crystalloids present on physiologically mature fruit, playing a role in light reflection (F & G). Lamellar wax crystalloids formed on exposed cutin after fracturing of plunge frozen material (H).

Figure 5 Micrographs of the epicuticular surface structures of post-anthesis fruitlets. Thin, smooth, amorphous wax film on periclinal epidermal cell walls (A). The primary cell wall (large arrow) appeared 3 times thicker than the layer that consisted of cutin integrated with embryonic wax (small arrow) (B). Fruitlets, only hours older, had a more substantial wax layer that became more visible due to shrinkage (C). Older fruitlets also had a better developed cutin layer (D). Segmentation of the embryonic wax film took place on the cell wall boundaries (E). Rapid increase in wax thickness began by longitudinal deposition of an amorphous wax layer (F).

Figure 6 Chronological epicuticular development of the surfaces of post-anthesis mango fruitlets (4 - 10 µm diameter). Stomata, surrounded by an amorphous wax film, beginning to open (A). Pliable appearance of wax film dividing into segments (B). Compromised embryonic wax due to removal of soluble fraction after fixation in aldehyde (C). Wax initials on the raised cell wall sections underneath were made visible by disruption of the wax film (D). Secondary wax deposition assembled into complex structures (E). Impregnation of both the cell wall and cutin by cuticular wax revealed by removal of soluble fraction (F). Plunge frozen, freeze dried material showed the secondary wax deposition integrating with the embryonic wax film (G). Enlarging fruit (6- 8 mm), with cutin growing at a higher rate than that of the wax layer, overtaking it in physical dimensions (H).

Figure 7 Surface structures of fruit exceeding 15 mm in size. Disrupted segments of embryonic wax film revealed platelets forming from beneath (A). The irregular margins of wax segments are the template for development of dendritic structures (B). Overturned ‘cookie cup’ shapes due to the dendritic extensions radiating from each segment (C). Structural height increase due to longitudinal wax deposition (D). Cracking of the ‘cookie cups’ signifying the inelasticity of the embryonic film remnants (E). Despite increase in thickness, longitudinal wax structures remained simple (F). Fruit surface with furrows in the wax, giving the impression of fissured layers (G). Cutin thickness of fruit > 12 - 15 mm was up to five times thicker than the wax layer (H).

Figure 8 Surface structures of fruit sizes between 10 - 25 mm. A ‘fissure’ representing the boundary between two opposing directions of deposition in platelet formation (A). Increasingly irregular cutin layer (8 - 10 µm thick) in cross-sectional views, with distorted epidermal cells (B). Manifestation of pyramidal wax plates (C). Development of complex cutin architecture followed the distortion of the epidermal cells (D). Deposition of wax crystalloids that formed a barrier to protect the rapidly expanding cutin (E).

Increased thickness of epicuticular wax during displacement of epidermal cells (F).

Figure 9 Micrographs of continued surface expansion. Newly opened facets of the pyramidal structures were not as abundantly filled with platelets as those from earlier divisions (A). Cutin deposition was not limited to the apical sides of cells (B). Sequential tearing and splitting of embryonic wax remnants happened at almost right angles (C). Deformed and misaligned epidermal cells with a haphazard appearance (D). Filling of laterally expanded zones and epicuticular changes were visible in fruit sizes > 60 - 70 mm (E). Cross-section profile of fruit > 70 mm with displaced, deformed epidermal cells, a cutin thickness exceeding 10 μm, and a 1 - 2 μm thick wax layer (F). Abaxial view of isolated cuticular membrane section, illustrating the convoluted cutin layer, with no apparent cracks developing (G). Cutin protrusion corresponding to the cell wall convolutions (H).

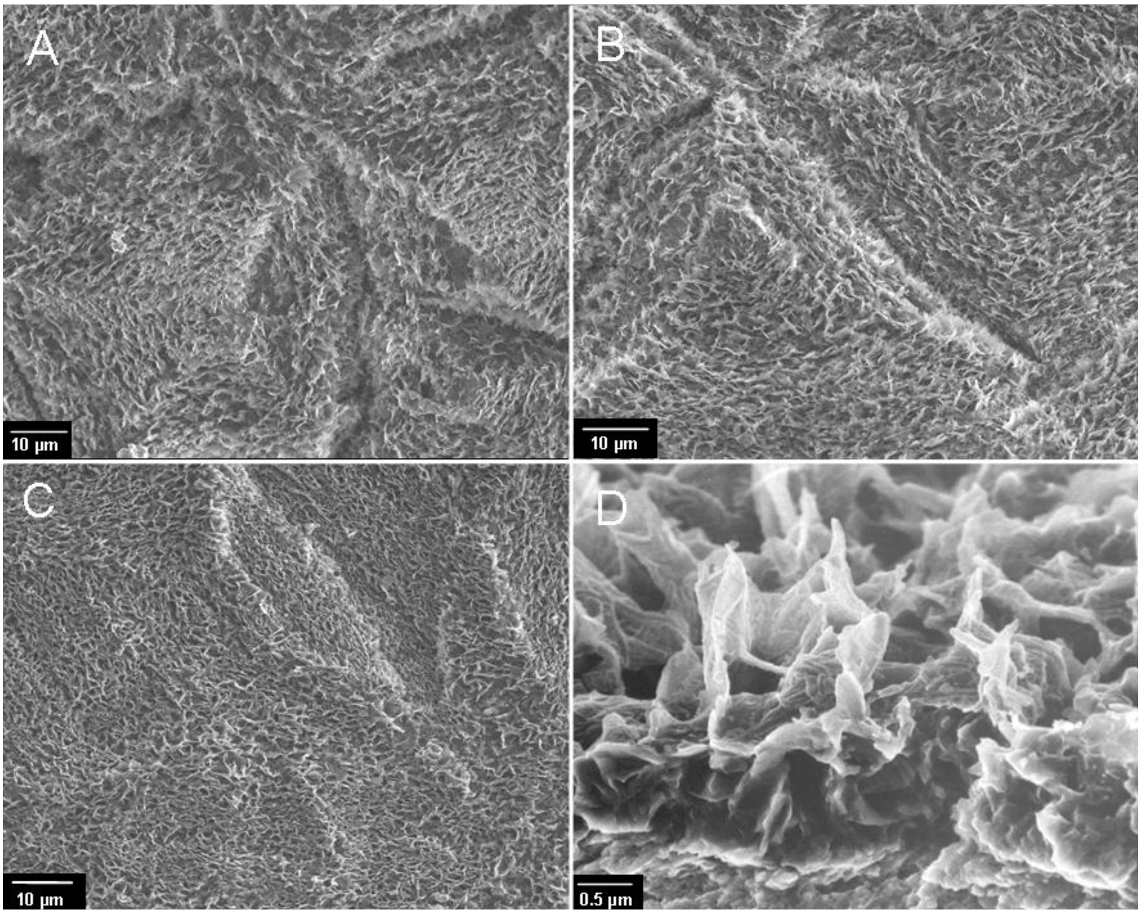
Figure 10 Thrip damage to the superficial layers of mango fruit was often observed as extending into several layers of exodermal cells, as illustrated by (A) and (B). Cell wall remnants covered by self assembling wax fractions (C). Individual mesophyll cells were often enclosed by cutin containing wax crystalloids (D). Interstitial space between two adjacent cutin layers, filled with lamellate wax crystalloids (E).

Figure 11 Surface structures of fruit close to physiological ripeness. Spontaneously self-assembled lamellar crystalloids on the exposed surfaces of cross-sectioned sample material (A). A ‘cookie cup’ structure developing on top of the parallel platelets due to internal gas build-up pushing liquid wax fractions outward (B). Cross-section of a deep fissure, with no indication of cutin cracks (C). Incomplete coalescence of cuticular wax left sections of cutin globules exposed (D).

Figure 12 (A) Micrograph of a newly formed cutin deposit not yet impregnated with cuticular wax. The localities indicated by numbers 1 - 7 correspond to Figures B - H. Transitional zone between cutin deposits of different ages (B). Several sections of cutin were observed to be apparently without wax impregnation (C). A section with more abundant wax still exhibited pores and holes in the cuticular wax layer (D). The proximity of older, established cutin contributed to the abundance of wax impregnating marginal sections of the new deposit (E). Observed pores represented incomplete flow of constituent wax fractions (F). An older section of cutin had similar pores and openings (G). Despite varying pore sizes, the presence of tubules could not be illustrated (H).

Figure 13 Adaxial view of a cutin membrane from which all epicuticular wax had been removed (A). Ridged plates formed during lateral expansion of the cutin. The geometric cuticular development corresponded to the geometric wax arrangement (B) & (C). Isolate cutin membrane sections (D) verified the role of mitotic cell division in the establishment of the cutin architecture. The ridges and valleys of the cuticular plates could not be matched indisputably to the placement of subcutaneous cells (E). Narrow furrows demarcated some wax plates (F), but no cuticular breaks or cracks could be illustrated (G).

2.8 FIGURES



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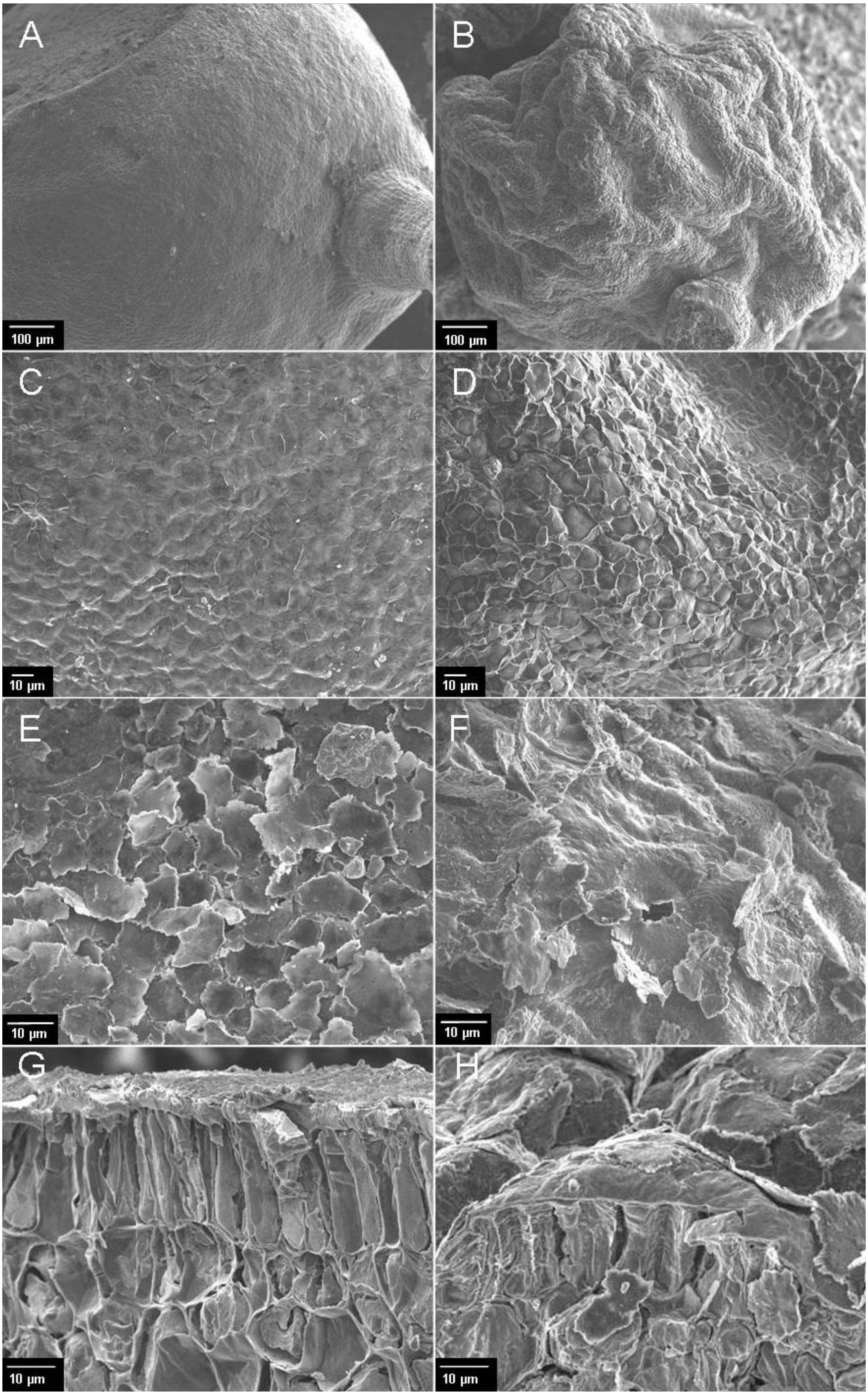

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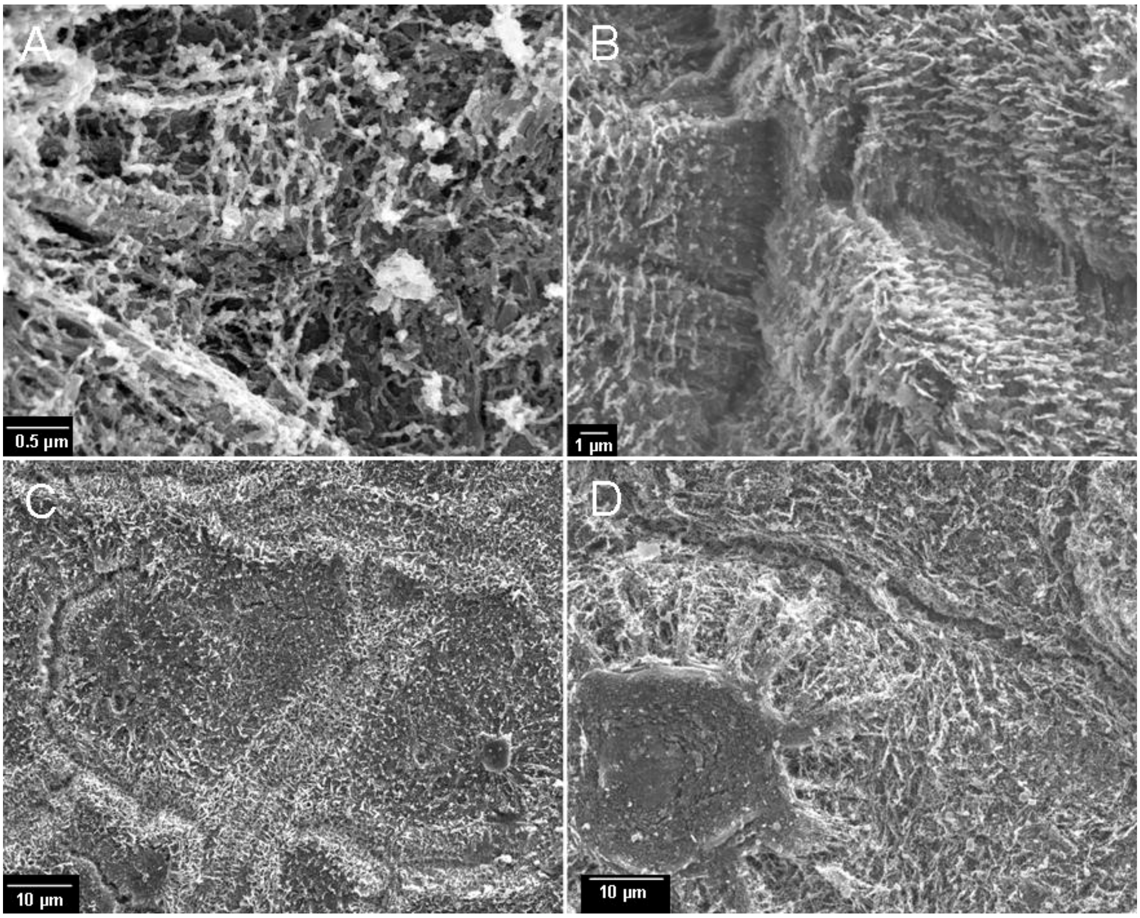

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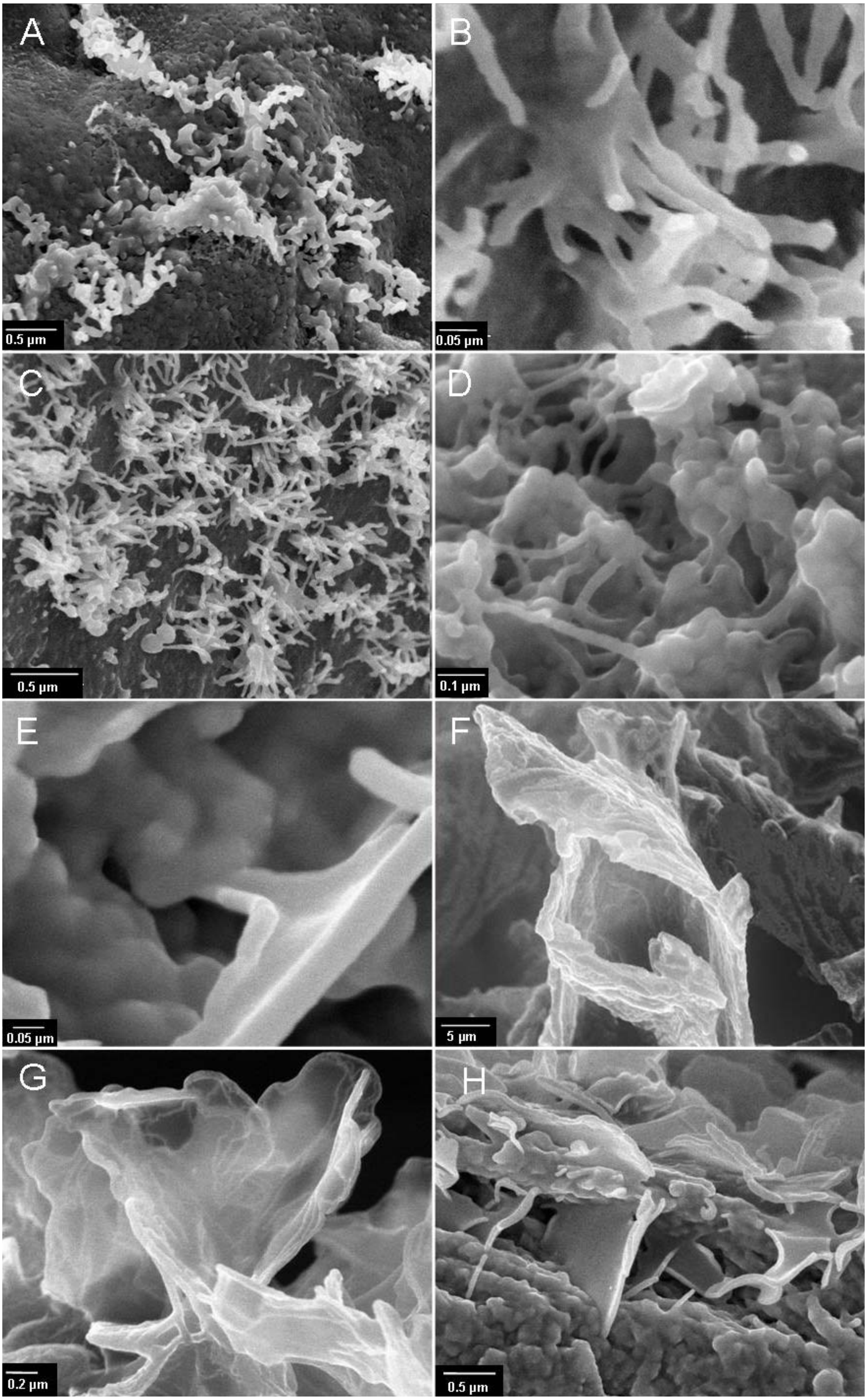

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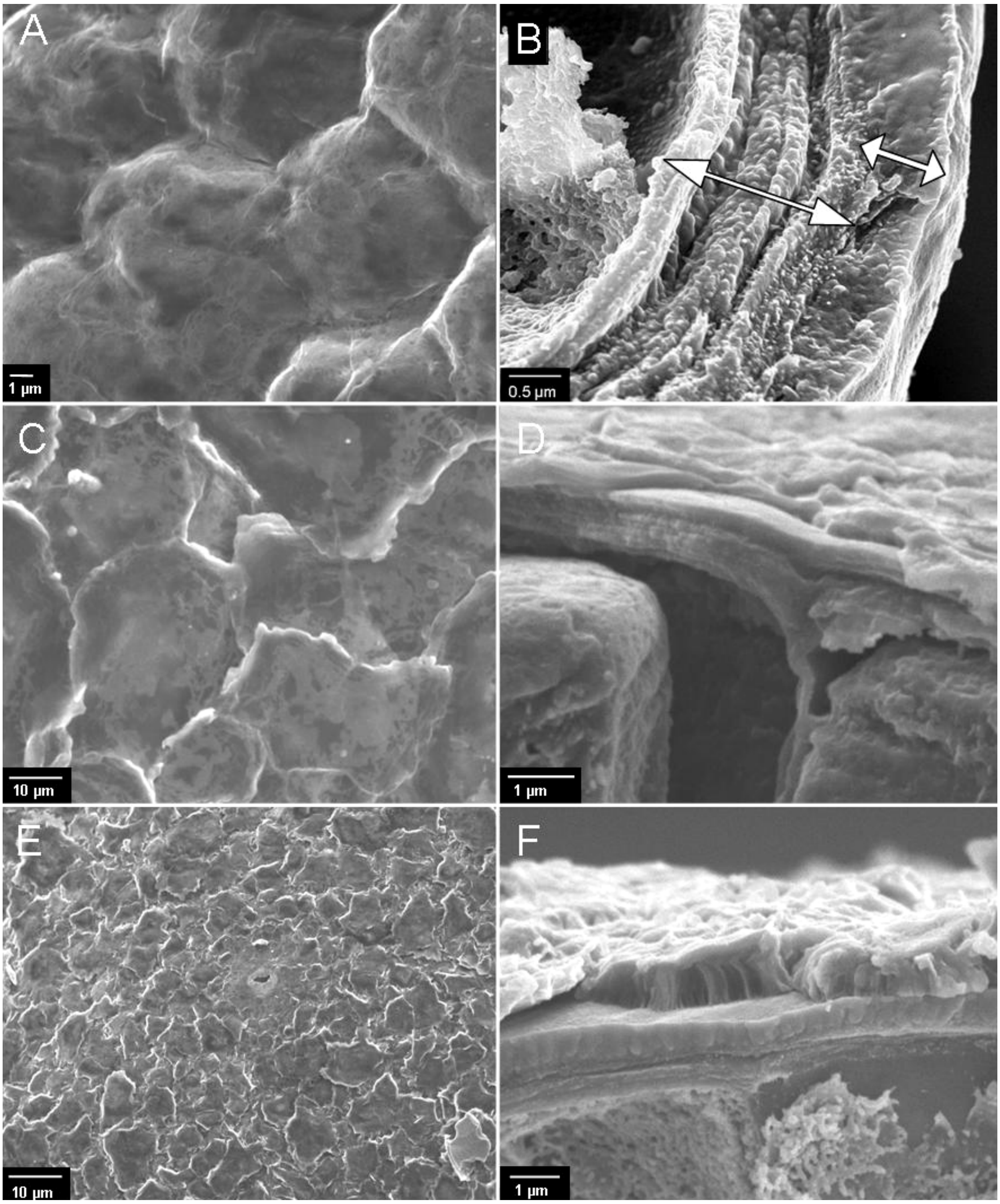

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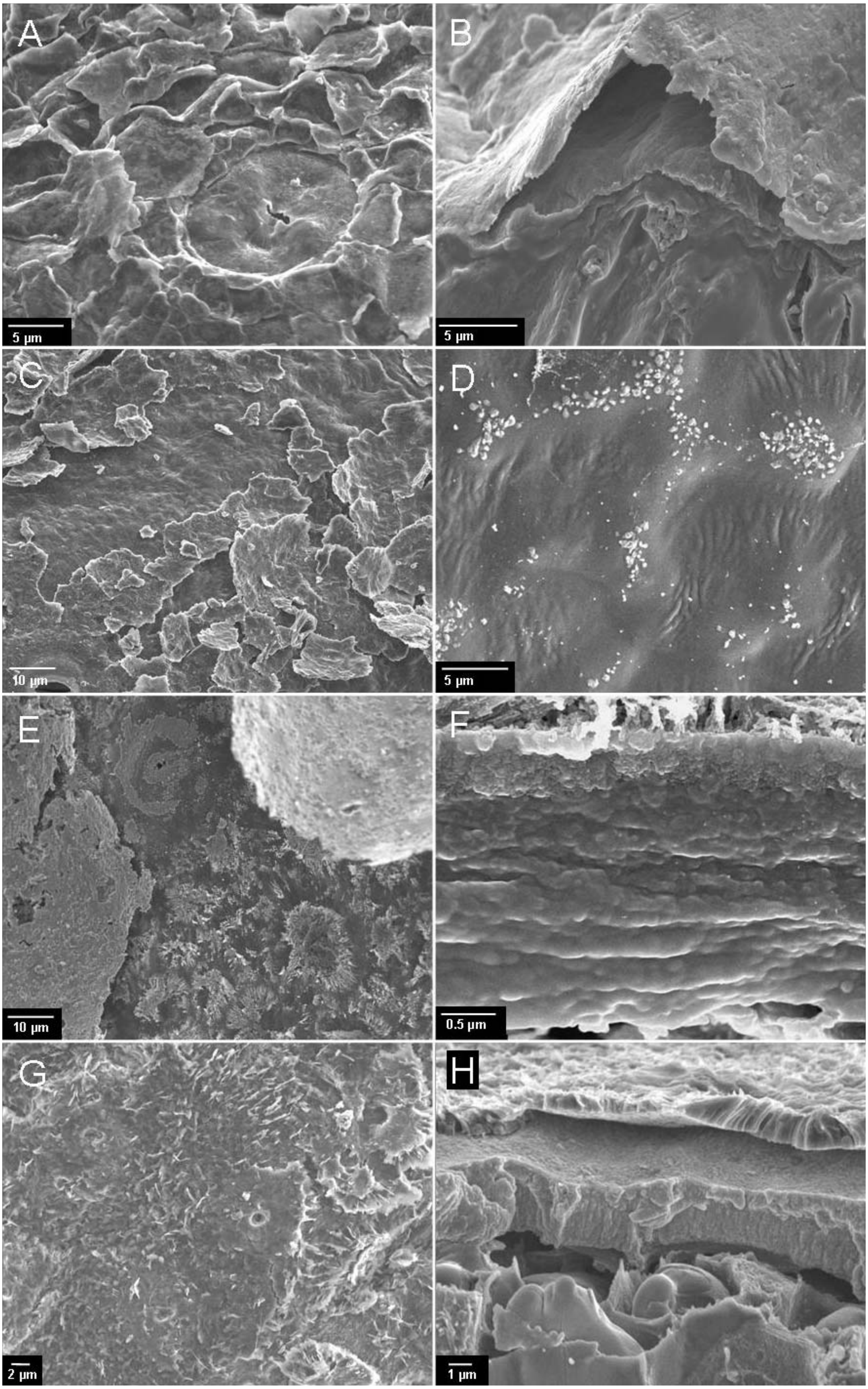

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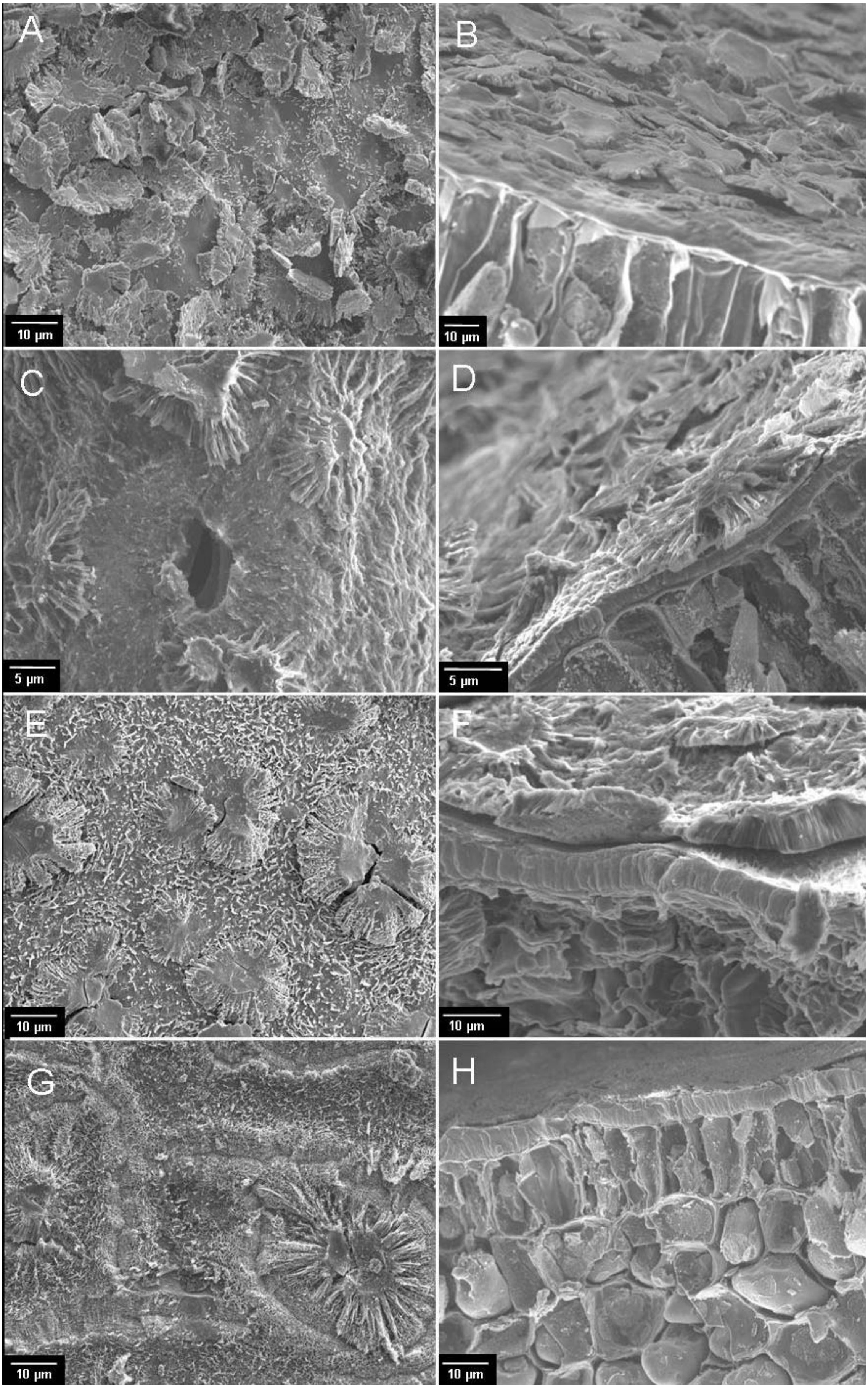

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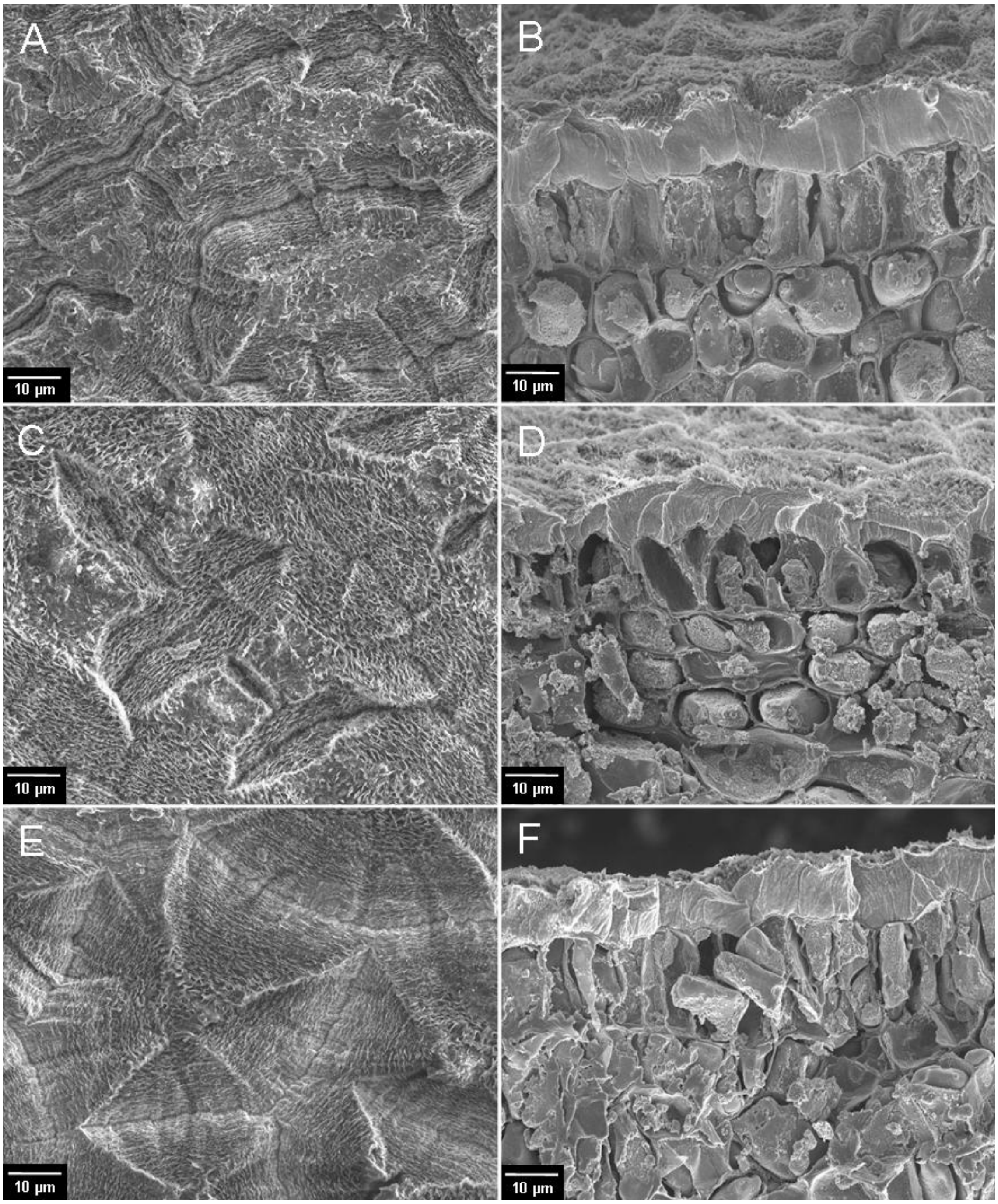

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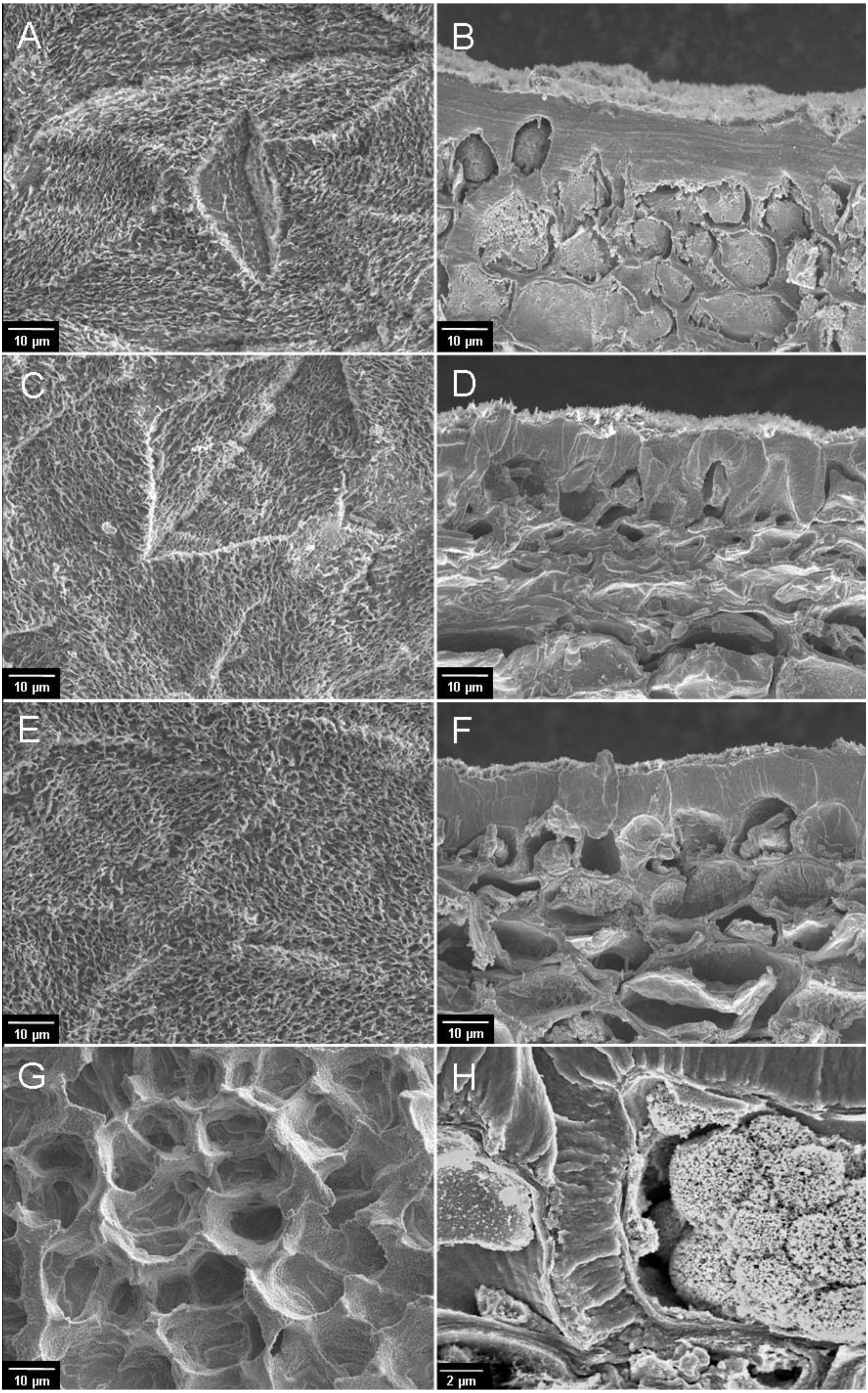

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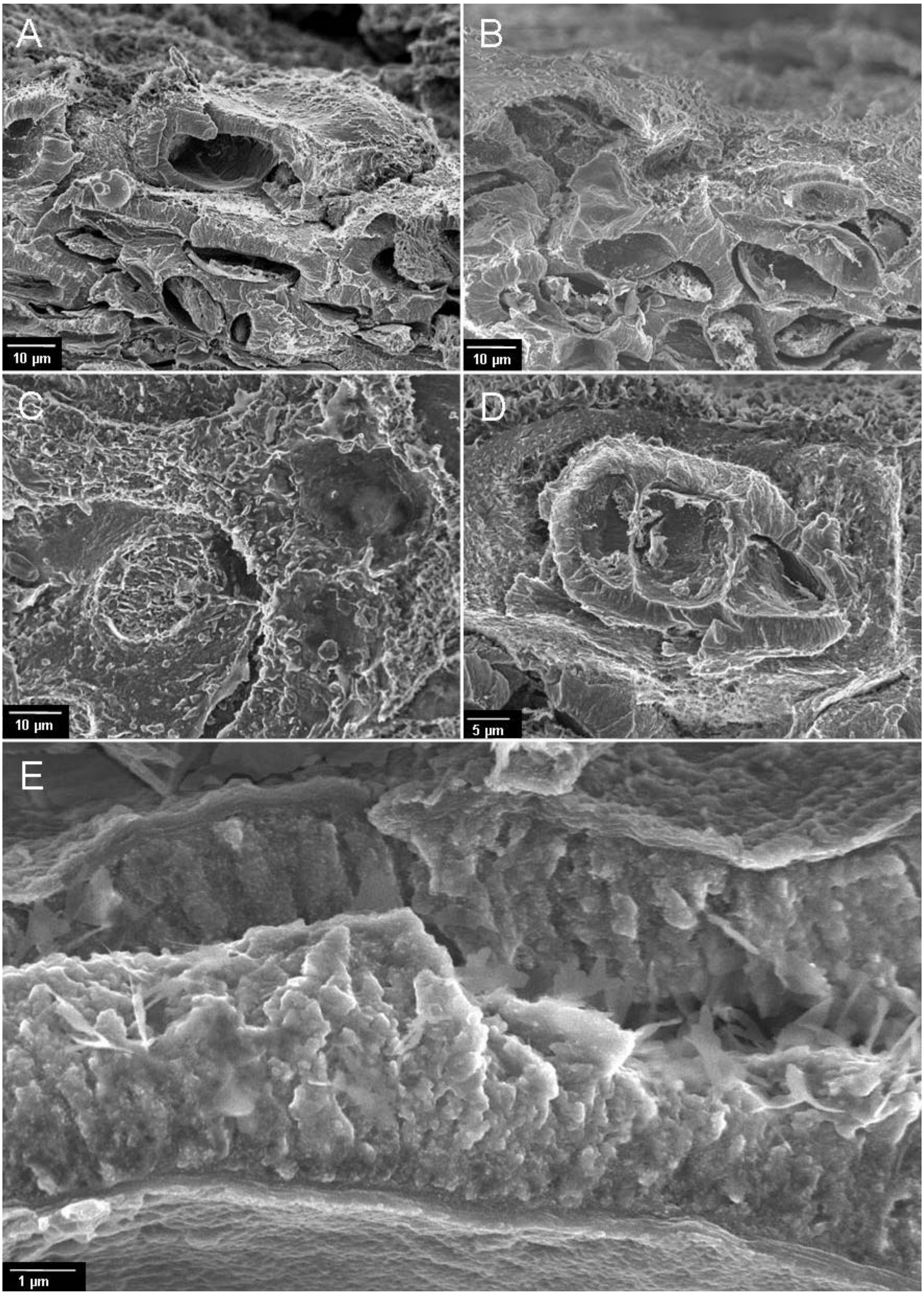

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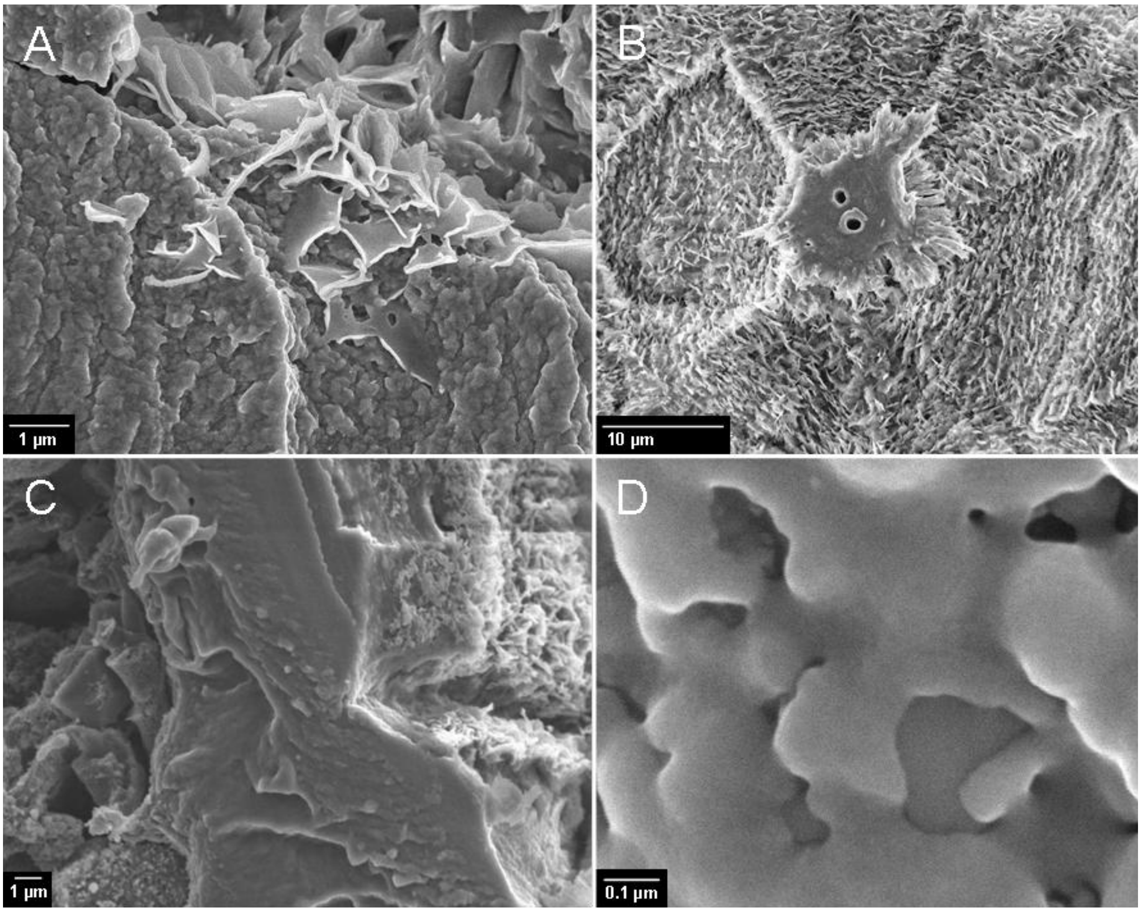

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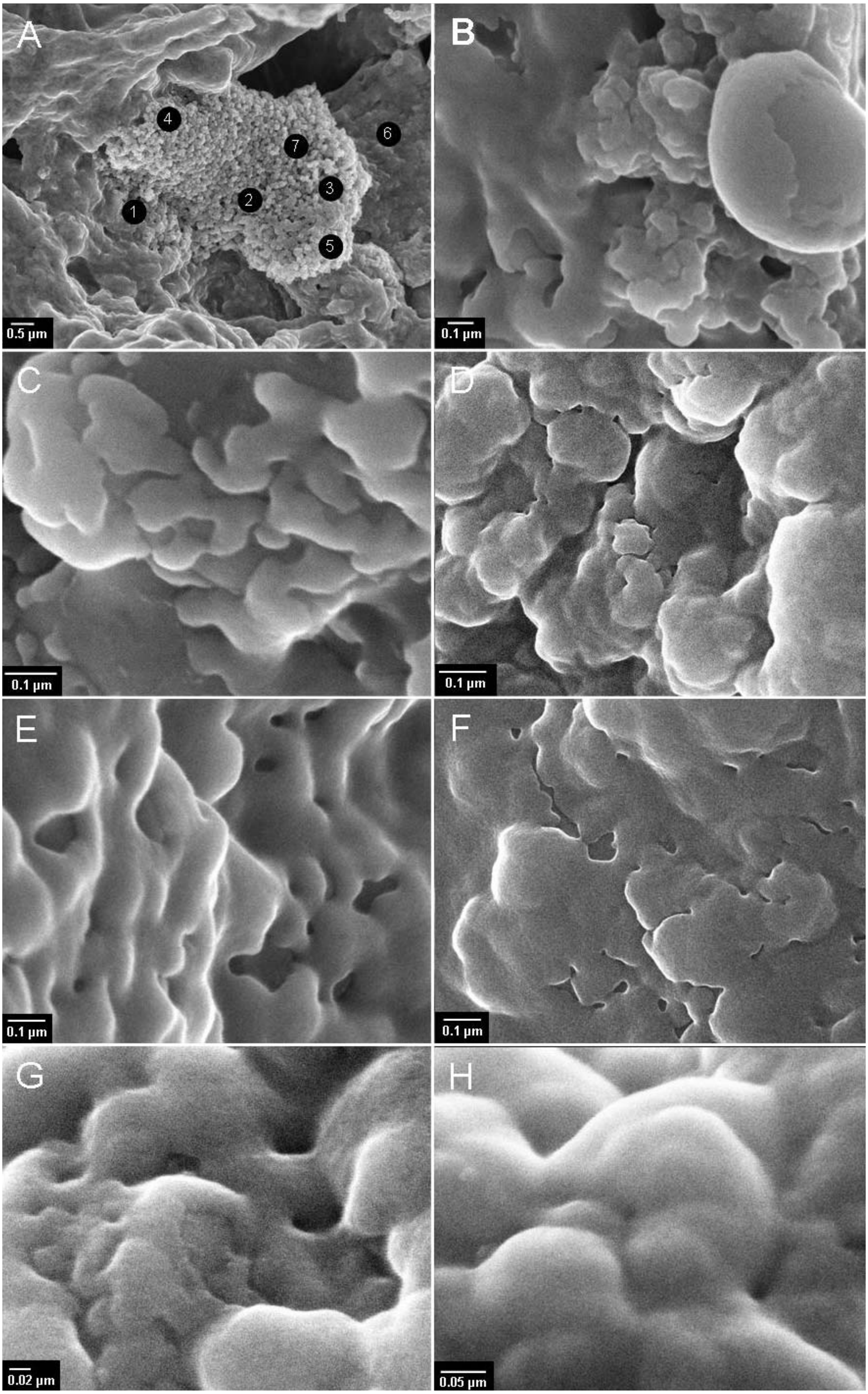

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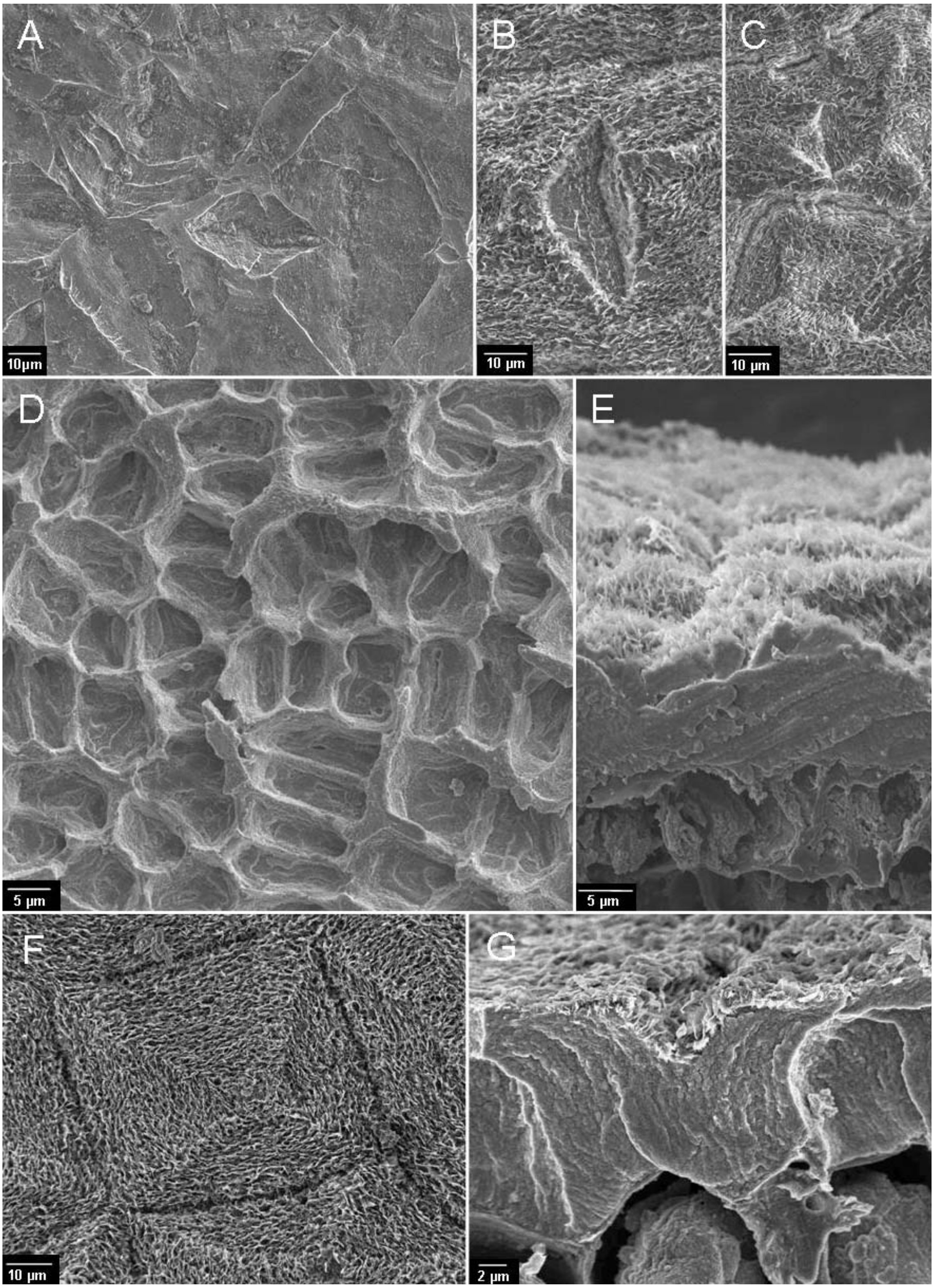

Figure 13